Cutting edge: thymic NK cells develop independently from T cell precursors.

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Thymic NK cells develop independently from T cell precursors

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Running title: Development of thymic NK cells

Abstract

While 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 GATA-3 dependent pathway. Characteristically, thymic NK cells express CD127, few Ly49 molecules and lack CD11b. Since 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 *Rorc*-expressing progenitors and do not express Rag2 or rearrange the TCRγ locus. We further demonstrate that thymic NK cells develop independently of the Notch signalling pathway, supporting the idea that thymic NK cells represent *bona fide* NK cells that can develop independently of all T cell precursors.

Introduction

It 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. While 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 ($CD127^{+}CD11b^{-}Ly49^{lo}$) and functional potential (higher cytokine secretion, lower cytotoxic potential (2)). Moreover, thymic NK cells are exported to the lymph nodes where they represent around 20% of the resident NK cell population (2).

While the thymic environment harbors other non-T cell lineage cells (including hematopoietic precursors (HPC), 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 (NKP) in the bone marrow encompasses cells with not only NK potential but T as well as NK/T bi-potent precursor cells (6). The relationship of thymic NK cells to classical NK cells, innate T lymphocytes (γδ T cells, NK-T cells) and mainstream αβ T cells remains unclear.

Environmental cues coordinate with specific transcription factors to orchestrate lymphocyte development. Essential cytokines for NK development (including thymic NK cells) includes IL-15, while 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, while 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 (ROR)γ (encoded by *Rorc*), following expression of the pre-TCR in committed pre-T cells (12). Here 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 (sharply limited)

Mice

C57BL/6J mice were purchased from Charles River. Rag2-GFP BAC transgenic, Mx-cre transgenic *Rbpj*$^{f/f}$, and Mx-cre transgenic *Notch1*$^{f/f}$, *Rorc(t)*-Cre^{TG} ROSA-YFP, CD3ε$^{-/-}$ and TCRβ$^{-/-}$ mice have been described previously (13-18). Mice were analyzed at 6-12 weeks of age. All experiments followed institutional guidelines (Animal Care and Use Committee of the Institut Pasteur) 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). Antibodies to Notch 1 (22E5.5) and Notch 2 (16F11) have been described (19). Stained single cell suspensions were acquired on a FACSCanto II (FACSDiva software 6.1; BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). Cells were sorted on a FACSAria II cell sorter (BD Biosciences). Dead cells were excluded using Live/Death fixable Aqua cell stain (Invitrogen).

PCR

Single NK cells were sorted from CD3ε$^{-/-}$ mice (thymus: CD127^{+} cells; spleen: CD127^{-} cells), and γδT cells from the thymus of TCRβ$^{-/-}$ mice as controls. Single-cell PCRs to detect the Vγ2-Jγ2 and Vγ4-Jγ1 rearrangements (according to the Heilig and Tonegawa nomenclature; ref 20) were performed as described (21).

Bone marrow chimeras

MX-cre Tg *Rbpj*$^{f/f}$ mice and MX-cre Tg *Notch1*$^{f/f}$ mice (both CD45.2) were injected 5 times at 2-day intervals with 150µg of poly(I)-poly(C) (Sigma-Aldrich). BM cells (where the deletion of the corresponding floxed alleles were verified as described (14, 15); supplemental Figure 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*$^{f/f}$ or *Notch1*$^{f/f}$ littermates were treated in the same way to generate control BM chimeras. Mice were analysed twelve weeks post-graft.

