Journal of Autoimmunity 105 (2019) 102307

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

A novel spleen-resident immature NK cell subset and its maturation in a T-bet-dependent manner

Baohui Wang[a,b], Jing Zhou[a,b], Yawen Chen[a,b], Haiming Wei[a,b], Rui Sun[a,b], Zhigang Tian[a,b,**], Hui Peng[a,b,*]

[a] Division of Molecular Medicine, Hefei National Laboratory for Physical Sciences at Microscale, The CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences, University of Science and Technology of China, China
[b] Institute of Immunology, University of Science and Technology of China, China

ARTICLE INFO

Keywords:
Immature NK cell
Conventional NK cell
Tissue residency
T-bet

ABSTRACT

NK cells are thought to develop primarily in the bone marrow during adult life. However, increasing evidence shows that NK cell developmental intermediates can be found in different peripheral tissues with unique characteristics. Here, we identified a unique NK cell subset with the CD49a$^-$CD49b$^-$ phenotype in the spleen. These cells displayed an immature phenotype and weak abilities in cytotoxicity and cytokine production. Adoptive transfer experiments revealed that they could develop into mature conventional NK (cNK) cells. Transcriptome analysis further confirmed their immature features. Parabiosis experiments revealed that these cells maintained tissue-resident properties in the spleen. Moreover, T-bet deficiency intrinsically impaired the ability of these cells to develop into mature cNK cells. Thus, our study identified a spleen-resident immature NK cell subset that could undergo extramedullary maturation in a T-bet dependent manner.

1. Introduction

Natural killer (NK) cells are important innate lymphocytes, serving as the first line of defense against infection and cancer. After being activated, NK cells can secrete perforin and granzymes to initiate apoptosis of the target cells [1–3]. Death ligands on NK cells, e.g., FasL and TRAIL, can also induce cell apoptosis by interacting with their corresponding receptors on target cells [4,5]. Additionally, NK cells also secrete cytokines, notably IFN-γ, and participate in shaping both innate and adaptive immunity [6–8].

NK cells are widely distributed all around the body, consisting of phenotypically and functionally distinct subsets, and the composition of NK cell subsets varies with tissue distribution. It is generally accepted that conventional NK (cNK) cells are generated mainly in the bone marrow and migrate through the circulation to peripheral tissues. In the hematopoietic lineage hierarchy, NK cell precursors (NKPs) represent the branching point of NK cell- and other lymphocyte-lineages. Although the precise phenotypic characteristics of NKPs is still controversial, it is believed that NKPs are within the cell population that lack linage markers, including pan-NK cell markers such as NK1.1 and NKp46, but possess CD122 expression [9–12]. NKPs give rise to immature NK (iNK) cells following acquisition of pan-NK cell markers [9]. iNK cells further undergo progressive phenotypic changes in expression of multiple surface molecules, such as CD49b, CD11b, CD27, Ly49s and KLRG1, and functional maturation to become mature NK cells [13–16].

CD49b$^-$ NK cells have long been regarded as iNK cells, as they could give rise to CD49b$^+$ NK cells and acquire other NK cell maturation markers under certain conditions [17–19]. However, recent studies found that the liver CD49b$^-$ NK cells represent a developmentally distinct lineage from cNK cells and could be distinguished from cNK cells by their positive expression of CD49a [20,21]. These CD49a$^+$CD49b$^-$ NK cells were liver-resident and did not convert into CD49b$^+$ cNK cells at steady state [20]. CD49a$^+$CD49b$^-$ tissue-resident NK are also found in other tissues such as uterus and skin, in addition to the liver [21]. Meanwhile, a common progenitor to all helper-like innate lymphoid cell (ILC) lineages has been defined, which could give rise to type 1 ILCs (ILC1s) including liver CD49a$^+$CD49b$^-$ NK cells and small intestine ILC1s with low CD49b expression, but do not develop into cNK cells [22]. In addition, the progenies of bone marrow CD49b$^-$ NK cells include both cNK cells and ILC1s [12]. Thus, the "CD49b$^-$"

Abbreviations: NK, natural killer; cNK, conventional NK; NKP, NK cell precursor; iNK, immature NK; DN NK, double negative NK; ILC, innate lymphoid cell; MNC, mononuclear cell; TLR, toll-like receptor

* Corresponding author. School of Life Sciences, University of Science and Technology of China, 443 Huang-Shan Road, Hefei, Anhui, 230027, China.
** Corresponding author. School of Life Sciences, University of Science and Technology of China, 443 Huang-Shan Road, Hefei, Anhui, 230027, China.
E-mail addresses: tzg@ustc.edu.cn (Z. Tian), huipeng@mail.ustc.edu.cn (H. Peng).

https://doi.org/10.1016/j.jaut.2019.102307
Received 2 April 2019; Received in revised form 15 July 2019; Accepted 17 July 2019
Available online 24 July 2019
0896-8411/

phenotype cannot precisely define the iNK cells. How to phenotypically distinguish iNK cells from ILC1s need to be determined.

Although bone marrow is considered the major site for NK cell generation, increasing evidence has shown that NK cells could develop in the peripheral organs, such as liver [20], thymus [23,24] and lymph nodes [25]. As an important secondary lymphoid organ, the spleen contains a large number of NK cells [26]. Moreover, spleen stromal cells express IL-15 [27], a cytokine essential for NK cell development, thus providing necessary signals in promoting NK cell development. Consistent with this, spleen fibroblasts could promote the NK cell development from blood progenitors via producing IL-15 [28]. Despite these findings, few studies focused on the NK cell extramedullary development in the spleen. In this study, we found an iNK cell population with tissue-resident features in the spleen. These iNK cells were $CD49a^-CD49b^-$ and could develop into mature cNK cells via a T-bet dependent manner.

2. Methods

2.1. Mice

Mice were bred in a specific-pathogen-free facility in accordance with the guidelines for the use of experimental animals at the University of Science and Technology of China. Wild-type C57BL/6 (WT B6, $CD45.2^+$) mice were bought from the Shanghai Experimental Animal Center (Shanghai, China). $CD45.1^+$ mice (B6 background) and *Tbx21*$^{-/-}$ mice (T-bet deficient, B6 background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). *Tbx21*$^{-/-}$, *Tbx21*$^{+/-}$, *Tbx21*$^{+/+}$, and $CD45.1^+CD45.2^+$ mice were bred in house. Age- and gender-matched 6-12-week-old mice were used for experiments.

2.2. Cell preparation

Liver mononuclear cells (MNCs) were obtained via the 40%–70% percoll density gradient centrifugation method as previously described [29]. The spleen was pressed and washed through a 200-gauge mesh. The cell suspension was centrifugated, and then the splenocytes were collected after lysing red blood cells. To collect BM cells, a femur of each mouse was flushed, followed by lysing red blood cells. Peripheral blood MNCs were prepared by lysing red blood cells. Inguinal lymph node cells were collected by pressing and washing the lymph nodes through a mesh. The lung MNCs were obtained as described previously [30]. Briefly, the minced lung was digested at 37 °C for 60 min in DMEM medium (Hyclone, South Logan, UT, USA) with 0.1% collagenase I (Sigma–Aldrich, Saint Louis, MO, USA) and 5% fetal calf serum; the supernatant was collected and the MNCs were obtained by percoll gradient centrifugation.

2.3. Cell purification and adoptive transfer

Splenocytes were incubated successively with biotin-NKp46 antibodies (Abs) and anti-biotin microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and then spleen $NKp46^+$cells were enriched by using magnetic cell sorting (MACS) columns (Miltenyi Biotech). Next, spleen $NKp46^+$cells were incubated with fluorescent Abs, and a FACS Aria cell sorter (BD Biosciences, San Jose, CA, USA) was used to purify spleen $CD49a^-CD49b^-$ NK cells and spleen $CD49a^-CD49b^+$ cNK cells. The purity was above 95%, as detected by post-sort flow cytometry. Purified cells were adoptively transferred into sublethally irradiated recipient mice (6.5 Gy irradiated, 1 day before transfer) via tail-vain injection.

