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A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127

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Natural killer (NK) cell development is thought to occur in the bone marrow. Here we identify the transcription factor GATA-3 and CD127 (IL-7Rα) as molecular markers of a pathway of mouse NK cell development that originates in the thymus. Thymus-derived CD127$^+$ NK cells repopulated peripheral lymphoid organs, and their homeostasis was strictly dependent on GATA-3 and interleukin 7. The CD127$^+$ NK cells had a distinct phenotype (CD11b^{lo}CD16$^-$CD69^{hi}Ly49^{lo}) and unusual functional attributes, including reduced cytotoxicity but considerable cytokine production. Those characteristics are reminiscent of human CD56^{hi}CD16$^-$ NK cells, which we found expressed CD127 and had more GATA-3 expression than human CD56$^+$CD16$^+$ NK cells. We propose that bone marrow and thymic NK cell pathways generate distinct mouse NK cells with properties similar to those of the two human CD56 NK cell subsets.

Natural killer (NK) cells were first identified based on their ability to lyse tumor cells without prior sensitization[1], but have since been well recognized for their importance in the defense against microbial agents and viruses. NK cells have an arsenal of effector molecules (cytotoxic granules and the capacity to produce inflammatory cytokines and chemokines) that allow them to accomplish their functions. Thus, the factors that orchestrate their development from hematopoietic precursors and that regulate their differentiation into mature effector lymphocytes are of great interest.

Stages of NK cell development have been distinguished based on phenotypic and functional analyses of NK cell precursors in the fetal liver and adult bone marrow[2,3]. Committed NK cell progenitors express CD122 (interleukin 2 receptor-β (IL-2Rβ)) but no other lineage-specific markers, which signals the first stage of NK cell development[4]. Subsequently, immature NK cells express NK1.1 (NKR-P1C or CD161c; on the C57BL/6 background) followed by DX5 (CD49b) and acquire an NK cell receptor 'repertoire' consisting of inhibitory and activating Ly49 receptors and CD94-NKG2 family members involved in target cell recognition[5]. NK cell maturation is completed after further changes in CD11b and CD43 expression and the acquisition of full functional competence, including natural cytotoxicity and the ability to secrete inflammatory cytokines[6].

The molecular mechanisms that drive NK cell development and guide the differentiation of these cells into effector lymphocytes have been partially elucidated[2]. Cytokines are essential regulators of NK cell development, and IL-15 is the dominant common γ-chain cytokine for NK cell generation, promoting the survival, population expansion and differentiation of immature and mature NK cells from both mouse and human NK cell progenitors[7–10]. In contrast, the common γ-chain cytokine IL-7 seems to be dispensable for NK cell development, although it is crucial for the development of adaptive B lymphocytes and T lymphocytes[11–14]. Through transcription factor–mediated expression of cytokine receptor genes, developing lymphocyte precursors gain the capacity to respond to environmental cues[15,16]. For example, PU.1, Ets-1, Id2 and Ikaros control the expression of several different growth factor and cytokine receptors (such as CD117, CD122 and CD137) that are crucial for early stages of NK cell generation[2]. The zinc-finger transcription factor GATA-3 controls many aspects of NK cell differentiation[17]. In the absence of GATA-3, immature bone marrow NK cells, with an incomplete Ly49 repertoire and lower expression of CD11b and CD43, seed the spleen. *Gata3*$^{-/-}$ splenic NK cells are poor producers of interferon-γ (IFN-γ) but nevertheless retain normal cytotoxic potential. Such characteristics are also found in NK cells deficient in interferon-regulatory factor 2 or the transcription factor

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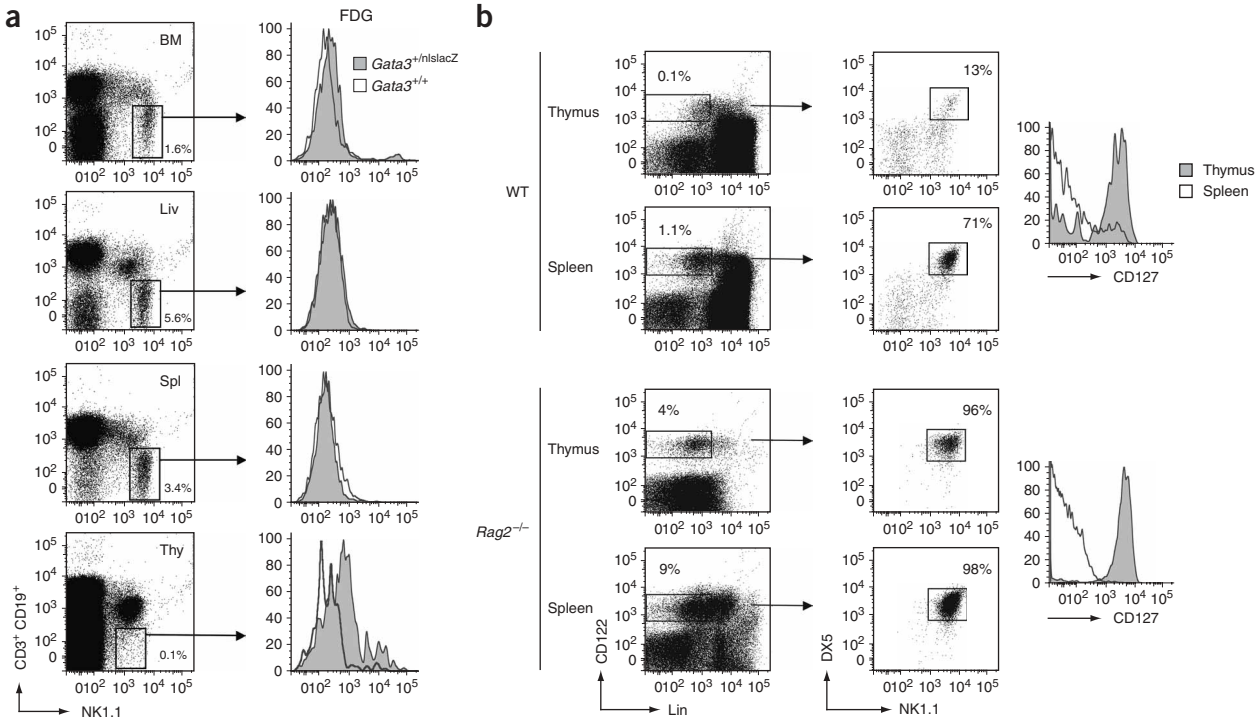

Figure 1 GATA-3 and CD127 expression by NK cells from various lymphoid tissues. (**a**) Left, flow cytometry of single-cell suspensions from *Gata3*$^{+/nlslacZ}$ and *Gata3*$^{+/+}$ mice, stained with antibodies to CD3, CD19 and NK1.1. Right, GATA-3 expression in CD3$^-$CD19$^-$NK1.1$^+$ cells from the flow cytometry at left, assessed indirectly with the lacZ substrate FDG. BM, bone marrow; Liv, liver; Spl, spleen; Thy, thymus. (**b**) Flow cytometry of lymphocytes from thymi and spleens of C57BL/6 (WT) and *Rag2*$^{-/-}$ mice, stained with antibodies to lineage markers (CD3, CD4, CD8, CD19 and Gr1; Lin) and to CD122, DX5, NK1.1 and CD127. Far right, expression of CD127 by CD122$^+$DX5$^+$NK1.1$^+$ cells from spleen and thymus. Numbers beside or above outlined areas indicate percent positive cells in each. Data are one representative experiment of four.