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 (DP) thymocytes (22). To identify whether thymic NK cells (identified as either $CD3^-NKp46^+$ 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)-Cre*^{TG} mice) were crossed to mice where 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 less than 8% of NK cells in the thymus of adult mice were progeny of *Rorc*-expressing progenitors (Figure 1A and supplemental Figure 2) indicating that the vast majority of these cells do not derive from DP cells. Moreover, only a small percentage (less than 3%) of thymic NK cells expressed intracellular CD3ε (Figure 1B). These data are inconsistent with the idea that thymic NK cells represent 'masquerading' $TCR\alpha\beta^+$ 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 (Figure 2A and (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 recombination activating genes (Rag)1 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. While 11% of DN1 cells, 80% of DN2 and all DN3 cells expressed high levels of GFP (Figure 2B), less than 1% of thymic NK cells were GFP^+ (Figure 2B). Moreover, the level of GFP expression by thymic NK cells was considerably lower compared to GFP^+ DN1 and DN2 cells (Figure 2B). These data indicate that essentially all thymic NK cells are not actively rearranging their antigen receptor loci, however, it can not 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 using freshly isolated splenic NK cell from B6 or *in vitro* expanded splenic NK cells from TCRβ$^{-/-}$δ$^{-/-}$ double-deficient mice only very few NK cells (about 1%) were found to have TCRγ rearrangements (4, 5). We therefore sorted single Lin^{-}NK1.1^{+}CD127^{+} thymic NK cells from CD3ε$^{-/-}$ mice (to avoid mature T cell contamination) and directly performed single-cell PCR to detect Vγ2-Jγ2 or Vγ4-Jγ1 rearrangements as these gene segments have been demonstrated to undergo the highest rate of rearrangements (25). Importantly, early T cell precursors from CD3ε$^{-/-}$ mice have been shown to undergo normal TCR rearrangements (17). We found 1/159 thymic NK cells had both Vγ2-Jγ2 and Vγ4-Jγ1 rearrangements, while none of the sorted thymic NK cells carried either only Vγ2-Jγ2 or only Vγ4-Jγ1 rearrangements. This represents a frequency of less than 1%, which is in agreement with the absence of Rag2 expression by thymic NK cells (Figure 2). As a control, we found that 30/30 single γδ T cells sorted from TCRβ$^{-/-}$ mice and 0/22 sorted splenic CD127^{-} 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 TCRγ locus, which is inconsistent with their development from aborted T cell precursors that had previously expressed Rag genes.

Thymic NK cells develop in the absence of Notch signalling

The transcription factor Notch plays an essential role in T cell development by instructing early lymphoid progenitors to adopt a T versus B cell fate (26). Notch signaling is critically dependent on the transcription factor RBPJ (26) and the absence of Notch1 or RBPJ has been shown to result in a complete absence of T cells (14, 15) due to an absence of the earliest T cell progenitors (ETPs) (8). We hypothesized that if thymic NK cells would derive from ETPs they should equally depend on Notch signalling for their development. We first determined whether thymic NK cells expressed any of the 4 Notch family members. We found that thymic NK cells as well as CD25^{+} DN thymocytes expressed Notch 1 and Notch 2 while only the latter population expressed Notch 3 (Figure 3A and data not shown). The expression of Notch-proteins by splenic CD127^{-}NK cells was similar to that observed on thymic NK cells (Figure 3A and data not shown). Thymic NK cells and CD25^{+} DN thymocytes expressed similar levels of Notch 2 at the cell surface while Notch 1 was expressed at around 10-fold higher levels by CD25^{+} DN thymocytes than by thymic NK cells (Figure 3A). These data suggested that thymic NK cells might derive from a Notch 1 and 2 expressing ETP. To assess the role for Notch signalling 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 Figure 3A and B). The numbers of splenic RBPJ-deficient and control $CD127^-CD3^-NKp46^+$ NK cells were comparable in the respective BM chimeras (*Rbpj*$^{-/-}$ NK cells: $1.4*10^5 \pm 7.5*10^4$ cells versus controls: $2.2*10^5 \pm 10^5$ 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). While thymic cellularity was comparable in both types of BM chimeras (*Rbpj*$^{-/-}$: $4.2*10^7 \pm 3.3*10^7$ versus controls: $3.5*10^7 \pm 2.1*10^7$) CD45.2 cells were clearly reduced in the absence of RBPJ (*Rbpj*$^{-/-}$ cells: $9.8*10^4 \pm 2.6*10^4$ versus controls: $2.1*10^6 \pm 2.9*10^6$). Nevertheless, thymic NK cells were present in normal percentages among total thymocytes when compared to control BM chimeras (Figure 3B) and their phenotype (Figure 3C) and absolute numbers (Figure 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). While $CD127^+$ NK cells can be generated from BM NKPs and ETPs *in vitro* using co-cultures 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*.