2.4. Antibody staining and flow cytometry

Monoclonal Abs (mAbs) against CD49a (Ha31/8), CD49b (DX5), CD107a (1D4B) and IFN-γ (XMG1.2) were purchased from BD Biosciences. mAbs against Eomes (Dan11mag), KLRG1(2F1), perforin (eBioOMAK-D) and granzyme B (16G6) were purchased from eBioscience (San Diego, CA, USA). mAbs against CD3ε (145-2C11), CD11b (M1/70), CD19 (1D3/CD19), CD27 (LG.3A10), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD62L (MEL-14), NK1.1 (PK136), NKp46 (29A1.4) and T-bet (4B10) were purchased from BioLegend (San Diego, CA, USA). Isolated cells were first incubated with rat serum to block Fc receptors, and then stained with fluorescently labeled mAbs against surface molecules. For intracellular transcription factor staining, cells were fixed, permeabilized (using a FoxP3/Transcription Factor Buffer Set; eBioscience) after staining for surface molecules, and then stained with mAbs against the intracellular molecules. For intracellular staining of IFN-γ, granzyme B and perforin, cells were stimulated with 30 ng/ml PMA (CalBioChem), 1 μg/ml ionomycin (Sigma–Aldrich) and 2.5 μg/ml monensin (Sigma–Aldrich) for 4 h at 37 °C. Then the cells were fixed, permebilized and stained for the intracellular molecules. For CD107a staining, anti-CD107a mAb was added at the beginning of the stimulation. All data were collected using the flow cytometers (LSR II, LSRFortessa X-20, FACS Aria; BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Parabiosis

WT ($CD45.2^+$) and $CD45.1^+$ B6 mice (age- and size-matched) were parabiosed for 4 weeks, according to a published protocol [31]. The mice were co-housed before operations. First, we made longitudinal skin incisions to the shaved lateral aspect of each mouse. Then, we used surgical sutures to join the mice with knots at the elbow and knee, and the wound was closed with surgical sutures. Parabiotic mice were treated with supplementary antibiotics (Sulfatrim) in drinking water after surgery.

2.6. RNA sequencing and analysis

RNA was isolated from sorted splenic $CD49a^-CD49b^-$ NK cells and $CD49a^-CD49b^+$ cNK cells using Direct-zol™ RNA Miniprep (Zymo Research Corp) and then sequenced by Illumina Novaseq™ 6000. To analyze the sequencing data, we aligned reads of samples to the reference genome (ftp://ftp.ensembl.org/pub/release-90/fasta/mus_musculus/) using HISAT package. The mapped reads of each sample were assembled using StringTie. Then all transcriptomes from the samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and edgeR was used to estimate the expression levels of all transcripts. StringTie was used to perform expression level for mRNAs by calculating FPKM. The differentially expressed mRNAs and genes were selected with log2 (fold change) ≥ 1 or log2 (fold change) ≤ -1 and with statistical significance (P value < 0.05) by R package.

The RNA-sequencing data have been deposited in NCBI's Gene Expression Omnibus and are accessible through accession number GEO: GSE133950 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE133950).

2.7. Statistics

Statistical significance of differences was determined by Student's t tests for 2 groups. P values less than 0.05 were considered significant.

3. Results

3.1. A unique $CD49a^-CD49b^-$ NK cell subset in the spleen

The $CD45^+CD3^-CD19^-NK1.1^+NKp46^+$ cells ($NK1.1^+NKp46^+$ cells) were previously defined as NK cells. However, with the in-depth study in recent years, in this group of cells, CD49a becomes the marker of tissue-resident NK cells and ILC1s, while CD49b is the marker of cNK

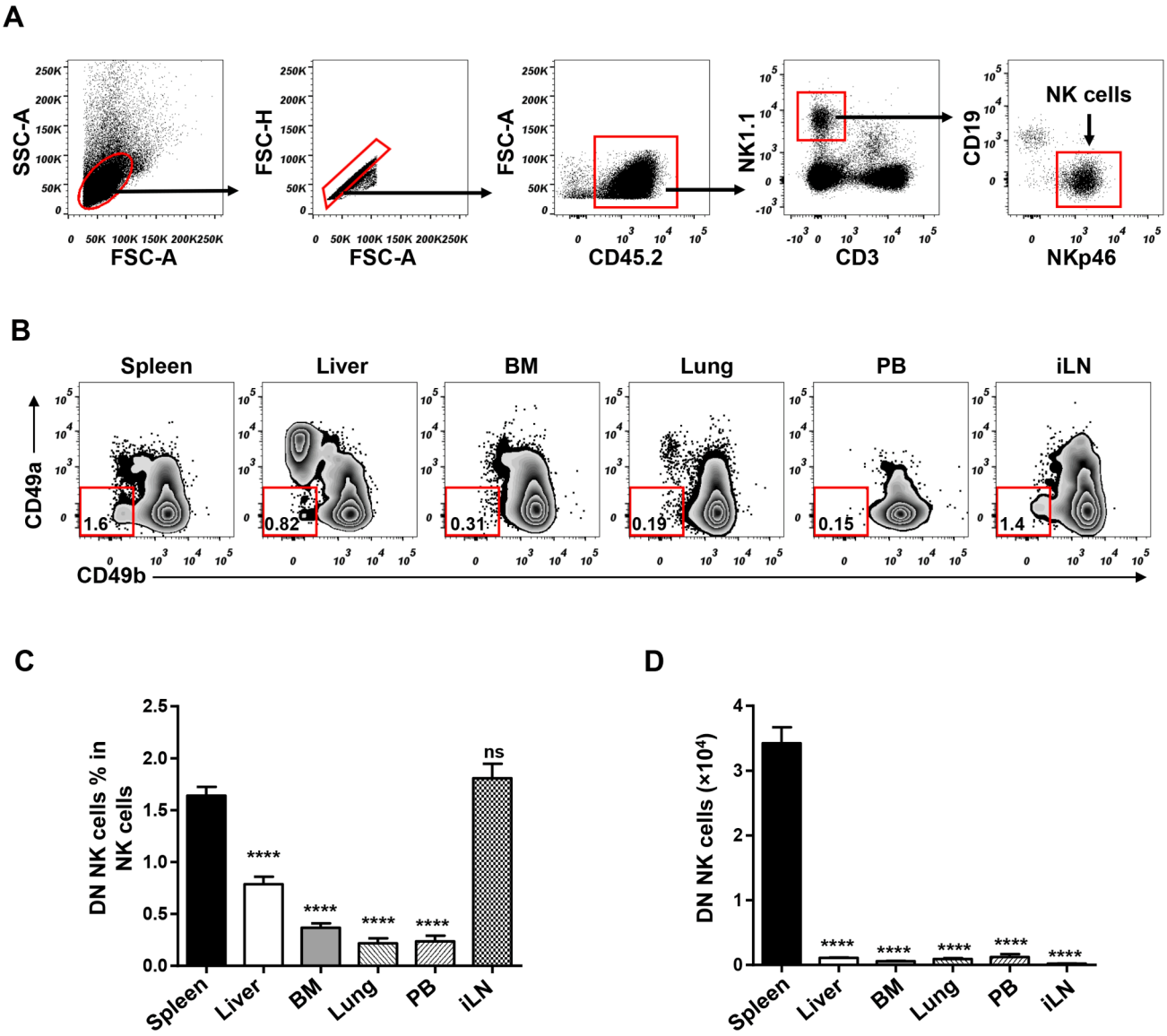
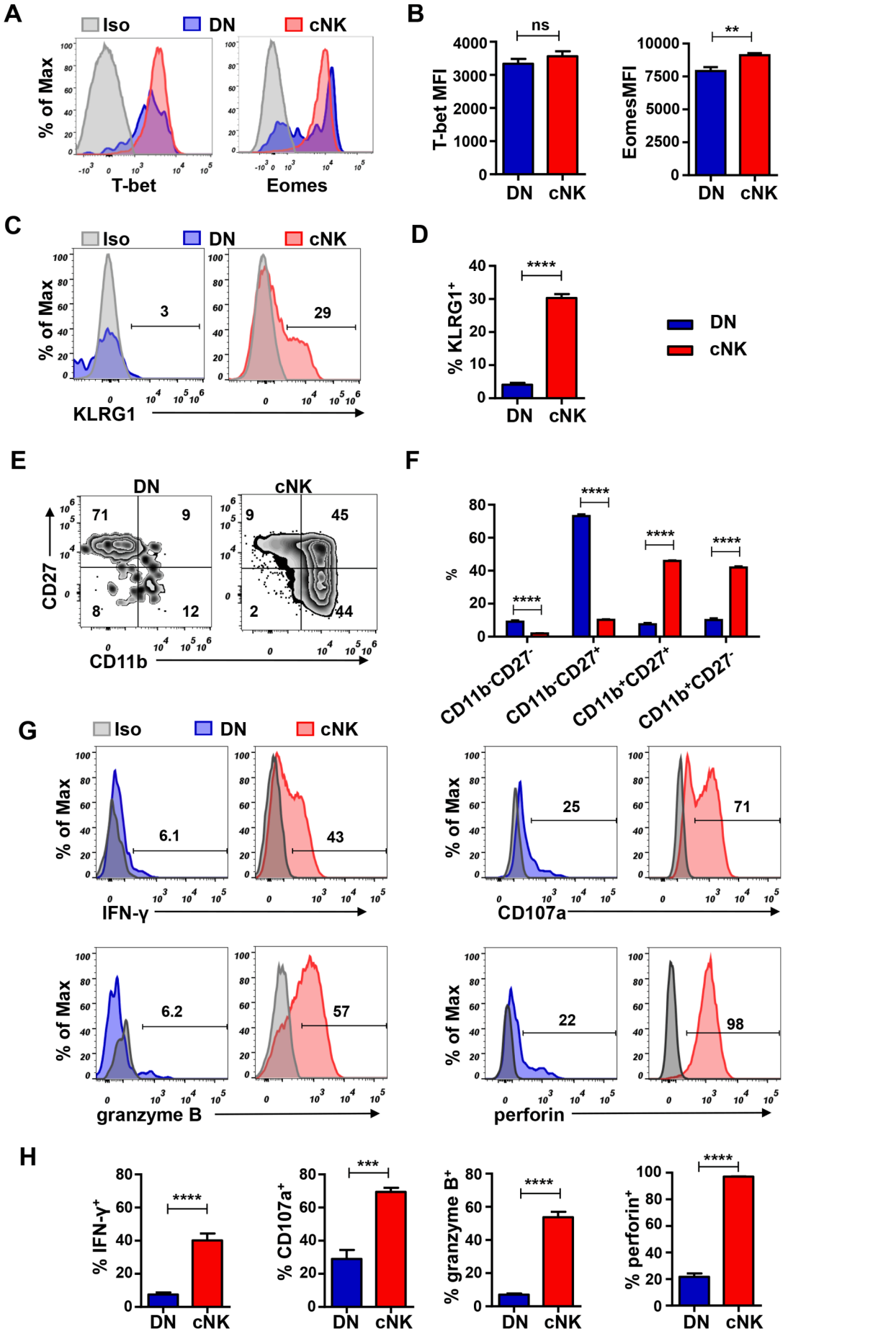