T-bet[18,19], suggesting a linked differentiation program through those different transcription factors[3].

NK cells are also found in the liver, lymph nodes and thymus, although the relationships among those NK cells are unclear. One possibility is that NK cells in those organs are simply recirculating cells[20], although some data suggest that NK cells found at different anatomical sites in the mouse are not derived from a unique peripheral pool. For example, mouse liver contains an atypical NK cell subset lacking expression of the NK cell marker DX5 and constitutively expressing the immune effector molecule TRAIL which seems to be involved in immunosurveillance for tumors[21]. Nevertheless, it is not apparent if those features result from a local differentiation process or, alternatively, if NK cells in different tissues originate from different precursor pools. Although it is generally accepted that developing NK cells emerge mainly from the bone marrow[22,23], 'bipotent' T cell–NK cell progenitors have been described in the thymus[24–26], suggesting the existence of many types of NK precursors, as well as a relationship between developing T cells and NK cells. Still, little evidence has been provided for phenotypically and functionally distinct subsets of mature NK cells in the adult mouse.

Here we have reassessed the developmental origins of thymic NK cells. NK cells originating in the thymus differed from bone marrow–derived NK cells by having more expression of GATA-3 and CD127 (IL-7Rα). CD127$^+$ thymic NK cells were exported to the peripheral lymphoid organs, where they retained unique phenotypic and functional properties closely resembling those of a subset of human NK cells (CD56^{hi}CD16$^-$ cells) with specific immunoregulatory properties[27]. We have thus identified an alternative thymic pathway of NK cell development that generates a distinct subset of mouse NK cells.

RESULTS

Thymic NK cells express GATA-3 and CD127

The developmental origin of the small population of NK cells found in the fetal and adult thymus remains obscure. Because GATA-3 is essential for the generation of the earliest T cell progenitors in the thymus[28], expression of GATA-3 in NK cells may provide some clues to their developmental origins. We investigated the expression of GATA-3 in NK cells derived from various anatomical sites using reporter mice in which expression of the gene encoding β-galactosidase is under control of the GATA-3 regulatory sequences (*Gata3*$^{+/nlslacZ}$)[28]. We found low GATA-3 expression in NK cells (CD3$^-$NK1.1$^+$) from the bone marrow and liver (**Fig. 1a**), whereas GATA-3 expression was essentially absent from splenic NK cells. Those results are consistent with published studies showing involvement of GATA-3 in NK cell differentiation, in particular during bone marrow maturation and in liver-specific homing[17]. In contrast, all thymic NK cells had substantial GATA-3 expression (**Fig. 1a**), exceeding that in other tissues. That result suggested that thymic NK cells differentiated *in situ* and differed from the recirculating peripheral NK cell pool.

The transcriptional program 'dictated' by GATA-3 is mostly unknown, although GATA-3 consensus DNA-binding sites have been identified in immunologically relevant target genes, including those encoding IL-4, IL-5, IL-13 (ref. 29), IFN-γ[30] and T cell receptor-α (TCRα) TCR β and TCR δ[31]. While characterizing irradiation

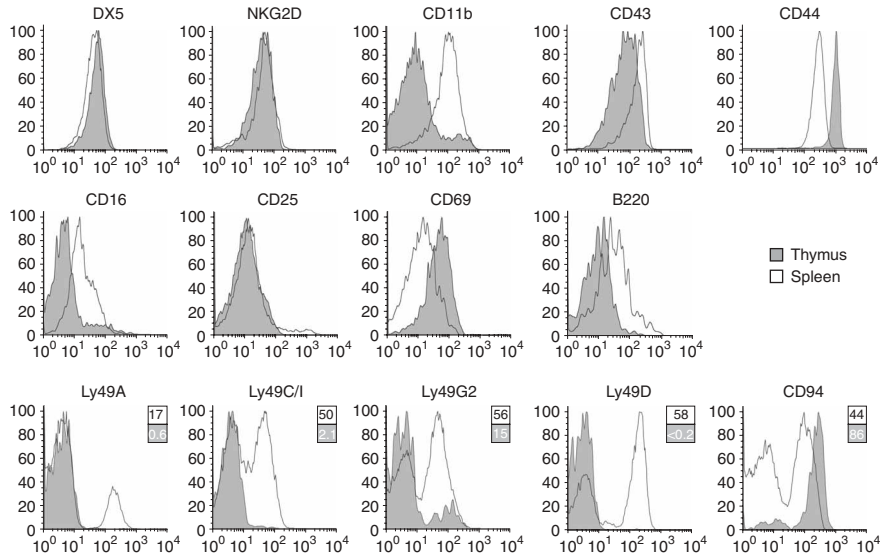

Figure 2 Phenotype of thymic NK cells. Flow cytometry for the surface marker phenotypes of thymic and splenic CD3$^-$NK1.1$^+$ NK cells. Numbers in boxes indicate the frequency of cells expressing Ly49 among NK1.1$^+$ cells. Each marker was analyzed on NK cells from three to six individual mice; data are representative.

chimeras generated after the transfer of fetal liver hematopoietic precursors from *Gata3*$^{+/-}$ or *Gata3*$^{-/-}$ embryos to alymphoid recipient mice deficient in recombination-activating gene 2 (*Rag2*$^{-/-}$) and IL-2Rγ (*Il2rg*$^{-/Y}$)[17], we identified CD127 as a potential target of GATA-3 in early double-negative thymocytes (M.G.O. *et al.*, unpublished results). Notably, most thymic NK cells expressed CD127, in contrast to peripheral splenic NK cells, which were almost exclusively CD127$^-$ (**Fig. 1b**).

