Tbet promotes NK cell egress from the bone marrow and CXCR6 expression in immature NK cells

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Tbet-deficient mice have reduced NK cells in blood and spleen, but increased NK cells in bone marrow and lymph nodes, a phenotype that is thought to be due to defective migration. Here, we revisit the role of Tbet in NK cell bone marrow egress. We definitively show that the accumulation of NK cells in the bone marrow of Tbet-deficient (Tbx21<sup>−/−</sup>) animals occurs because of a cell-intrinsic migration defect. We identify a profile of gene expression, co-ordinated by Tbet, which affects the localisation of NK cells in the bone marrow. Tbet promotes Cxcr6 expression and immature NK cells accumulate in the bone marrow of CXCR6-deficient mice. This suggests that CXCR6 is among the mediators of migration, controlled by Tbet, that co-ordinate NK cell bone marrow egress.

NK cells | bone marrow | migration | Tbet | CXCR6
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Introduction

Tbet was originally described as the key transcription factor directing Th1 lineage commitment (Szabo et al., 2000). More recently, it has become clear that Tbet also drives differentiation and memory cell generation in a number of lymphocyte lineages (Kallies and Good-Jacobson, 2017) as well as being required for the development and survival of ILC1 (Spits et al., 2013). There is still debate about the extent to which ILC1 form a separate lineage from NK cells (Vivier et al., 2018; Gao et al., 2017; Cortez et al., 2017; Park et al., 2019; Xu et al., 2019), with one key factor that distinguishes NK cells from ILC1 being the greater extent to which ILC1 depend upon Tbet for their development (Sojka et al., 2014; Daussy et al., 2014). Nevertheless, Tbet-deficient mice do display defects in NK cell number, maturation status and function (Townsend et al., 2004; Jenne et al., 2009). Tbet-deficient mice have reduced NK cells in blood and spleen, but increased NK cells in the bone marrow and lymph nodes (Townsend et al., 2004; Jenne et al., 2009). These observations led to the suggestion that Tbet is required for NK cells to leave the bone marrow or lymph nodes and enter the blood (Jenne et al., 2009). Tbet-deficient NK cells express lower levels of S1pr5 mRNA than their wild type counterparts, and S1pr5 knockout mice phenocopy Tbet-deficient animals, suggesting that Tbet mediates bone marrow and lymph node egress by upregulating S1PR5 expression (Jenne et al., 2009). We were prompted to revisit the role of Tbet in bone marrow egress by the unexpected finding that a conditional knockout of Tbet in NK cells did not display the accumulation of NK cells in the bone marrow that has been reported in other Tbet-deficient strains of mice. Here, we report that in the absence of Tbet, NK cells display a cell-intrinsic defect in their ability to leave the bone marrow, and in their ability to differentiate to the final stage of NK cell development. We find that, in the absence of Tbet, CXCR6-expressing bone marrow NK cells are lost. We also observe an accumulation of immature NK cells in the bone marrow in the absence of CXCR6, although this is smaller than that observed in the absence of Tbet, and a reduced ability of CXCR6-deficient bone marrow to reconstitute peripheral NK cell compartments, pointing to a minor role for CXCR6 in NK cell trafficking.

Results

Tbet-deficient NK cells accumulate in the bone marrow

NK cells have previously been reported to accumulate in the bone marrow of Tbet-deficient strains of mice (Townsend et al., 2004; Jenne et al., 2009). We examined the frequencies of Lineage-negative NK1.1<sup>+</sup> cells within the bone marrow of both Tbx21<sup>−/−</sup> and Ncr1<sup>Cre</sup> Tbx21<sup>−/−</sup> mice, compared to appropriate controls (Fig. 1a). Within the lineage-negative NK1.1<sup>+</sup> compartment, we defined CD11b<sup>+</sup> cells as immature (iNK) cells, further subdividing the CD11b<sup>+</sup> mature (mNK) compartment by their expression of CD27 into a CD27<sup>+</sup> subset of intermediate maturity, which for simplicity is sometimes called “mNK1”, and a more mature CD27<sup>−</sup> subset called “mNK2” (Kim et al., 2002; Chiossone et al., 2009; Goh and Huntington, 2017). Eomes<sup>−</sup> CD49a<sup>+</sup> cells, suggested to represent ILC1, have previously been reported within the Lineage-negative NK1.1<sup>+</sup> bone marrow compartment (Klose et al., 2014), although other studies have failed to find a prominent ILC1 population within this gate (Turchinovich et al., 2018; Wang et al., 2018). In the bone marrow of our mice, we did not find a significant Eomes<sup>−</sup> CD49a<sup>+</sup> population (Fig. 1a – the liver, in which a substantial ILC1 population is present, is shown as a positive control). This confirms that, in these animals, this gating strategy identifies NK cells.