Fig. 1. Abundant DN NK cells exist in murine spleen. (A) The gating strategy of NK cells. The NK cells were gated as $CD45^+CD3^-CD19^-NK1.1^+NKp46^+$ cells. (B) Representative flow cytometry plots showing expression of CD49a and CD49b on NK cells in the liver, spleen, bone marrow (BM), lung, peripheral blood (PB) and inguinal lymph node (iLN) of WT C57BL/6 (B6) mice. (C-D) The percentages (C) and numbers (D) of DN NK cells in different organs are shown (n = 4–5 mice per group). BM: cell numbers in one femur; PB: cell numbers in 1 ml blood. All plots are representative of two or three independent experiments. Means ± SEM are shown (unpaired two-tailed Student's *t*-test; ns, not significant; $^{****}P < 0.0001$; *P* values were calculated between the indicated group and the spleen group).

cells. Here, we examined the expression of CD49a and CD49b on $NK1.1^+NKp46^+$ cells from different organs of WT mice (Fig. 1A and B). Consistent with previous findings, the $CD49a^-CD49b^+$ subset, defined as cNK cells, was abundant in all examined organs (Fig. 1B) [20]. The $CD49a^+CD49b^-$ subset was enriched in the liver, defined as liver-resident NK cells [20]. We noticed that there was a small subset in $NK1.1^+NKp46^+$ cells: $CD49a^-CD49b^-$ cells, which we defined as double negative (DN) NK cells. By comparing the frequency and absolute numbers of DN NK cells in different tissues, we found that the frequency of splenic DN NK cells within NK cells is higher than that of their counterparts in the liver, BM, lung and peripheral blood, but comparable to that of lymph node DN NK cells (Fig. 1C). The total count of DN NK cells in the spleen is much higher than that in the lymph node and other tissues (Fig. 1D), suggesting a relative abundance of DN NK cells in the spleen.

To identify the development stage of the splenic DN NK cells, we further analyzed the expression of transcription factors and surface markers. Transcription factors T-bet and Eomes are master regulators of NK cell development, maturation, and function [32]. We compared the expression of T-bet and Eomes between splenic DN NK cells and cNK cells (Fig. 2A and B). Both DN NK cells and cNK cells were T-bet positive, but MFI of Eomes in DN NK cells was lower than that in cNK cells, due to a small fraction of DN NK cells were Eomes negative. The expression of KLRG1 represents a terminal stage of NK cell differentiation associated with reduced proliferation and effector functions [15]. Interestingly, DN NK cells were mostly negative for KLRG1 (Fig. 2C and D). CD11b and CD27 constituted a classical 4-stage model of NK-cell maturation: $CD11b^{low}CD27^{low} \rightarrow CD11b^{low}CD27^{high} \rightarrow CD11b^{high}CD27^{high} \rightarrow CD11b^{high}CD27^{low}$ [16]. We also found that DN NK cells were mainly $CD11b^{low}CD27^{high}$ (Fig. 2E and F). These data suggest that the murine splenic DN NK cells are phenotypically immature.

We next sought to compare the effector function of DN NK and cNK cells. Upon PMA and ionomycin stimulation, splenic DN NK cells produced significantly less IFN-γ, and expressed markedly lower levels of cytotoxic molecules, including granzyme B and perforin, and the



(caption on next page)

Fig. 2. Splenic DN cells are phenotypically and functionally immature. (A) Representative histograms of T-bet (left) and Eomes (right) staining in DN NK (blue) and cNK (red) cells. (B) The mean fluorescence intensity (MFI) of T-bet and Eomes in DN NK and cNK cells was quantified (n = 5 mice per group). (C) Representative histograms of the maturation marker KLRG1 expression on DN NK (blue) and cNK (red) cells. (D) The percentages of KLRG1$^+$ cells among the indicated NK cell subsets are shown (n = 5 mice per group). (E) Representative flow cytometric analysis of CD27 and CD11b expression by splenic DN NK and cNK cells. (F) Frequency of the indicated NK cell subsets in DN NK and cNK cells (n = 5 mice per group). (G) Representative histograms of IFN-γ, CD107a, Granzyme B and perforin staining in DN NK (blue) and cNK (red) cells. (H) The percentages of cells expressing the indicated molecules were quantified (n = 5 mice per group). All plots are representative of two or three independent experiments. Means ± SEM are shown (unpaired two-tailed Student's *t*-test; ns, not significant; ***$P < 0.001$; ****$P < 0.0001$).