We further characterized thymic CD127$^+$ NK cells from C57BL/6 mice using a panel of monoclonal antibodies and compared their phenotype to that of mature splenic CD127$^-$ NK cells. Like their peripheral splenic counterparts, thymic NK cells had uniform expression of several NK cell markers, including CD122, NK1.1, DX5 and NKG2D (**Figs. 1b** and **2**). Thymic NK cells, however, differed from their splenic counterparts with lower expression of CD11b, CD43 and B220, no CD16 expression and upregulated expression of CD44 and CD69 (**Fig. 2**). That phenotype was associated with a distinct receptor repertoire. Thus, whereas splenic NK cells express a repertoire of activating and inhibitory C-type lectin receptors of the Ly49 family[5], most thymic CD127$^+$ NK cells lacked the inhibitory Ly49 receptors A, C/I and G2 and all failed to express the activating receptor Ly49D (**Fig. 2**). A greater proportion of thymic NK cells were CD94$^+$ (with higher expression of CD94) than were splenic NK cells. The thymi of C57BL/6 mice contained about 5×10^4 NK cells; their phenotype and absolute numbers were unaffected by mutations in the genes encoding CD3ε or RAG-2 (**Fig. 1b** and data not shown) and were independent of changes in thymic cellularity (CD127$^+$ NK cell numbers were constant between 10 d and 6 months of age). Those results distinguished thymic CD127$^+$ NK cells from splenic NK cells and suggested that expression of GATA-3 and CD127 may provide defining characteristics of this NK cell subset.

Function of IL-7 in thymic NK cell homeostasis

Studies have demonstrated a redundant function for IL-7 in the generation of NK cell precursors and immature NK cells in the bone marrow and for normal homeostasis of mature NK cells in the spleen[10,12,14]. We thus compared thymic NK cell generation in *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice. IL-7 deficiency had no effect on the absolute numbers or phenotype of bone marrow or splenic NK cells[14]. In contrast to *Rag2*$^{-/-}$ mice, *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice had almost no CD127$^+$ thymic NK cells, demonstrating that IL-7 was critical for the homeostasis of NK cells in the thymus (**Fig. 3a**,**b**). The few thymic NK cells in *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice were CD127$^-$ and had a 'splenic' NK cell phenotype, including expression of Ly49D (**Fig. 3a**). Those observations indicated that true thymic NK cells are IL-7 dependent and suggested that there are two independent pathways of NK cell development, intrathymic and bone marrow derived, in mice.

Thymic export of CD127$^+$ NK cells

We next sought to determine whether thymic CD127$^+$ NK cells contribute to the peripheral NK cell pool by assessing the presence of these cells in various tissues. CD127$^+$ NK cells were poorly represented (less than 5%) in the bone marrow, liver and spleen but comprised a sizeable proportion (15–30%) of NK cells in the axillary, abdominal, inguinal and mesenteric lymph nodes (**Fig. 4a**). We compared CD127$^+$ and CD127$^-$ NK cells from the lymph node and found that the former had a receptor repertoire overlapping that of thymic CD127$^+$ NK cells, a repertoire characterized by less expression of Ly49A, C/I and G2 and an absence of Ly49D (**Fig. 4b** and data not shown). Furthermore, CD127$^+$ lymph node NK cells had less expression of CD11b and were CD69^{hi}, consistent with a 'thymic' phenotype. In contrast, CD127$^-$ lymph node NK cells were identical to mature splenic NK cells (CD11b^{hi} and CD69$^-$) and had a 'splenic' Ly49 repertoire (**Fig. 4c**). Those results identified two subsets

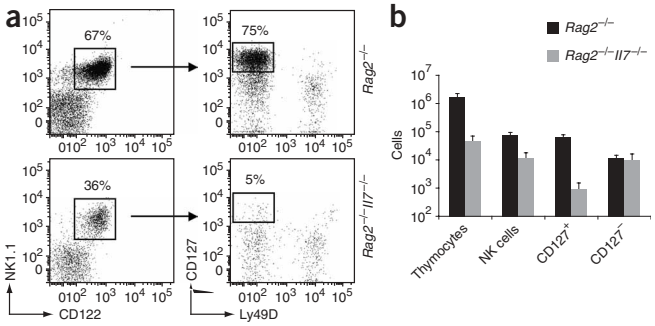

Figure 3 Function of IL-7 in the homeostasis of thymic NK cells. (**a**) Flow cytometry of thymocytes from *Rag2*$^{-/-}$and *Rag2*$^{-/-}$*Il7*$^{-/-}$mice, stained for CD122, NK1.1, CD127, Ly49D and CD25. Left, NK1.1 versus CD122 expression on CD25$^-$ thymocytes; numbers above boxed areas indicate percent NK1.1$^+$CD122$^+$ cells (boxed). Right, CD127 versus Ly49D expression on gated CD25$^-$NK1.1$^+$CD122$^+$ NK cells; numbers above boxed areas indicate percent CD127$^+$Ly49D$^-$ cells (boxed). Data are one representative experiment of three. (**b**) Absolute numbers (mean and s.d.) of total thymocytes, thymic NK cells and CD127$^+$ and CD127$^-$ thymic NK cells in *Rag2*$^{-/-}$mice ($n = 3$) and *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice ($n = 4$). $P < 0.001$ for all cell types except CD127$^-$ thymic NK cells, for which $P = 0.65$. Data are representative of four experiments.