Concluding remarks

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 bi-potent NK/T progenitor present in the bone marrow (6). In contrast, DN2 thymocytes, while 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 physiologic conditions, as the latter can develop in absence of all T cell precursors and show little evidence of antigen-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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Footnotes

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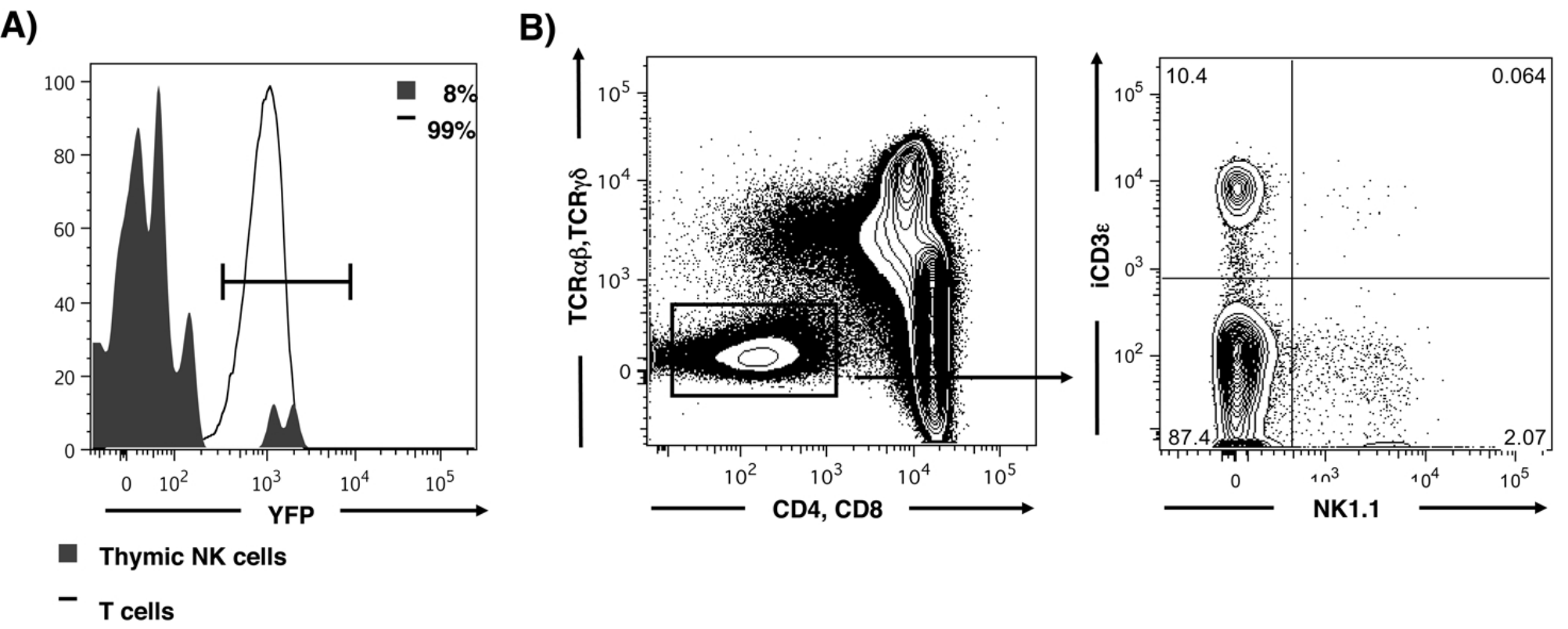
Figure Legends

Figure 1. *Rorc* expression 'fate mapping' and intracellular CD3ε expression by T cells and thymic NK cells. A) Expression of YFP by CD3+NKp46- (black line) and CD3-NKp46+ thymocytes (shaded grey) from *Rorc(t)*-Cre^{TG} ROSA-YFP mice. B) Viable thymocytes from adult C57BL/6 mice were stained with the indicated antibodies (left). Expression of intracellular CD3ε versus NK1.1 (middle) on gated cells as indicated on the left. Percentages indicate the frequencies of the gated cells.