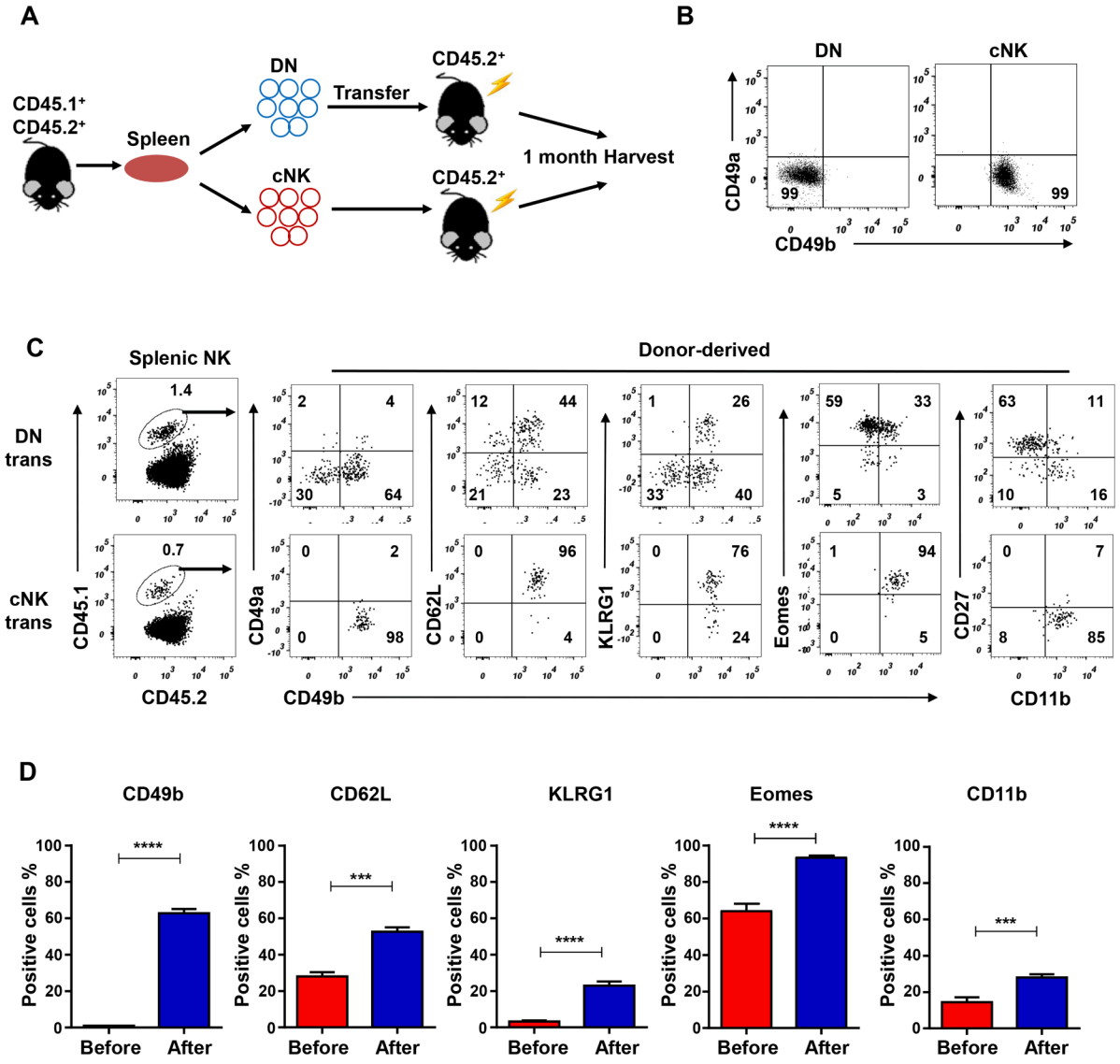


Fig. 3. Splenic DN NK cells can differentiate into mature cNK cells after adoptive transfer. (A) Schematic of experiment. Sublethally irradiated CD45.2$^+$ B6 mice received 2 × 10^5 splenic DN NK or cNK cells from CD45.1$^+$CD45.2$^+$ mice. One month after transfer, the recipient spleens were harvested. (B) CD49b and CD49a expression on sorted DN NK and cNK cells before transfer is shown. (C) Representative flow cytometry plots showing the expression of cell surface markers and the transcription factor Eomes on donor-derived (CD45.1$^+$CD45.2$^+$) NK cells in the recipient spleen. (D)The percentages of cells expressing the indicated molecules among donor-derived cells (before transfer: red; after transfer: blue). Data are representative of two independent experiments, with n = 3–5 mice per group. Means ± SEM are shown (unpaired two-tailed Student's *t*-test; ***$P < 0.001$; ****$P < 0.0001$).

degranulation marker CD107a, as compared to cNK cells (Fig. 2G and H). These results suggest that splenic DN NK cells are not functionally mature.

3.2. Splenic CD49a$^-$CD49b$^-$ NK cells give rise to mature cNK cells

Since splenic DN NK cells were phenotypically and functionally immature, we wondered whether they could develop into mature cNK cells. We adoptively transferred splenic DN NK and cNK cells from CD45.1$^+$ mice into sublethally irradiated CD45.2$^+$ mice, which were harvested one month later (Fig. 3A and B). We found that donor-derived DN NK cells could migrate back to the spleen. About 60% of donor-derived DN NK cells converted into CD49b$^+$ cNK cells while the remaining cells maintained CD49a$^-$CD49b$^-$ phenotype. Nearly all donor-derived cNK cells still kept CD49a$^-$CD49b$^+$ phenotype (Fig. 3C and D). Furthermore, the donor-derived DN NK cells up-regulated the expression of NK cell maturation markers, including the surface molecules CD62L, KLRG1 and CD11b and the transcription factor Eomes, in the recipient spleen after transfer (Fig. 3C and D). These data indicate that the DN NK cells are at the immature stage during cNK cell

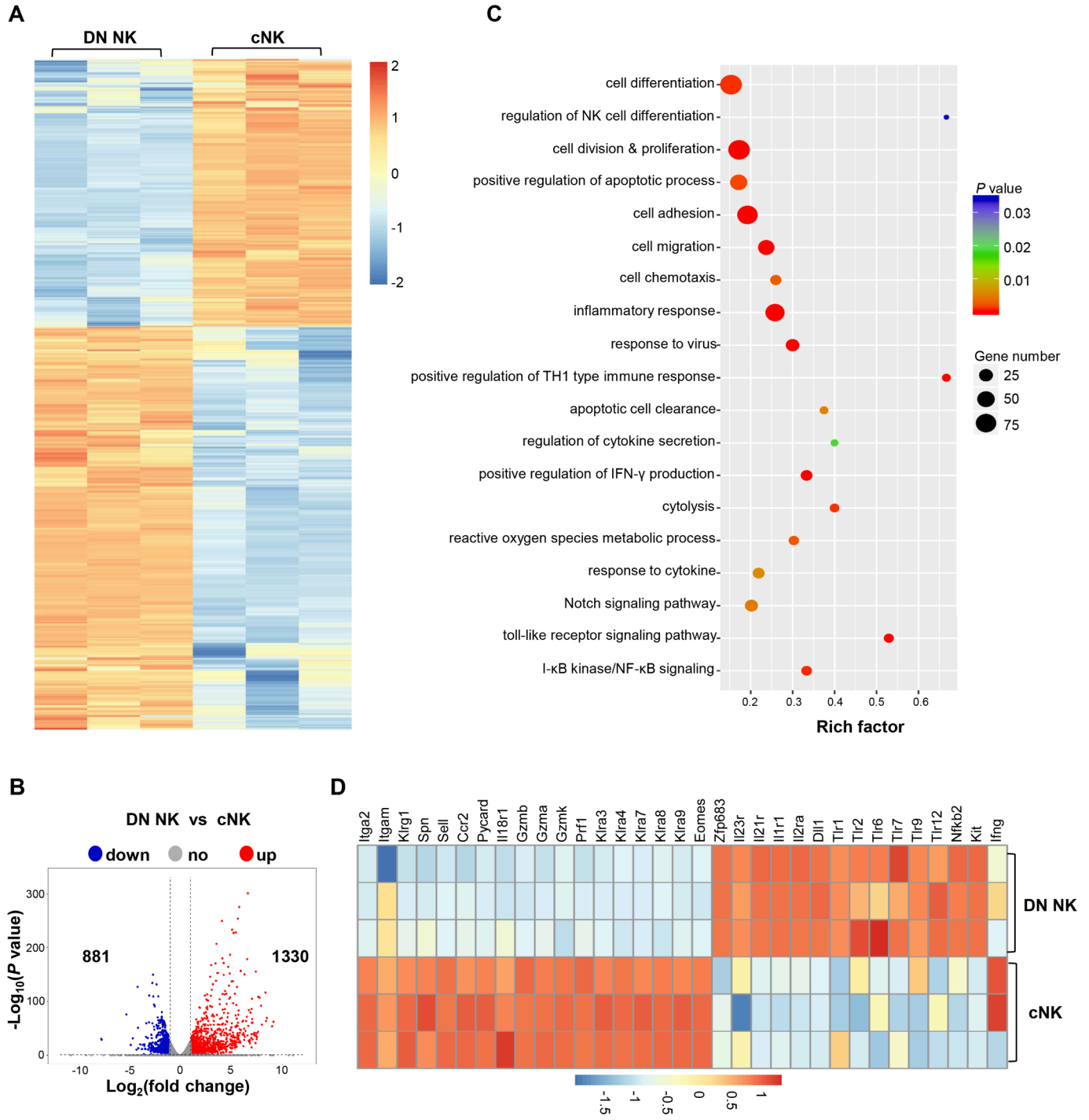


Fig. 4. Comparative transcriptome analysis of splenic DN NK cells and cNK cells. (A) Heat maps of the differentially expressed genes in splenic DN NK cells and cNK cells. The differentially expressed genes were selected with log2 (fold change) ≥ 1 or log2 (fold change) ≤ −1 and with statistical significance (*P* value < 0.05). (B) Comparative transcriptome analysis between splenic DN NK cells and cNK cells using RNA sequencing. The differentially expressed genes are highlighted in red (up) or blue (down). The numbers represent the number of up-regulated genes (left) or down-regulated genes (right). (C) Gene Ontology (GO) analysis of the differentially expressed genes. (D) Heat maps of the selected genes.

development process and have the potential to develop into mature cNK cells.