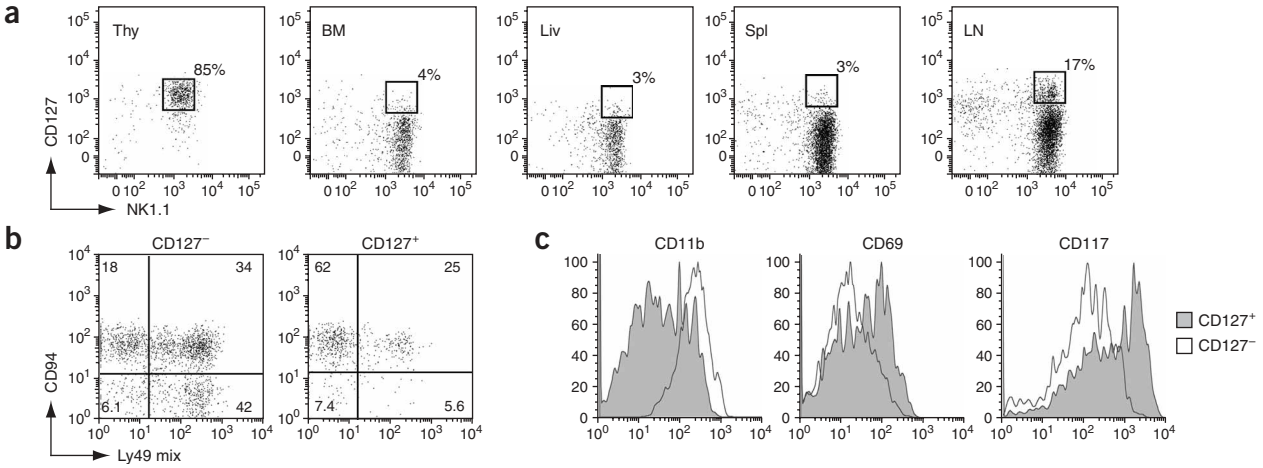

Figure 4 Identification of 'thymic' NK cells in various lymphoid organs. (**a**) Flow cytometry of the NK1.1 and CD127 profiles of gated CD3⁻CD122⁺ cells from C57BL/6 mice. Numbers above boxed areas indicate the percent CD127⁺NK1.1⁺ cells (boxed) among all NK cells. LN, lymph node. Data are one representative experiment of six. (**b**) Flow cytometry of receptor repertoire expression on CD127⁺ lymph node NK cells. CD122⁺NK1.1⁺ NK cells (CD3⁻CD19⁻) from lymph node were electronically gated, followed by analysis of expression of CD94 and a 'Ly49 mix' (Ly49A, Ly49C, Ly49D, Ly49G2 and Ly49I) on CD127-expressing NK cell subsets. Numbers in quadrants indicate percent cells in each. Data are representative of three experiments. (**c**) Flow cytometry of expression of CD69, CD11b and CD117 by CD127⁺ and CD127⁻ NK cells from the lymph node. Data are representative results from one experiment of four.

of lymph node NK cells and suggested that the peripheral CD127⁺ NK cell subset derive from export of a thymic precursor.

To assess the contribution of thymic NK cell export to the peripheral lymphoid tissues, we grafted 'allotype-marked' newborn thymus from CD45.2⁺ *Tcra*$^{-/-}$ mice under the kidney capsule of CD45.1⁺ *Rag2*$^{-/-}$ recipient mice. Then, 3 weeks later we assessed the presence of CD45.2⁺ NK cells in recipient mice and characterized their CD127 expression. We recovered thymus graft–derived cells (CD45.2⁺) from bone marrow, spleen, liver and lymph node in percentages ranging from 2% to 50%. Most thymus-derived cells were γδ T cells (98%), with the remaining 2% being mature NK cells (CD3⁻NK1.1⁺). Most thymus graft–derived NK cells were CD127⁺ and closely matched the thymic NK cell phenotype (**Fig. 5a**). We detected a higher percentage of thymus-derived NK cells in lymph node than in spleen (2% of lymph node NK cells were CD45.2⁺ versus less than 0.5% in the spleen). Notably, that strong bias in lymph node–homing of thymic NK cells was in recipient *Rag2*$^{-/-}$ mice with a full, steady-state peripheral NK cell compartment. Those results demonstrated that the thymus exports CD127⁺ NK cells that then localize to peripheral lymphoid organs.

Analyses of athymic nude *Foxn1*$^{-/-}$ mice were consistent with a thymic origin of CD127⁺ NK cells. *Foxn1*$^{-/-}$ and wild-type mice had similar absolute numbers of splenic and lymph node CD3⁻NK1.1⁺ NK cells (data not shown). However, the lymph nodes of *Foxn1*$^{-/-}$ mice had percentages of CD127⁺ NK cells that were 10% those in euthymic mice, and the small percentages of splenic CD127⁺ NK cells were even lower in the absence of a thymus (**Fig. 5b**). Thus, our data supported

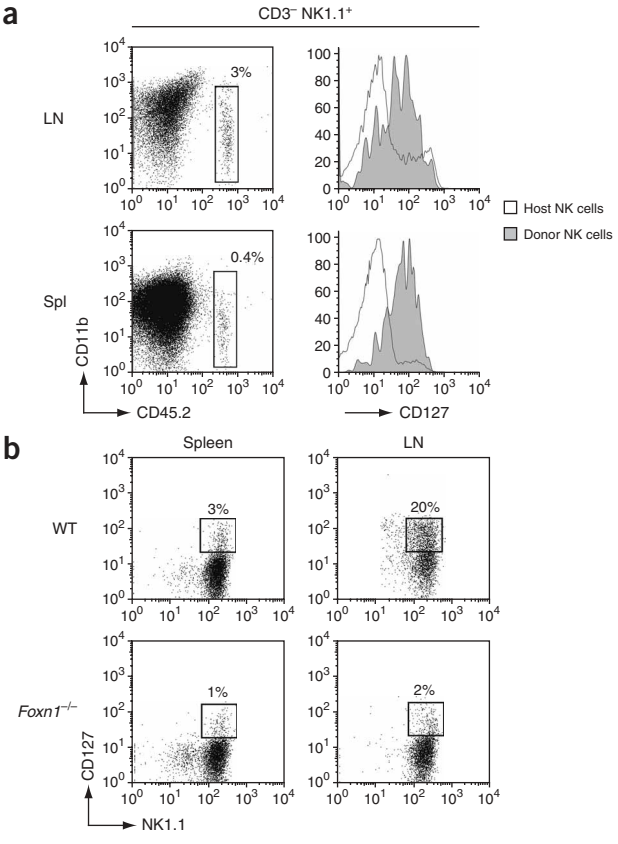

Figure 5 Thymic NK cells are exported to the periphery. (**a**) Flow cytometry of lymph node cells (LN) and spleen cells (Spleen) from CD45.1⁺ *Rag2*$^{-/-}$ mice grafted under the kidney capsule with thymi from newborn CD45.2⁺ *Tcra*$^{-/-}$ mice. Recipient mice were killed 3 weeks after grafting and cells were stained for CD45.2, CD3, NK1.1, CD11b and CD127. Left, CD45.2 versus CD11b profiles of gated CD3⁻NK1.1⁺ cells; numbers above boxed areas indicate percent donor-derived NK cells (boxed). Right, CD127 expression on the donor and host NK cells. Similar results were obtained with five grafted mice. (**b**) Flow cytometry of NK1.1 versus CD127 profiles of gated CD3⁻CD122⁺ splenocytes (Spleen) and lymph node cells (LN) from C57BL/6 control mice (WT) and athymic *Foxn1*$^{-/-}$mice, analyzed for CD127⁺ NK cells. Numbers above boxed areas indicate the frequency of CD127⁺ NK cells (boxed) in the NK compartment. Data are one representative experiment of three.