Figure 2. Rag2-GFP expression by double-negative thymocytes and thymic NK cells. A) CD44 versus CD25 profile on gated $CD3^-CD4^-CD8^-Gr\text{-}1^-CD19^-$ thymocytes from adult Rag2-GFP BAC transgenic mice. The percentages give the frequencies of the double-negative (DN) subsets (DN1: $CD44^+CD25^-$; DN2: $CD44^+CD25^+$; DN3: $CD44^-CD25^+$; 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 Notch 1 (shaded grey) and Notch 2 (grey line) by $CD3^-CD4^-CD8^-Gr\text{-}1^-CD19^-CD122^+NKp46^+CD127^+$ (left) and $CD3^-CD4^-CD8^-Gr\text{-}1^-CD19^-CD122^-CD25^+$ thymocytes (middle) and $CD3^-CD4^-CD8^-Gr\text{-}1^-CD19^-CD122^-NKp46^+CD127^-$ splenocytes (right). Controls are in black (shaded). B) 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. C) Chimerism among $CD3^-NKp46^+$ thymocytes from the different BM chimeras (left: control; right: *Rbpj*-deficient) 12 weeks after reconstitution is given in percentages. D) Absolute numbers (mean and s.d.) of $CD45.2^+CD127^+$thymic NK cells in controls (littermate n=3) and $Rbpj^{-/-}$ BM chimeras (n=3). p>0,4. NS=not statistically significant.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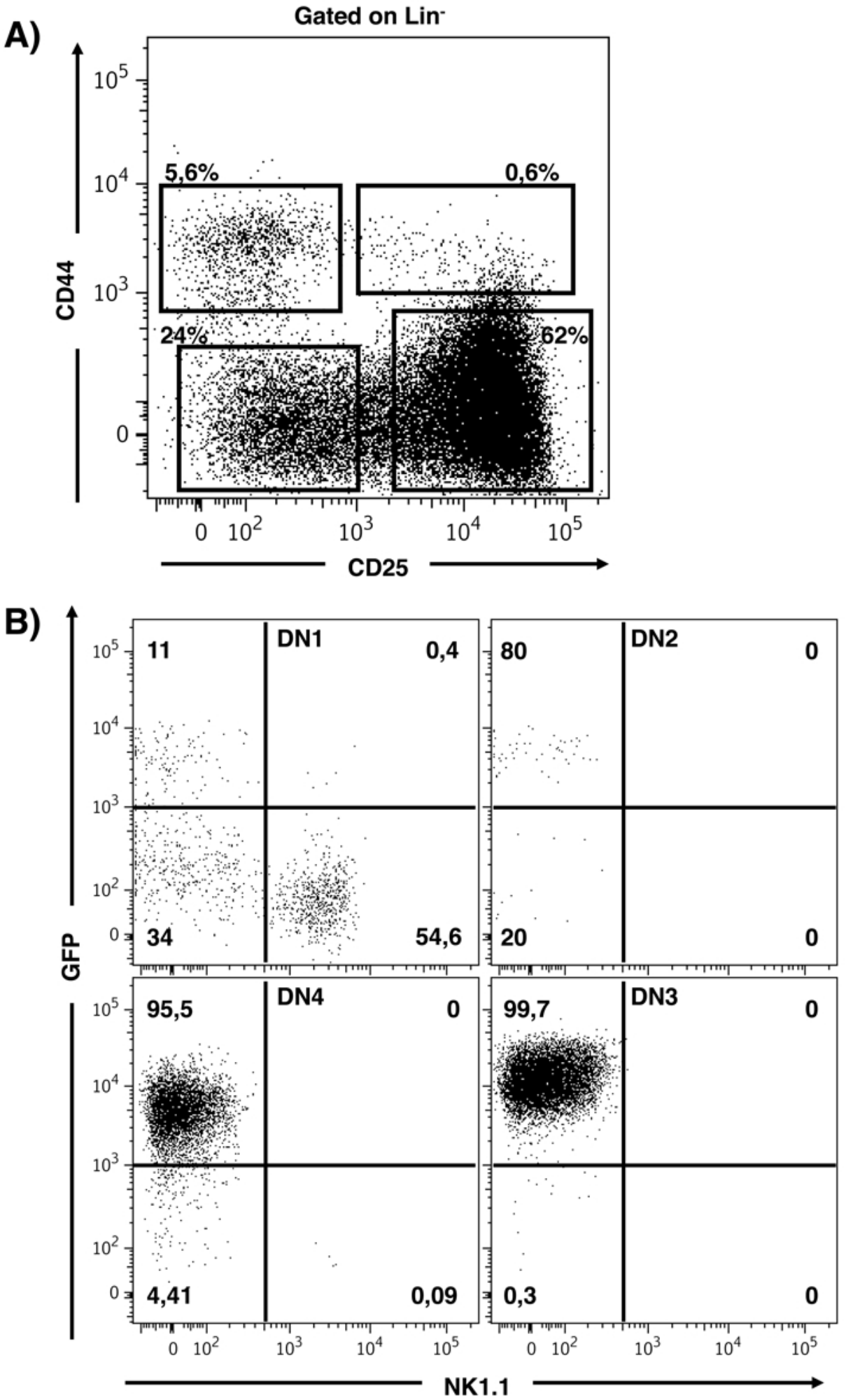
2.07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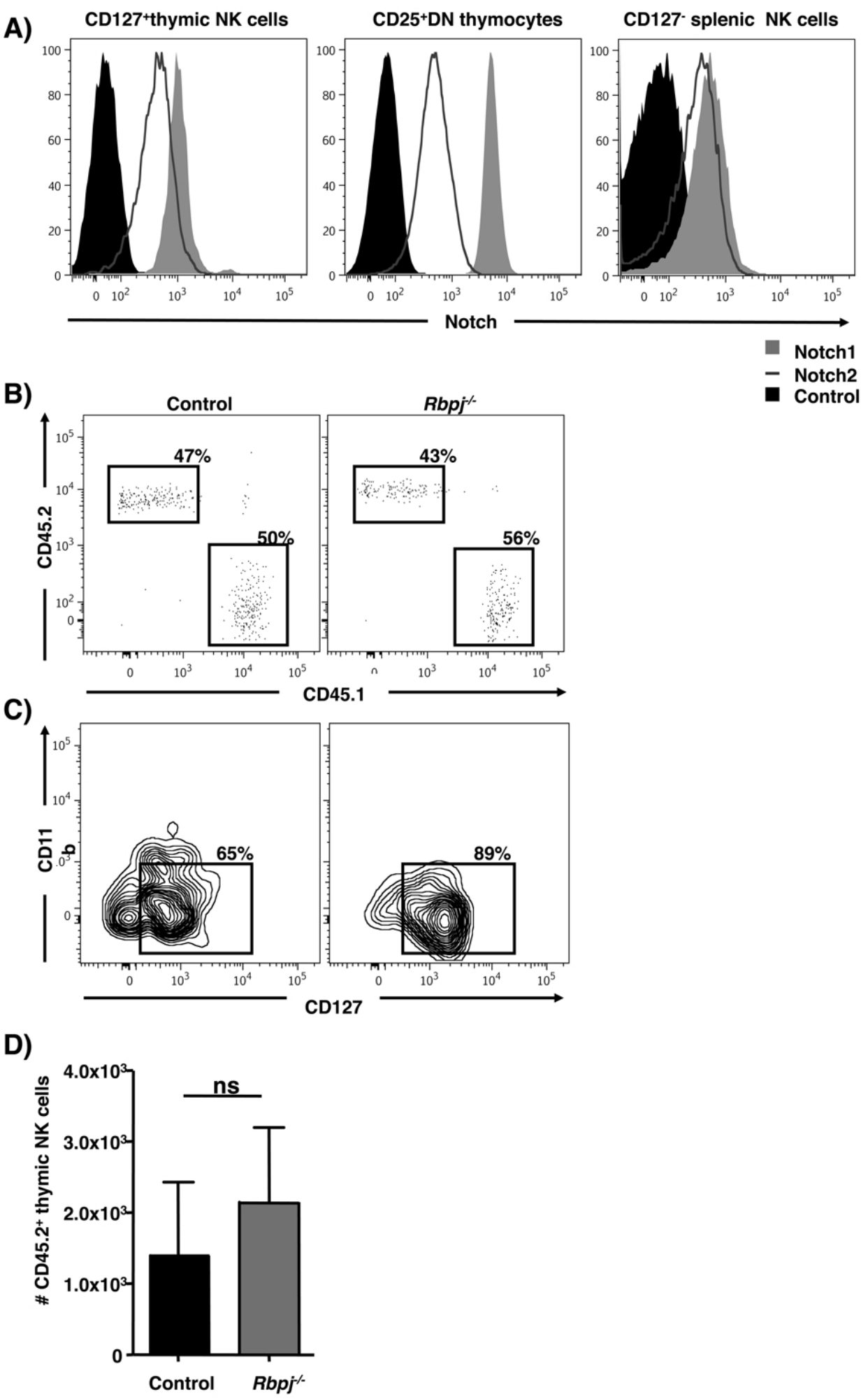
A)
CD127+thymic NK cells
CD25+DN thymocytes
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Control
B)
Control
Rbpj-/-
47%
50%
43%
56%
CD45.2
CD45.1
C)
65%
89%
CD11b
CD127
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CD45.2+ thymic NK cells
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Ribeiro *et al.* Supplemental Figure 1