3.3. Splenic CD49a^{-}CD49b^{-} NK cells are transcriptionally different from cNK cell

To deeply understand the differences between splenic DN NK and cNK cells, we conducted high-throughout RNA sequencing. Comparative transcriptome analysis of DN NK and cNK cells revealed that there were 2211 differentially expressed genes (fold change ≥ 2 and *P* value < 0.05), among which 1330 genes were up-regulated in DN NK cells (Fig. 4A and B). The differentially expressed genes included those involved in cell differentiation, responses to stimuli, cell trafficking, cell proliferation and effector functions (Fig. 4C).

Consistent with our findings that DN NK cells are relatively immature, DN NK cells had lower expression of *Itga2* (CD49b), *Itgam*

(CD11b), *Klra3* (Ly49C), *Klra4* (Ly49D), *Klra7* (Ly49G), *Klra8* (Ly49H), *Klra9* (Ly49I), and *Eomes*, but higher expression of *Kit* (c-kit) (Fig. 4D). In accordance with the flow cytometric results (Fig. 2G and H), cytolysis genes such as *Gzmb*, *Prf1*, *Gzma*, and *Gzmk* were down-regulated in DN NK cells (Fig. 4C and D). Moreover, DN NK cells had lower expression of *Il18r1* (Fig. 4D), which might be a factor influencing their IFN-γ production [33,34]. We also observed that *Il1r1*, *Il2ra, Il21r*, *Il23r*, and genes encoding toll-like receptors (TLRs) were expressed at higher levels in DN NK cells than in cNK cells (Fig. 4D), implying that DN NK cells might be more sensitive to certain cytokine stimuli and respond directly to pathogen components. Notably, in comparison with cNK cells, DN NK cells expressed higher levels of *Zfp683* (Hobit), a transcription factor critical for tissue-resident lymphocytes [35], and lower levels of *Sell* (CD62L) and *Ccr2*, suggesting that these two populations may differ in migratory properties.

3.4. Splenic CD49a$^-$CD49b$^-$ NK cells are tissue-resident

Since DN NK cells were rarely found in other tissues (Fig. 1D), and they had a distinct transcription profile impacting their trafficking compared to cNK cells (Fig. 4C and D), we wondered whether DN NK cells do or not move out from the spleen. To address this, we analyzed splenic DN NK cells in congenic CD45.1$^+$ and CD45.2$^+$mice that were surgically joined by parabiosis (Fig. 5A). Surprisingly, we found that DN NK cells expressing CD45.1$^+$ were confined to the CD45.1$^+$spleens, which contained few DN NK cells from the CD45.2$^+$ parabiont, and vice versa (Fig. 5B and C). Nevertheless, cNK cells from each parabiont were found in the spleens of both parabionts (Fig. 5B and C), consistent with previous reports [20,21]. These results indicate that the majority of murine splenic DN NK cells reside in the spleen and do not participate in the peripheral circulation.

3.5. T-bet intrinsically drives maturation of splenic CD49a$^-$CD49b$^-$ NK cells

Previous reports demonstrated the decrease in number of peripheral cNK cells when T-bet was deficient [18,36], but the mechanism is not fully understood. Considering high expression of T-bet on DN NK cells, we wonder whether T-bet affects the developmental potential of DN NK cells. Here, we noticed that the percentage and the number of murine splenic DN NK cells were significantly increased in T-bet-deficient mice, while the splenic cNK cell number was reduced (Fig. 6A, C, D and E). In accordance with previous reports [37], the percentage of terminally mature cells (CD11b$^+$KLRG1$^+$ cells) declined in cNK cells (Fig. 6B and F). These data suggest that T-bet deficiency may impede the development of DN NK cells into mature cNK cells.

To confirm this, we respectively sorted splenic DN NK cells from CD45.1$^+$ WT mice and CD45.2$^+$ T-bet-deficient mice, mixing them at a ratio of 1:1, and then adoptively transferred them into sublethally irradiated CD45.1$^+$CD45.2$^+$ recipient mice (Fig. 7A). One month after transfer, T-bet-deficient donor cells mostly remained the CD49a$^-$CD49b$^-$ phenotype while about half of the WT donor cells converted into CD49a$^-$CD49b$^+$ cNK cells (Fig. 7B and C). In contrast to the finding that WT donor cells substantially became KLRG1$^+$CD11b$^+$, T-bet-deficient donor cells could hardly generate these terminally mature cells (Fig. 7B and D). These data indicate that T-bet deficiency impedes the maturation process of splenic DN NK cells converting into mature cNK cells.

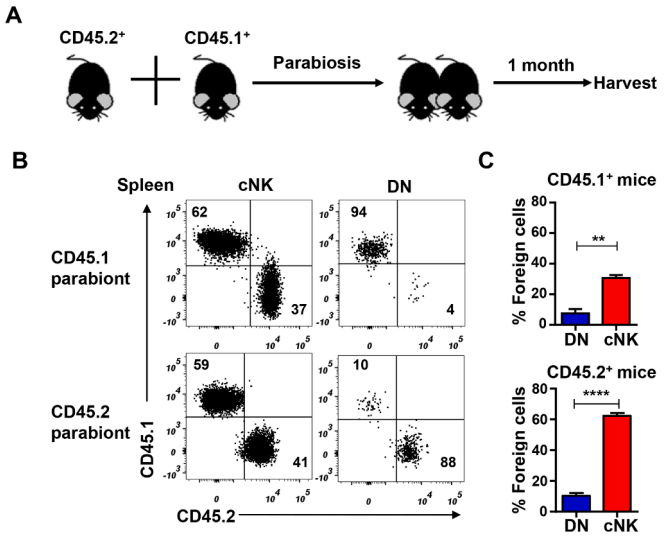


Fig. 5. Splenic DN NK cells rarely exchange between parabiotic mice. (A) Schematic of experiment. WT B6 (CD45.2$^+$) mice were parabiosed to congenic B6-CD45.1$^+$ mice. 4 weeks later, the spleen was harvested. (B) Representative dot plots show the expression of CD45.1 and CD45.2 on splenic DN NK and cNK cells from each parabiont as indicated. (C) The percentages of foreign–derived cells among the splenic DN NK (blue) and cNK (red) cells in the parabiotic CD45.1$^+$ (left) or CD45.2$^+$ mice (right). Data are representative of three pairs of parabiotic mice. Means ± SEM are shown (unpaired two-tailed Student's *t*-test; $^{**}P < 0.01$; $^{****}P < 0.0001$).

4. Discussion

NK cells are a heterogeneous population. Bulk NK cells that have been extensively studied actually represent a mixture of distinct cell subsets. For example, bulk NK cells in the liver are divided into cNK cells and liver-resident NK cells [20,21]. The picture is more complicated in the intestine, where NK1.1$^+$NKp46$^+$ cells are comprised of cNK cells, ILC1s and NKp46$^+$ ILC3s [38]. Generally, ILC1s express low levels of cNK cell maturation-associated molecules, such as CD49b, CD11b and Ly49s, but express relatively high levels of CD49a, CD69 and CD200R [39,40]. In this study, we found that splenic CD49b$^-$ NK cells, unlike their counterparts in the liver, were substantially CD49a negative. Since these DN NK cells express the pan-NK cell markers NK1.1 and NKp46, they are likely to lie downstream of NKPs. Compared to CD49b$^+$ cNK cells, they displayed an immature phenotype, and were functionally incompetent as evidenced by their weak abilities in IFN-γ production and cytotoxicity. After adoptive transfer, splenic DN NK cells could acquire CD49b expression and up-regulate expression of NK cell maturation-markers including CD11b, CD62L and KLRG1, but remained CD49a negative in the spleen. These findings suggest that splenic DN NK cells represent iNK cells.