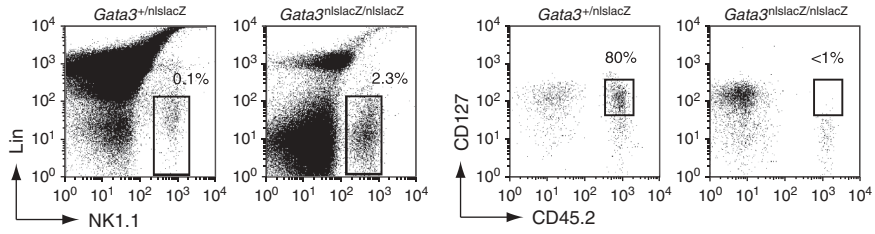

Figure 6 GATA-3 expression is required for the development of CD127$^+$ thymic NK cells. Flow cytometry of cells from CD45.1$^+$ *Rag2*$^{-/-}$ mice reconstituted with wild-type (*Gata3*$^{+/nlslacZ}$) or GATA-3-deficient (*Gata3*$^{nlslacZ/nlslacZ}$) CD45.2$^+$ hematopoietic precursor cells. Recipient mice were killed 6 weeks after transfer and thymocytes were stained for lineage markers (CD3, CD4 and CD8; Lin), NK1.1, CD127, CD45.2 and CD11b. Left, NK1.1 versus lineage marker expression on gated lymphocytes. Right, CD127 versus CD45.2 expression on electronically gated Lin$^-$NK1.1$^+$ cells. Numbers above boxed areas indicate percent Lin$^-$NK1.1$^+$ cells (boxed on left) or CD127$^+$CD45.2$^+$ cells (boxed on right). Similar results were obtained in three independent experiments.

the idea that the thymus exports CD127$^+$ NK cells and selectively contributes CD127$^+$ NK cells to the lymph node.

GATA-3 required for the generation of CD127$^+$ NK cells

Because GATA-3 expression was higher in the CD127$^+$ subset of lymph node NK cells (data not shown), we next assessed whether CD127$^+$ lymph node NK cells required GATA-3 expression for their development. We transferred fetal liver cell samples containing hematopoietic stem cell precursors from wild-type or GATA-3-deficient embryos into irradiated CD45.1$^+$ *Rag2*$^{-/-}$ recipients. Then, 6 weeks later, we analyzed the CD127 expression of the donor-derived thymic and lymph node NK cells. We recovered approximately equal numbers of donor and recipient thymic NK cells from the *Gata3*$^{+/-}$ chimeras; both donor and recipient NK cells were CD127$^+$. In contrast, donor CD127$^+$ NK cells were absent from the thymi of irradiation chimeras generated with *Gata3*$^{-/-}$ hematopoietic stem cells (**Fig. 6**). The CD127$^-$ NK cells in this context probably represented peripheral *Gata3*$^{-/-}$ splenic NK cells that re-entered the thymus. In the lymph nodes of chimeras generated with *Gata3*$^{+/-}$ fetal liver–hematopoietic stem cells, a subset of NK cells (about 8%) expressed CD127, whereas the lymph nodes of *Gata3*$^{-/-}$ chimeras were essentially devoid of CD127$^+$ NK cells (less than 0.2%). Those results indicated that GATA-3 expression is required for the generation of CD127$^+$ thymic NK cells, whereas bone marrow–derived NK cells can develop in the absence of GATA-3 expression.

Functional attributes of CD127$^+$ NK cells

Cytotoxic NK cells are present among IL-2- and IFN-α/β-cultured thymocytes from mice bearing the severe combined immunodeficiency mutation[20]. We reassessed the relative capacity of thymic (CD127$^+$) and splenic (CD127$^-$) NK cells to lyse the NK cell–sensitive target YAC-1 (mouse thymoma cells). Using unprimed *Rag2*$^{-/-}$ mice as donors, we found that freshly isolated thymic NK cells were cytolytic for YAC-1 cells but were less active than freshly isolated splenic NK cells from the same mice (**Fig. 7a**). Consistent with that finding, granzyme B expression was lower in thymic NK cells than in their splenic counterparts (**Fig. 7b**).

One possible expanation for the lower cytotoxic activity of thymic NK cells may relate to their state of differentiation, as these cells were CD11b^{lo} and had a poorly developed NK cell receptor repertoire (**Fig. 2**). We therefore compared the cytokine production profiles of thymic and splenic NK cells in response to an inflammatory cytokine (IL-12) or pharmacological activators. Despite their 'immature' CD11b^{lo} phenotype, thymic CD127$^+$ NK cells were strong cytokine producers (**Fig. 7c**). Thymic NK cells produced more IFN-γ than did splenic NK cells and also produced granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF), whereas splenic NK cells were poor producers or nonproducers of the last two cytokines. We obtained similar results when we compared CD127$^+$ and CD127$^-$ lymph node NK cells (**Fig. 7c**). CD127$^+$ and CD127$^-$ NK cell subsets had low expression of IL-12Rβ1 (data not shown), suggesting that differences in IL-12 signaling probably did not account for their different cytokine-production capacities. The CD127$^+$ lymph node NK cell subset produced a range of cytokines

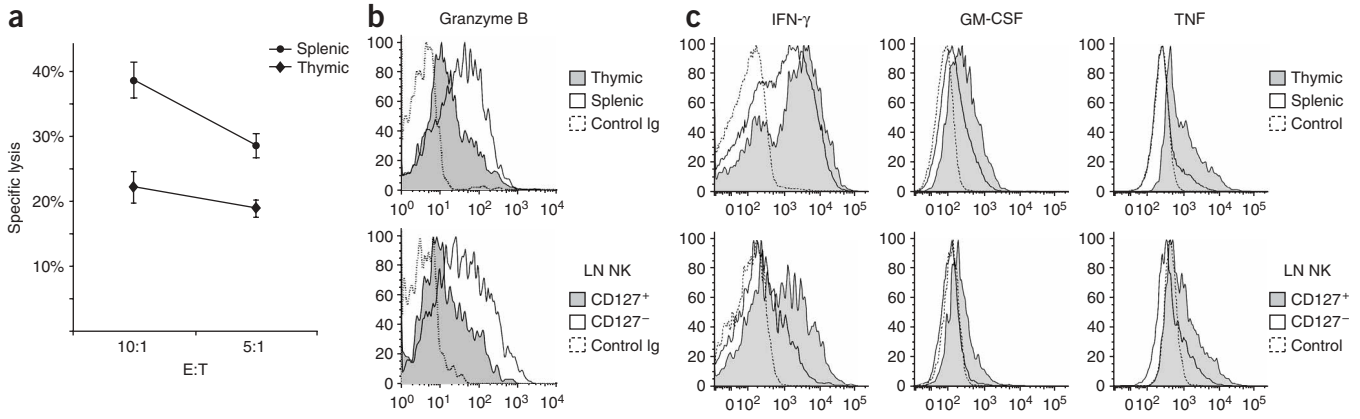

Figure 7 Functional properties of CD127-expressing NK cell subsets. (**a**) *In vitro* killing assay of YAC-1 target cells with thymocytes or splenocytes from *Rag2*$^{-/-}$ mice as effector cells. E:T, effector/target. Data are one representative result of two experiments. (**b**) Flow cytometry of granzyme B expression in thymic and splenic NK cells (top) and in CD127$^+$ and CD127$^-$ lymph node NK cells (bottom). Control Ig, staining with control immunoglobulin. Data are one representative result of two experiments. (**c**) Flow cytometry of intracellular expression of IFN-γ, GM-CSF and TNF by thymic (CD127$^+$) versus splenic (CD127$^-$) NK cells (top row) or electronically gated CD127$^+$ versus CD127$^-$ lymph node NK cells. Cells were stimulated with IL-12 (for IFN-γ and GM-CSF analysis) or with phorbol 12-myristate 13-acetate plus ionomycin (for TNF analysis). Control, CD127$^+$ thymic NK cells (top) or CD127$^+$ lymph node NK cells (bottom) cultured in IL-2 alone. Data are one representative experiment of three.