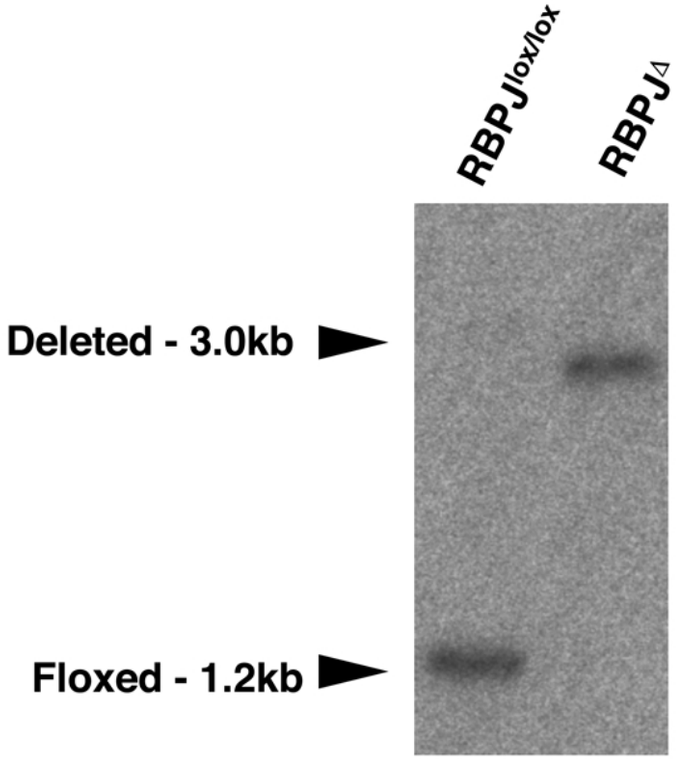

Figure 1. Conditional deletion of Rbpj in early hematopoietic precursors. A) Southern blot of whole bone marrow genomic DNA of control *Rbpj*$^{flox/flox}$ (left) and *Rbpj*$^{-/-}$ mice (right).