Although splenic DN NK cells display poor effector functions, they have more abundant transcripts for some inflammatory cytokine receptors and TLRs, as compare to cNK cells, suggesting that they might be sensitive to pathogen and cytokine stimuli. In fact, we observed an expansion of DN NK cells in the spleen following lymphocytic choriomeningitis virus infection (data not shown). Since previous studies have shown that TLR3 ligand stimulation induces NK cell egress from the spleen to inflamed tissues [41], it raises a possibility that the differentiation of splenic DN NK cells may replenish the pool of cNK cells during infection.

Tissue-resident features of lymphocytes have attracted much interest of many investigators during recent years. Tissue-resident lymphocytes share a number of characteristics, including high expression of several surface molecules associated with lymphocyte retention within tissues [42]. Splenic DN NK cells lack expression of the tissue-resident markers CD49a and CD103, and only a few of them express CD69 or CXCR6 (data not shown). However, parabiosis experiments revealed that they were mostly host-derived, thus suggesting their tissue-resident feature. Transcriptome analysis of DN NK and cNK cells also revealed that many differentially expressed genes were associated with cell adhesion and migration, which may provide clues to molecular mechanisms underlying retention of DN NK cells in the spleen. Tissue residency of splenic DN NK cells raises a question about the origin of these cells.

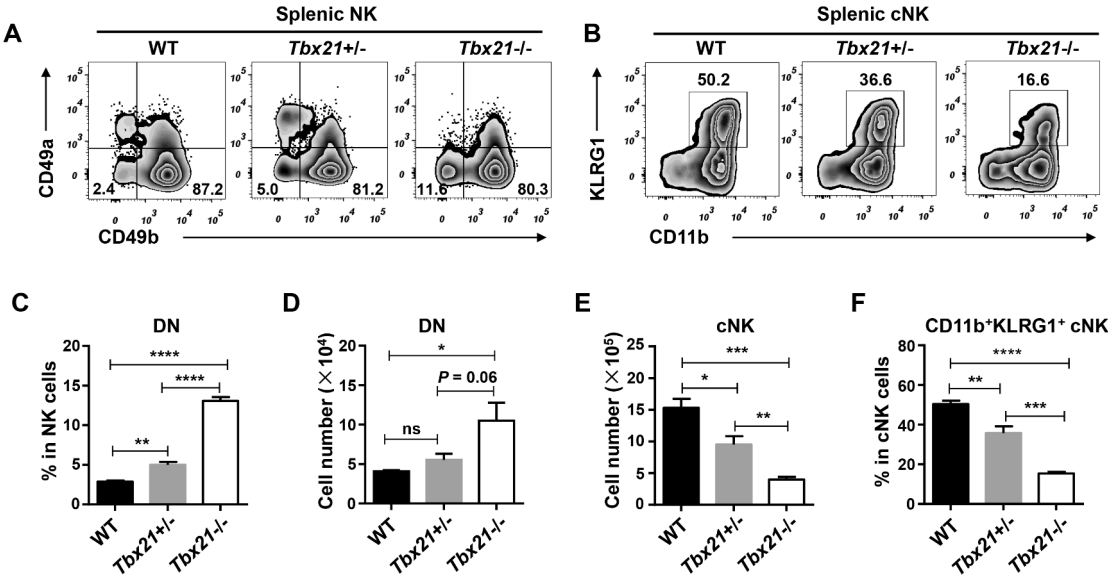


Fig. 6. Splenic DN NK cells are increased in T-bet-deficient mice. (A–F) Splenocytes were isolated from WT, *Tbx21* (T-bet) $^{+/-}$, and *Tbx21*$^{-/-}$ mice, and analyzed by flow cytometry. Representative dot plots show the expression of the indicated molecules on splenic bulk NK (A) or cNK (B) cells. Bar graphs display the percentages (C) and absolute numbers (D) of splenic DN NK cells. (E) The absolute numbers of splenic cNK cells are shown. (F) The percentages of splenic CD11b^{+}KLRG1^{+} cells in cNK cells are shown. Data are representative of two independent experiments, with n = 3–5 mice per group. Means ± SEM are shown (unpaired two-tailed Student's *t*-test; **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

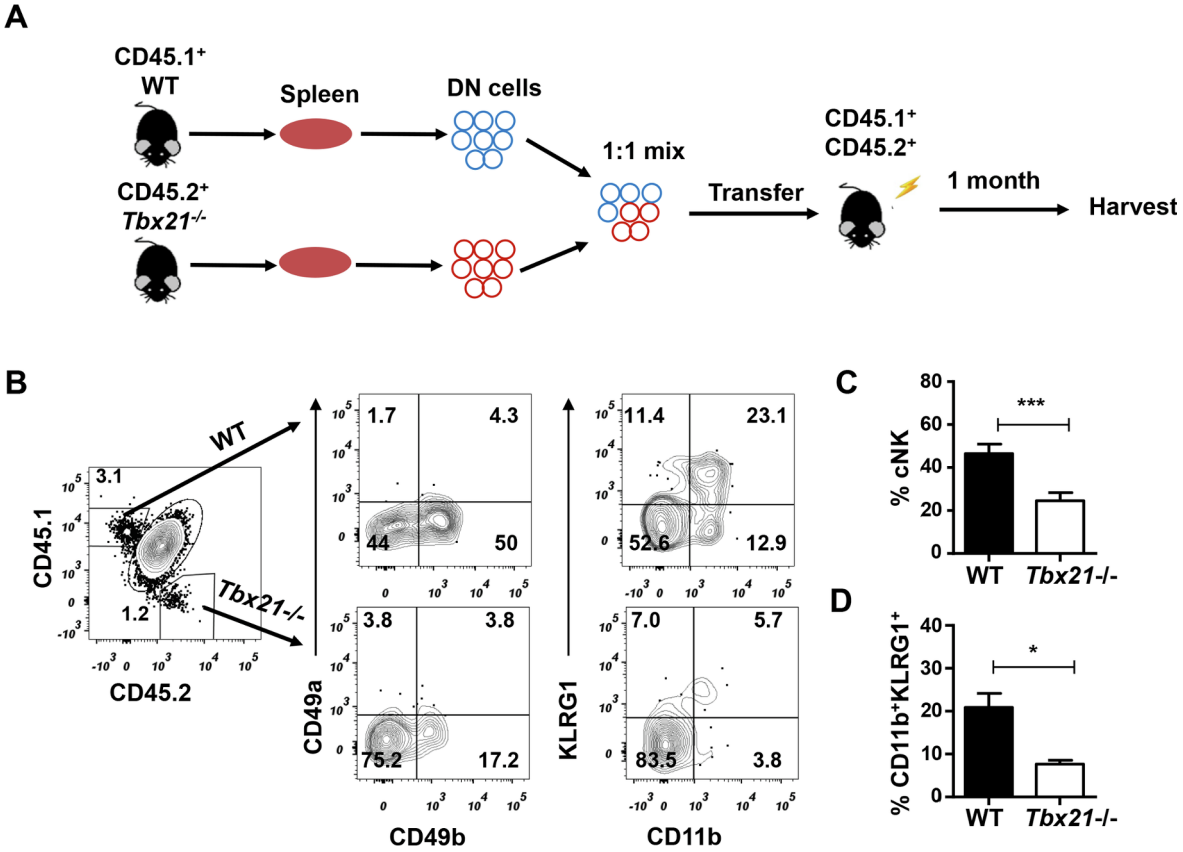


Fig. 7. T-bet deficiency impedes the maturation of splenic DN NK cells. (A–D) Sublethally irradiated CD45.1^{+}CD45.2^{+} B6 mice received 2 × 10^{5} CD45.1^{+}WT splenic DN NK cells mixed with 2 × 10^{5} CD45.2^{+}*Tbx21*$^{-/-}$ splenic DN NK cells, and donor-derived cells in recipients were analyzed one month after transfer. (A) Schematic of experiment. (B) The representative flow cytometry plots showing the expression of the indicated molecules on WT- or *Tbx21*$^{-/-}$-donor derived NK cells in recipient spleens. (C)The percentages of cNK cells in the WT donor-derived (black) and *Tbx21*$^{-/-}$ donor-derived (white) cells. (D) The percentages of the CD11b^{+}KLRG1^{+} cells in the WT donor-derived (black) and *Tbx21*$^{-/-}$ donor-derived (white) cells. Data are representative of two independent experiments, with n = 4–5 mice per group. Means ± SEM are shown (paired two-tailed Student's *t*-test; **P* < 0.05; ****P* < 0.001).