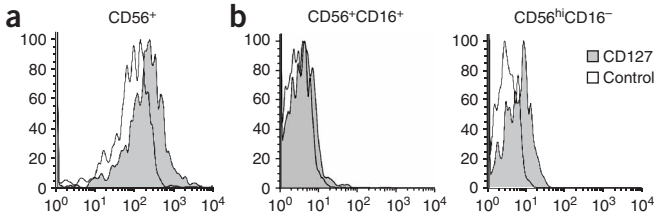

Figure 8 Expression of CD127 in human thymic NK cells and peripheral blood NK cell subsets. (**a**) Flow cytometry of CD127 expression in thymic NK cells from a 2-year-old patient undergoing invasive cardiac surgery. Thymocyte samples were depleted of CD4 cells and were stained with antibodies to CD3, CD56 and CD127; data represent CD127 expression or control isotype staining (Control) on $CD3^-CD56^+$ thymocytes. (**b**) Flow cytometry of CD127 expression by peripheral blood mononuclear cells (PBL) collected from normal donors. Cell samples were depleted of CD4 T cells by magnetic-activated cell sorting and then were stained with antibodies to CD3, CD16, CD56 and CD127; data represent CD127 expression or control isotype staining (Control) on $CD3^-CD56^{hi}CD16^-$ and $CD3^-CD56^+CD16^+$ cells. Data are one representative experiment of four.

quantitatively and qualitatively different from that of their $CD127^-$ counterparts. These results collectively indicated that $CD127^+$ NK cells are mature and that their biological effector functions are distinct from those elicited by $CD127^-$ NK cells.

Human NK cells differentially express GATA-3 and CD127

The phenotypic and functional differences between the mouse $CD127^+$ and $CD127^-$ NK cell subsets, such as different CD16 expression, cytolytic activity and cytokine-production capacity, bear similarities to those of human NK cell subsets[27]. Human $CD3^-CD56^+$ NK cells can be distinguished based on their expression of CD16 and CD56: $CD56^{hi}CD16^-$ NK cells are poorly cytotoxic, yet produce larger amounts of cytokines, including IFN-γ, TNF, GM-CSF and IL-10; $CD56^+CD16^+$ NK cells, in contrast, are highly cytotoxic but relatively poor cytokine producers. We therefore considered whether the two human subsets were analogous to the CD127 mouse NK cell subsets. We first evaluated the expression of surface markers on human NK cells subsets that are differentially expressed on the mouse NK cell subsets. We found that human $CD56^{hi}CD16^-$ NK cells expressed CD127, whereas $CD56^+CD16^+$ NK cells were $CD127^-$ (**Fig. 8**). Moreover, *Gata3* transcript expression by $CD56^{hi}CD16^-$ NK cells was 3.6-fold higher than that of $CD56^+CD16^+$ NK cells (based on quantitative PCR normalized to expression of the gene encoding glyceraldehyde phosphate dehydrogenase). Finally, like their mouse counterparts, most NK cells in human thymus were $CD127^+$ (**Fig. 8**). Those observations were consistent with the conclusion that the two human and two mouse NK cells subsets may have similar developmental and functional properties.

DISCUSSION

Although it is known that mature NK cells are present in the human and mouse thymus, their developmental origins and biological functions remain unclear. Early studies demonstrated that thymic NK cells from mice with severe combined immunodeficiency could lyse classical NK cell–sensitive targets (although their cytokine production potential was not assessed)[20]. Nevertheless, little evidence was provided that thymic NK cells from adult mice were different from their splenic counterparts, and thus it was proposed that thymic NK cells were recirculating mature splenic NK cells[20].

We have identified here a thymic pathway of NK cell development in mice characterized by expression of GATA-3 and CD127. Thymic NK cells expressed more GATA-3 than did splenic NK cells, and most thymic but not splenic NK cells expressed CD127. Additional phenotypic differences, such as more CD69 expression and a narrower Ly49 receptor repertoire, distinguished thymic NK cells from their splenic counterparts. The observation that CD127 is not expressed on Notch-stimulated *Gata3*$^{-/-}$ early thymocyte precursors suggests that most $CD127^+$ NK cells are generated intrathymically from hematopoietic progenitors, most likely early T cell precursors, through a GATA-3-dependent pathway. That hypothesis is consistent with published studies demonstrating that NK cells can be derived from bipotent T cell–NK cell precursors present in human and mouse fetal thymus[24–26]. Therefore, both thymic NK cell generation and T cell generation are strictly GATA-3 dependent, indicating their close developmental relationship.

We found that CD127 expression was a functional and distinguishing feature of thymic NK cells, as their generation was much lower in the absence of IL-7, whereas bone marrow NK cell development was normal[14]. Accordingly, thymic NK cells resemble adaptive mouse lymphocytes (T cell and B cells) in their IL-7 dependence[32]. The few thymic NK cells in IL-7-deficient mice differed from most thymic NK cells in normal mice, closely resembling splenic $CD127^-$ NK cells. Although it remains possible that the 'splenic' $CD127^-$ NK cells develop intrathymically in the absence of IL-7, we favor the hypothesis that the minor thymic $CD127^-$ NK cell subset represents recirculating mature splenic NK cells. Thus, both a major intrathymic and a minor extrathymic pathway of NK cell development can contribute to the thymic NK cell pool. Nevertheless, all NK cell developmental pathways are IL-15 dependent[8,14].

Thymic NK cells, like newly generated naive T cells, are exported to the peripheral lymphoid tissues and circulate through the lymph (data not shown). Many and varied interactions between dendritic cells and NK cells in the lymph node can potentially result in reciprocal activation and immune activation, or alternatively, in dendritic cell elimination and immune tolerance[33]. In addition, multifunctional interferon-producing 'killer' dendritic cells (IKDCs) have been identified in lymphoid tissues[34,35]. One possibility is that $CD127^+$ NK cells are related to IKDCs. However, that possibility seems unlikely, given that $CD127^+$ NK cells are $Ly49D^-$ and $B220^-$, whereas IKDCs express these markers, and $CD127^+$ NK cells are IL-7 dependent, whereas IKDCs are not[34,35]. Pathogen-activated dendritic cells recruit NK cells to the lymph node, where they can produce cytokines (including IFN-γ) that participate in the efficient generation of T helper type 1 (T_H1)–polarized T cell responses[36]. Moreover, NK cells and IKDCs in the lymph node have potent antitumor activity, which can be augmented by dendritic cell activation[34,35,37]. Whether those immune effects are mediated through or perhaps regulated by $CD127^+$ or $CD127^-$ NK cells (or both) remains to be determined.