Ribeiro *et al.* Supplemental Figure 2

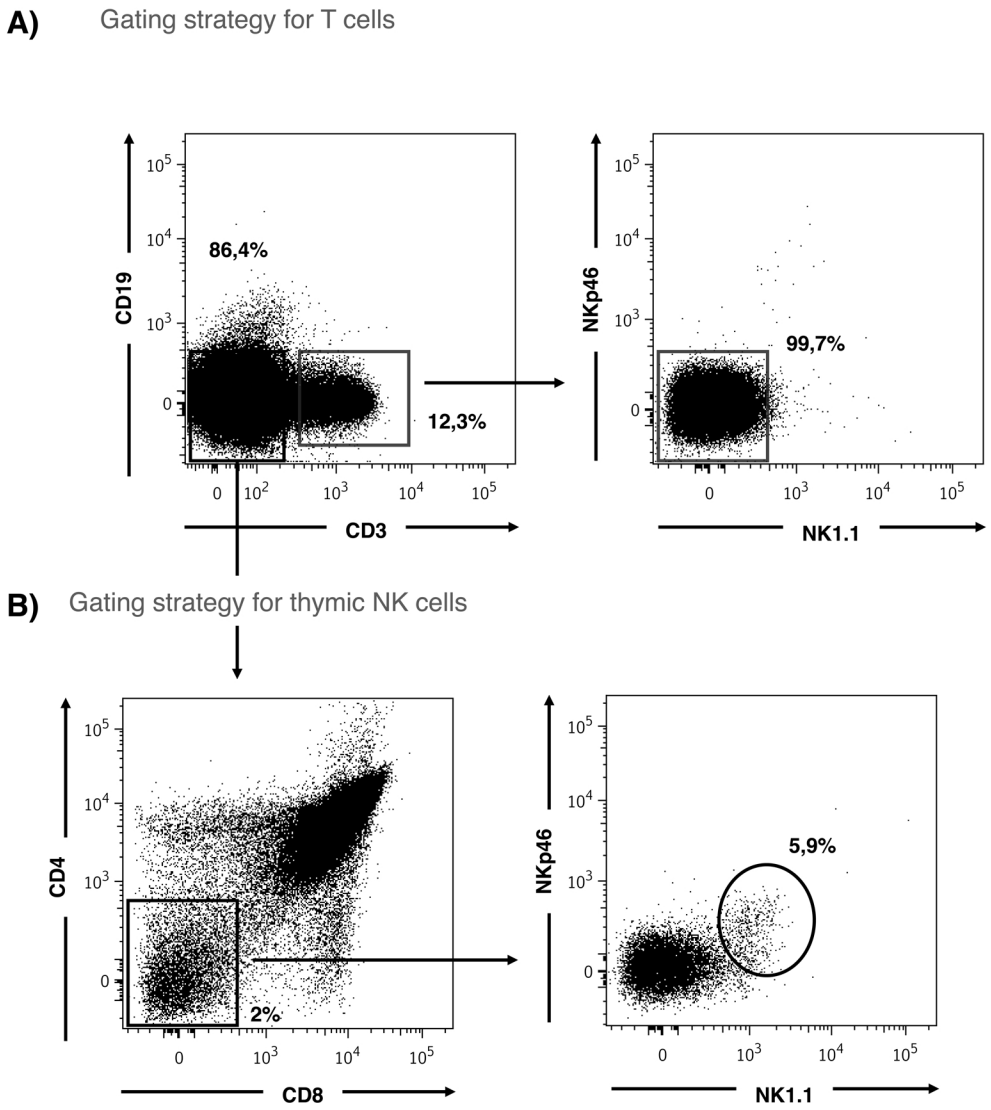

Figure 2. Gating strategies to identify CD3+NKp46- and CD3-NKp46+ thymocytes. Thymocytes were stained with the indicated antibodies. A) For the identification of CD3+NKp46- thymocytes cells were gated gated on CD19-CD3- cells (left) and then on NKp46-NK1.1- cells (right). B) For the identification of 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).