Although spleen can conduct extramedullary hematopoiesis in pathological conditions [43,44], whether the progenitors of NK cells exist in the spleen of healthy adult mice remains unclear. Since these DN NK cells were still detectable in the recipients > 2 months after transfer (data not shown), they may migrate from hematopoiesis site to the spleen during a certain stage of ontogeny and keep self-renewal. Notably, there were a small proportion of DN NK cells that could come from the parabiont mice, which implied a possibility that splenic DN NK cells might partly come from the bone marrow via blood circulation. Overall, the origin of splenic DN NK cells needs further investigation.

By intravenous injection of splenic DN NK cells into recipient mice, donor cells were also found in tissues outside the spleen. In particular, transferred DN NK cells partially converted into $CD49a^+$ liver-resident NK cells; however, splenectomy in normal WT mice did not influence the number of liver-resident NK cells (data not shown), thus excluding the contribution of splenic DN NK cells to the liver-resident NK cell pool. This is likely due to the tissue-resident feature of splenic DN NK cells. At steady state, they have no chance to participate in circulation to get access to the liver, and their confinement to the spleen makes them exclusively give rise to cNK cells, suggesting that environmental niches are critical in determining DN NK cell differentiation.

T-bet has been demonstrated to be essential for NK cell terminal maturation [37]. T-bet-deficiency results in reduced expression of CD27 and c-kit and increased expression of KLRG1on NK cells [18,37,45,46]. T-bet also controls the $S1P_5$ expression, which helps NK cell egress from the bone marrow and lymph nodes [47]. Accordingly, NK cell numbers are increased in the bone marrow and lymph nodes but reduced in the periphery of T-bet deficient mice [47]. It appears that T-bet is central in the transcription factor network governing NK cell maturation. Transcription factors ETS1, TOX1 and TOX2 act upstream of T-bet by positively regulating T-bet expression during NK cell development [48–50], whereas Foxo1 downregulates T-bet expression to inhibit NK cell maturation [51]. T-bet further controls expression of transcription factors Blimp1 and Zeb2 to promote NK cell maturation [46,52]. In addition to these intrinsic factors, T-bet deficiency can extrinsically impede NK cell final maturation by regulating IL-15Rα expression on monocytes [53]. In striking contrast to cNK cells, our study found that splenic DN NK cell numbers were significantly increased in T-bet deficient mice. Moreover, T-bet deficient DN NK cells could hardly generate mature cNK cells, suggesting that the development process from DN NK cells to mature cNK cells was intrinsically dependent on T-bet. Since these DN NK cells were mainly spleen-resident, this developmental step occurred in the spleen locally, and block of such step might be responsible for splenic cNK cell reduction observed in T-bet deficient mice. It would be of interest to further investigate whether, or to what extent, the development from splenic DN to cNK cells contributes to the total cNK cell pool in periphery.

Collectively, we have identified a spleen-resident NK cell subset characterized by $CD49a^-CD49b^-$ phenotype, which were phenotypically and functionally immature and can undergo extramedullary maturation in a T-bet dependent manner. The impeded development step from DN NK cells to cNK cells caused by T-bet deficiency may explain the reduction of cNK cells in the periphery tissues. This study also sheds light on the extramedullary development.

Author contributions

BW, HP designed the experiments; BW, JZ, YC performed the experiments; BW, HW, RS, ZT, HP analyzed/interpreted the data; BW, ZT, HP wrote the manuscript.

Conflicts of interest

There is no conflict of interest.

Funding source

This work was supported by the Natural Science Foundation of China (#81788101, 81571522, 81821001, 91642105, 91842307, 91542000).

References

[1] M.P. Henkart, P.A. Henkart, Lymphocyte mediated cytolysis as a secretory phenomenon, Adv. Exp. Med. Biol. 146 (1982) 227–247.
[2] T.A. Fehniger, S.F. Cai, X. Cao, A.J. Bredemeyer, R.M. Presti, A.R. French, et al., Acquisition of murine NK cell cytotoxicity requires the translation of a pre-existing pool of granzyme B and perforin mRNAs, Immunity 26 (2007) 798–811.
[3] J. Thiery, D. Keefe, S. Boulant, E. Boucrot, M. Walch, D. Martinvalet, et al., Perforin pores in the endosomal membrane trigger the release of endocytosed granzyme B into the cytosol of target cells, Nat. Immunol. 12 (2011) 770–777.
[4] H. Arase, N. Arase, T. Saito, Fas-mediated cytotoxicity by freshly isolated natural killer cells, J. Exp. Med. 181 (1995) 1235–1238.
[5] L. Zamai, M. Ahmad, I.M. Bennett, L. Azzoni, E.S. Alnemri, B. Perussia, Natural killer (NK) cell-mediated cytotoxicity: differential use of TRAIL and Fas ligand by immature and mature primary human NK cells, J. Exp. Med. 188 (1998) 2375–2380.
[6] A. Martin-Fontecha, L.L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, et al., Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H) 1 priming, Nat. Immunol. 5 (2004) 1260–1265.
[7] L. Moretta, G. Ferlazzo, C. Bottino, M. Vitale, D. Pende, M.C. Mingari, et al., Effector and regulatory events during natural killer-dendritic cell interactions, Immunol. Rev. 214 (2006) 219–228.
[8] E. Vivier, E. Tomasello, M. Baratin, T. Walzer, S. Ugolini, Functions of natural killer cells, Nat. Immunol. 9 (2008) 503–510.
[9] E.E. Rosmaraki, I. Douagi, C. Roth, F. Colucci, A. Cumano, J.P. Di Santo, Identification of committed NK cell progenitors in adult murine bone marrow, Eur. J. Immunol. 31 (2001) 1900–1909.
[10] J.W. Fathman, D. Bhattacharya, M.A. Inlay, J. Seita, H. Karsunky, I.L. Weissman, Identification of the earliest natural killer cell-committed progenitor in murine bone marrow, Blood 118 (2011) 5439–5447.
[11] S. Carotta, S.H. Pang, S.L. Nutt, G.T. Belz, Identification of the earliest NK-cell precursor in the mouse BM, Blood 117 (2011) 5449–5452.
[12] M.G. Constantinides, H. Gudjonson, B.D. McDonald, I.E. Ishizuka, P.A. Verhoef, A.R. Dinner, et al., PLZF expression maps the early stages of ILC1 lineage development, Proc. Natl. Acad. Sci. U. S. A. 112 (2015) 5123–5128.
[13] W. Goh, N.D. Huntington, Regulation of murine natural killer cell development, Front. Immunol. 8 (2017) 130.
[14] S. Kim, K. Iizuka, H.S. Kang, A. Dokun, A.R. French, S. Greco, et al., In vivo developmental stages in murine natural killer cell maturation, Nat. Immunol. 3 (2002) 523–528.
[15] N.D. Huntington, H. Tabarias, K. Fairfax, J. Brady, Y. Hayakawa, M.A. Degli-Esposti, et al., NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation, J. Immunol. 178 (2007) 4764–4770 Baltimore, Md : 1950.
[16] L. Chiossone, J. Chaix, N. Fuseri, C. Roth, E. Vivier, T. Walzer, Maturation of mouse NK cells is a 4-stage developmental program, Blood 113 (2009) 5488–5496.
[17] K. Takeda, E. Cretney, Y. Hayakawa, T. Ota, H. Akiba, K. Ogasawara, et al., TRAIL identifies immature natural killer cells in newborn mice and adult mouse liver, Blood 105 (2005) 2082–2089.
[18] S.M. Gordon, J. Chaix, L.J. Rupp, J. Wu, S. Madera, J.C. Sun, et al., The transcription factors T-bet and Eomes control key checkpoints of natural killer cell maturation, Immunity 36 (2012) 55–67.
[19] C.A. Vosshenrich, J.P. Di Santo, Developmental programming of natural killer and innate lymphoid cells, Curr. Opin. Immunol. 25 (2013) 130–138.
[20] H. Peng, X. Jiang, Y. Chen, D.K. Sojka, H. Wei, X. Gao, et al., Liver-resident NK cells confer adaptive immunity in skin-contact inflammation, J. Clin. Investig. 123 (2013) 1444–1456.
[21] D.K. Sojka, B. Plougastel-Douglas, L. Yang, M.A. Pak-Wittel, M.N. Artyomov, Y. Ivanova, et al., Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells, Elife 3 (2014) e01659.
[22] C.S.N. Klose, M. Flach, L. Mohle, L. Rogell, T. Hoyler, K. Ebert, et al., Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages, Cell 157 (2014) 340–356.
[23] C.L. Vargas, J. Poursine-Laurent, L. Yang, W.M. Yokoyama, Development of thymic NK cells from double negative 1 thymocyte precursors, Blood 118 (2011) 3570–3578.
[24] C.A. Vosshenrich, M.E. Garcia-Ojeda, S.I. Samson-Villeger, V. Pasqualetto, L. Enault, O. Richard-Le Goff, et al., A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127, Nat. Immunol. 7 (2006) 1217–1224.
[25] A.G. Freud, B. Becknell, S. Roychowdhury, H.C. Mao, A.K. Ferketich, G.J. Nuovo, et al., A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells, Immunity 22 (2005) 295–304.
[26] C. Gregoire, L. Chasson, C. Luci, E. Tomasello, F. Geissmann, E. Vivier, et al., The trafficking of natural killer cells, Immunol. Rev. 220 (2007) 169–182.
[27] G. Cui, T. Hara, S. Simmons, K. Wagatsuma, A. Abe, H. Miyachi, et al., Characterization of the IL-15 niche in primary and secondary lymphoid organs in vivo, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 1915–1920.