$CD127^+$ thymic NK cells in the lymph node have functional characteristics, such as higher cytokine production and lower cytotoxicity, that distinguish them from bone marrow–derived splenic $CD127^-$ NK cells. The two NK subsets in the mouse have homology to human $CD56^{hi}CD16^-$ and $CD56^+CD16^+$ NK cell subsets[27]. We found that like their mouse counterparts, human thymic NK cells were $CD127^+$. Moreover, the two human NK cell subsets in peripheral blood differed in CD127 expression, with $CD56^{hi}CD16^-$ human NK cells being $CD127^+$ and $CD56^+CD16^+$ human NK cells being $CD127^-$. Human $CD56^{hi}CD16^-$ NK cells had more GATA-3 expression than did $CD56^+CD16^+$ NK cells. Based on their phenotypic and functional similarities, we propose that the $CD127^+$ mouse NK subset we have identified corresponds to the $CD56^{hi}CD16^-$ human NK cell subset. Whether mouse $CD127^+$ NK cell subsets have unique

immunoregulatory functions, like human NK cell subsets, remains to be determined. Also, the capacity of IL-7 to modulate the proliferation and function of CD127$^+$ NK cells in both mice and humans should be evaluated. The molecular mechanisms accounting for the different functional attributes of these NK cell subsets remain unknown. Nevertheless, functionally heterogeneous NK cell subsets exist not only in humans but also in rodents. Also, evidence suggests that CD56^{hi}CD16$^-$ human NK cells can develop *in situ* in the lymph node[38]. The thymic dependence of mouse lymph node CD127$^+$ NK cells suggests that human CD56^{hi}CD16$^-$ NK cells could also be thymus derived.

Although most of our work here used mice on the C57BL/6 background, CD127$^+$ thymic NK cells are also present in other mouse strains, such as nonobese diabetic and BALB/c (data not shown), suggesting that GATA-3-driven CD127$^+$ thymic NK cell development represents a conserved, general mechanism for NK cell diversification. Thus, NK cells might have an important function in homeostasis of the thymus. Studies have suggested, for example, that NK cells could have an immunosurveillance function in the thymus, because early thymocyte precursors are targets for mature cytotoxic splenic NK cells[39]. Subsequent studies have suggested that NK cells can modulate intrathymic T cell development through a perforin-mediated mechanism[40]. In both of those studies, highly proliferating cells appeared as targets for NK cells. Analysis of mice deficient in thymic NK cells and a better understanding of the precise localization of CD127$^+$ and CD127$^-$ thymic NK cells may help provide clues to their biological functions.

Our data suggested that thymic NK cell development imparts particular functional characteristics to this subset of NK cells. Transcription factors are essential for establishing cytokine profiles in differentiating T cells[41]. GATA-3 promotes T_H2 cytokine production (IL-4, IL-5 and GM-CSF) while antagonizing T_H1–like cytokine production (IFN-γ and TNF). GATA-3 has a paradoxical function in NK cell differentiation in promoting IFN-γ production by mature NK cells[17]. We found that GATA-3-expressing CD127$^+$ NK cells were capable of producing both IFN-γ (T_H1) and GM-CSF (T_H2) and therefore again call into question the 'classical' T_H2 function of GATA-3 in the elaboration of NK cell cytokine profiles. Our results have also suggested that GATA-3 does not antagonize IFN-γ production in NK cells but instead suggested that it can function to diversify cytokine production capacities when expressed in this lymphoid cell subset. Thus, the mechanisms by which different transcription factors orchestrate NK cell cytokine production do not fit neatly into the T_H1-T_H2 paradigm. Furthermore, NK cell cytokine production may not be fixed and thus may be modified in a transcription factor–dependent way in a given biological context. Future studies should address the signals regulating the broad cytokine expression profile of GATA-3$^+$CD127$^+$ NK cells and to what extent these NK cells demonstrate plasticity in their cytokine profiles when conditioned in different stimulatory environments.

METHODS

Mice. C57BL/6 mice with a β-galactosidase reporter gene under control of the GATA-3 regulatory sequences (*Gata3*$^{+/nlslacZ}$) and *Rag2*$^{-/-}$ and *Rag2*$^{-/-}$*Il7*$^{-/-}$ mice on the C57BL/6 background have been described[14,28]. Control wild-type, *Tcra*$^{-/-}$ and athymic *Foxn1*$^{-/-}$ mice on the C57BL/6 background were purchased from Charles River, Jax or Centre de Distribution, Typage et d'Archivage. All mice were kept in specific pathogen–free conditions and all animal experiments were approved by the committee on animal experimentation of the Institut Pasteur (Paris, France) and were approved by the French Ministry of Agriculture. Human samples were obtained from healthy volunteers and patients who had provided written informed consent in accordance with procedures at the Institut Pasteur.

Generation of hematopoietic chimeras. *Gata3*$^{+/nlslacZ}$ mice were mated and the morning the vaginal plug was first present was considered embryonic day 0.5. For recovery of GATA-3-deficient fetal liver cells, gestating *Gata3*$^{+/nlslacZ}$ female mice were provided isoproterenol (100 μg/ml), L-phenylephrine (100 μg/ml) and ascorbic acid (2 mg/ml; all from Sigma) in the drinking water beginning 8.5 d after coitus. Embryos were recovered at embryonic day 14.5, and GATA-3-deficient embryos (*Gata3*$^{nlslacZ/nlslacZ}$; called '*Gata3*$^{-/-}$' here) were identified by PCR with the primers G3S (5′-CAGGAGTCCGCGGA CCTCC-3′), wild-type-AS (5′-CGTTGAGGACCGCGGGGTG-3′) and lacZ-AS (5′-CCAAGCTTGGACTCAAAAAACT-3′). PCR consisting of 30 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C yielded a 149–base pair fragment of the *Gata3*$^+$ allele and a 243–base pair fragment of the *Gata3*^{nlslacZ} allele. Single-cell suspensions of fetal liver were prepared and were transferred intravenously into CD45.1$^+$ *Rag2*$^{-/-}$ recipient mice. At 6 weeks after transfer, the presence of donor-derived NK cell subsets was assessed in recipient mice.