[28] D. Briard, D. Brouty-Boye, B. Azzarone, C. Jasmin, Fibroblasts from human spleen regulate NK cell differentiation from blood CD34(+) progenitors via cell surface IL-15, J. Immunol. 168 (2002) 4326–4332 Baltimore, Md : 1950.
[29] J. Wang, R. Sun, H. Wei, Z. Dong, B. Gao, Z. Tian, Poly I:C prevents T cell-mediated hepatitis via an NK-dependent mechanism, J. Hepatol. 44 (2006) 446–454.
[30] Y. Wang, T. Li, Y. Chen, H. Wei, R. Sun, Z. Tian, Involvement of NK cells in IL-28B–mediated immunity against influenza virus infection, J. Immunol. 199 (2017) 1012–1020.
[31] P. Kamran, K.I. Sereti, P. Zhao, S.R. Ali, I.L. Weissman, R. Ardehali, Parabiosis in mice: a detailed protocol, J. Vis. Exp. (2013) e50556.
[32] F. Simonetta, A. Pradier, E. Roosnek, T-bet and eomesodermin in NK cell development, maturation, and function, Front. Immunol. 7 (2016) 241.
[33] J.H. Pedra, F.S. Sutterwala, B. Sukumaran, Y. Ogura, F. Qian, R.R. Montgomery, et al., ASC/PYCARD and caspase-1 regulate the IL-18/IFN-γ axis during Anaplasma phagocytophilum infection, J. Immunol. 179 (2007) 4783–4791.
[34] C.A. Dinarello, G. Kaplanski, Indeed, IL-18 is more than an inducer of IFNγ, J. Leukoc. Biol. 104 (2018) 237.
[35] L.K. Mackay, M. Minnich, N.A. Kragten, Y. Liao, B. Nota, C. Seillet, et al., Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes, Science 352 (2016) 459–463.
[36] C. Daussy, F. Faure, K. Mayol, S. Viel, G. Gasteiger, E. Charrier, et al., T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow, J. Exp. Med. 211 (2014) 563–577.
[37] M.J. Townsend, A.S. Weinmann, J.L. Matsuda, R. Salomon, P.J. Farnham, C.A. Biron, et al., T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells, Immunity 20 (2004) 477–494.
[38] C.S. Klose, M. Flach, L. Mohle, L. Rogell, T. Hoyler, K. Ebert, et al., Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages, Cell 157 (2014) 340–356.
[39] L. Tang, H. Peng, J. Zhou, Y. Chen, H. Wei, R. Sun, et al., Differential phenotypic and functional properties of liver-resident NK cells and mucosal ILC1s, J. Autoimmun. 67 (2016) 29–35.
[40] O.E. Weizman, N.M. Adams, I.S. Schuster, C. Krishna, Y. Pritykin, C. Lau, et al., ILC1 confer early host protection at initial sites of viral infection, Cell 171 (2017) 795–808 e12.
[41] J. Wang, J. Xu, W. Zhang, H. Wei, Z. Tian, TLR3 ligand-induced accumulation of activated splenic natural killer cells into liver, Cell. Mol. Immunol. 2 (2005) 449–453.
[42] X. Fan, A.Y. Rudensky, Hallmarks of tissue-resident lymphocytes, Cell 164 (2016) 1198–1211.
[43] D. Visnjic, Z. Kalajzic, D.W. Rowe, V. Katavic, J. Lorenzo, H.L. Aguila, Hematopoiesis is severely altered in mice with an induced osteoblast deficiency, Blood 103 (2004) 3258–3264.
[44] C.H. Kim, Homeostatic and pathogenic extramedullary hematopoiesis, J. Blood Med. 1 (2010) 13–19.
[45] M. Malaisé, J. Rovira, P. Renner, E. Eggenhofer, M. Sabet-Baktach, M. Lantow, et al., KLRG1+ NK cells protect T-bet–deficient mice from pulmonary metastatic colorectal carcinoma, J. Immunol. 192 (2014) 1954–1961 Baltimore, Md : 1950.
[46] A. Kallies, S. Carotta, N.D. Huntington, N.J. Bernard, D.M. Tarlinton, M.J. Smyth, et al., A role for Blimp1 in the transcriptional network controlling natural killer cell maturation, Blood 117 (2011) 1869–1879.
[47] C.N. Jenne, A. Enders, R. Rivera, S.R. Watson, A.J. Bankovich, J.P. Pereira, et al., T-bet–dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow, J. Exp. Med. 206 (2009) 2469–2481.
[48] K. Ramirez, K.J. Chandler, C. Spaulding, S. Zandi, M. Sigvardsson, B.J. Graves, et al., Gene deregulation and chronic activation in natural killer cells deficient in the transcription factor ETS1, Immunity 36 (2012) 921–932.
[49] S. Yun, S.H. Lee, S.R. Yoon, M.S. Kim, Z.H. Piao, P.K. Myung, et al., TOX regulates the differentiation of human natural killer cells from hematopoietic stem cells in vitro, Immunol. Lett. 136 (2011) 29–36.
[50] Q.P. Vong, W.H. Leung, J. Houston, Y. Li, B. Rooney, M. Holladay, et al., TOX2 regulates human natural killer cell development by controlling T-BET expression, Blood 124 (2014) 3905–3913.
[51] Y. Deng, Y. Kerdiles, J. Chu, S. Yuan, Y. Wang, X. Chen, et al., Transcription factor Foxo1 is a negative regulator of natural killer cell maturation and function, Immunity 42 (2015) 457–470.
[52] M.J. van Helden, S. Goossens, C. Daussy, A.L. Mathieu, F. Faure, A. Marcais, et al., Terminal NK cell maturation is controlled by concerted actions of T-bet and Zeb2 and is essential for melanoma rejection, J. Exp. Med. 212 (2015) 2015–2025.
[53] K. Soderquest, N. Powell, C. Luci, N. van Rooijen, A. Hidalgo, F. Geissmann, et al., Monocytes control natural killer cell differentiation to effector phenotypes, Blood 117 (2011) 4511–4518.