Thymus transplantation. Thymi were isolated from newborn CD45.2$^+$ *Tcra*$^{-/-}$ mice and were grafted under the kidney capsules of CD45.1$^+$ *Rag2*$^{-/-}$ mice. Bone marrow, spleens, livers and lymph nodes from grafted recipients were collected 3 weeks later. The presence of donor-derived lymphocytes was assessed in single-cell suspensions prepared from those isolated tissues.

Flow cytometry. Cells isolated from thymus, spleen, bone marrow and perfused liver were prepared as described[17]. Human thymocytes and peripheral blood mononuclear cells were isolated as described[42]. Before being stained, cells were treated with Fc-Block (antibody to CD16-CD32; Becton Dickinson PharMingen) or purified mouse immunoglobulin G. Monoclonal antibodies to the following mouse antigens were conjugated to fluorescein isothiocyanate, phycoerythrin, phycoerythrin-indotricarbocyanine, allophycocyanin, allophycocyanin-indotricarbocyanine, peridinin chlorophyll protein–cyanine 5.5 or biotin: Ter119, CD19 (1D3), B220 (RA3-6B2), CD3 (145-2C11), γδTCR (GL3), CD90.2 (53-2.1), CD2 (RM2-5), CD44 (IM7), CD25 (PC61), CD43 (S7), CD11b (M1/70), CD49b (DX5), CD69 (H1.2F3), CD94 (18d3), CD122 (TM-β1), CD127 (A7R34), Ly49A (JR9), Ly49G2 (4D11), Ly49C/I (5E6), Ly49D (4E5), NK1.1 (PK136), IFN-γ (XMG1.2), GM-CSF (MP1-22E9), TNF (MP6-XT22), NKG2D (CX5), CD117 (2B8) and granzyme B (GB12). Human antibodies were to CD56 (B159), CD3 (SK7), CD16 (3G8) and CD127 (40131). Antibodies were purchased from Becton Dickinson PharMingen, eBioscience, Southern Biotechnologies Associates, Caltag, Biolegend and R&D Systems. Biotinylated antibodies were visualized with streptavidin-allophycocyanin-indotricarbocyanine (Becton Dickinson PharMingen and eBioscience). Stained cells were analyzed with a FACSCanto (Becton Dickinson) and FlowJo software (Treestar).

Analysis of GATA-3 transcriptional activity. The activity of β-galactosidase (an indicator of *Gata3* activity) can be monitored with the substrate FDG (fluorescein-di-β-galactopyranoside; Sigma), whose cleavage generates fluorescein. Cell suspensions (10×10^6 cells/ml) were prepared in prewarmed complete medium (Opti-MEM with 2% FCS, 10 μM β-mercaptoethanol, 100 mg/ml of streptomycin and 100 U/ml of penicillin); this was mixed for 1 min at 37 °C with an equal volume of a solution of FDG (2 μM in distilled water). FDG 'loading' was stopped by the addition of a tenfold excess of cold medium containing 300 μM chloroquine to inhibit lysosomal degradation of FDG. After 30 min of incubation on ice, cells were stained for cell surface markers and were analyzed by flow cytometry.

Quantification of GATA-3 mRNA in human NK cells. Total RNA was purified from sorted CD56^{hi}CD16$^-$ and CD56$^+$CD16$^+$ NK cells with RNeasy columns (Qiagen) and human *Gata3* mRNA transcripts were quantified by real-time PCR with Taqman probes as described (Assays-on-Demand; Applied Biosystems). Expression was were normalized to that of amplicons of the gene encoding glyceraldehyde phosphate dehydrogenase in each sample.

NK cell lytic activity. A standard ^{51}Cr-release assay was used to measure NK lytic activity *in vitro* as described[14]. YAC-1 cells (mouse thymoma; H-2^a) used

as target cells were maintained in complete medium (RPMI 1640 medium with 5% FCS and 10 μM β-mercaptoethanol). Target cells were labeled with 100 μCi of ^{51}Cr (ICN Pharmaceutical) and 5×10^3 cells were incubated for 4 h with 'graded' numbers of effector cells in 200 μl of medium. Freshly isolated total thymocytes or splenocytes were used as effector cells. After flow cytometry to determine the percentages of NK1.1$^+$ cells, effector/target ratios were established. More than 95% of thymic NK cells and less than 4% of splenic NK cells were CD127$^+$. The radioactivity released into the cell-free supernatant was measured and percent specific lysis was calculated as follows: 100 × (experimental release – spontaneous release) / (maximum release – spontaneous release). Spontaneous release was always less than 5%.

***In vitro* cytokine production.** Total splenocytes, thymocytes and lymph node cells were cultured in round-bottomed microtiter plates containing mouse IL-2 (1,000 U/ml) and were stimulated for 18 h at 37 °C with mouse IL-12 (2 ng/ml; Peprotech). Brefeldin A (10 μg/ml) was added during the last 4 h to raise intracellular cytokine stores by inhibiting their secretion. In some wells, phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (1 μg/ml) were added with the brefeldin A. Cells were then washed, were stained for NK1.1 and CD127 at the cell surface, were fixed for 10 min at 25 °C in 2% paraformaldehyde and then were stained intracellularly with fluorescein isothiocyanate–conjugated monoclonal antibody to TNF, phycoerythrin-conjugated monoclonal antibody to GM-CSF and phycoerythrin-indotricarbocyanine–conjugated monoclonal antibody to IFN-γ (all from Becton Dickinson Pharmingen) in 0.5% saponin. Control cells were incubated in medium with IL-2.

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AUTHOR CONTRIBUTIONS

C.A.J.V., M.E.G.-O., S.I.S.-V., L.E., D.G.-G., L.R., S.E. and J.P.D. did the experiments; V.P., O.R.-L. and E.C. provided technical assistance; B.R., A.C. and S.E. contributed to the experimental design and analysis; and C.A.J.V. and J.P.D. wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Erratum: T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α

Yuanyuan Zha, Reinhard Marks, Allen W Ho, Amy C Peterson, Sujit Janardhan, Ian Brown, Kesavannair Praveen, Stacey Stang, James C Stone & Thomas F Gajewski

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In the version of this article initially published, the equal contribution of the third author was omitted. The error has been corrected in the HTML and PDF versions of the article.

Erratum: A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127

Christian A J Vosshenrich, Marcos E García-Ojeda, Sandrine I Samson-Villéger, Valerie Pasqualetto, Laurence Enault, Odile Richard-Le Goff, Erwan Corcuff, Delphine Guy-Grand, Benedita Rocha, Ana Cumano, Lars Rogge, Sophie Ezine & James P Di Santo

Nature Immunology **7, 1217–1224 (2006); corrected after print 3 November 2006**

In the version of this article initially published, the symbols in the key of **Figure 7a** are incorrect. The filled ovals are Splenic; the filled diamonds are Thymic. The error has been corrected in the HTML and PDF versions of the article.