In line with previous reports, we found an accumulation of Lineage-negative NK1.1<sup>+</sup> cells in the bone marrow of Tbx21<sup>−/−</sup> mice, which predominantly affects the CD11b<sup>+</sup> mNK population (Fig. 1b). In the bone marrow of...
Ncr1Cre Tbx21fl/fl conditional knockout mice in which Cre recombinase-mediated excision of Tbx21 (Tbet) is driven by the Ncr1 (NKp46) promoter, we did not observe the accumulation of NK cells that we saw in Tbx21−/− mice (Fig. 1c). The most obvious explanation for this difference would be if the requirement for Tbet in NK cell egress from the bone marrow were not cell-intrinsic. In a previous study, mixed chimera experiments convincingly showed that the requirement for Tbet in NK cells leaving the lymph nodes is cell-intrinsic, but for bone marrow egress the results were less clear (Jenne et al., 2009). Therefore, we created mixed chimeras to determine whether the requirement for Tbet in bone marrow egress is cell-intrinsic (Fig. 1d,e). In the chimeras, we saw an increase in the ratio of Tbet-deficient:Tbet-sufficient cells within the CD11b+ mNK compartment of the bone marrow, but not within the iNK compartment. This mirrors our observations in Tbx21−/− mice, where an accumulation of NK cells is observed in the mNK, but not in the iNK compartment. These results therefore support the idea that the requirement for Tbet in NK egress from the bone marrow is cell-intrinsic.

In contrast to Tbx21−/− mice, in which Tbet is consistently absent, Tbet depletion in conditional knockout mice is dependent on Ncr1 expression. We examined the expression of NKp46 and Tbet in LMPPs, CLPs and NK progenitors (gat-
NK cells are less able to leave the bone marrow in the absence of Tbet

Previous reports attribute the bone marrow accumulation of Tbet-deficient NK cells to a defect in migration involving S1PR5, since S1pr5 mRNA is underexpressed in Tbet-deficient NK cells, S1pr5<sup>-/-</sup> mice phenocopy the accumulation in NK cells in the bone marrow of Tbet-deficient mice and in S1pr5<sup>-/-</sup> mice the accumulation is caused by a migration defect (Jenne et al., 2009; Walzer et al., 2007; Mayol et al., 2011). However, a defect in NK cell migration from the bone marrow in the absence of Tbet has not yet been formally shown. Therefore, we examined NK cell migration from the bone marrow in vivo. Recently-migrated cells translocate to the sinusoids before exiting the bone marrow, so sinusoidal leukocyte staining acts as an indicator of recent migration from the bone marrow. The procedure involves intravenous injection of fluorescently-labelled anti-CD45.2 monoclonal antibody followed by a 2-minute incubation to allow the circulation to carry the antibody to the sinusoids and stain leukocytes in the blood and sinusoids, while sparing parenchymal cells.

NKp46 (encoded by Ncr1) is not detectable at the protein level until the immature NK cell stage of development (Fig. 1g). Therefore we considered the possibility that transient Tbet expression early in development, before Ncr1-mediated Cre recombinase activity is switched on, could be sufficient to allow NK cell egress later in development. A similar situation occurs for the transcription factor Nfil3, whose expression is required before NK-lineage commitment for later development into functional NK cells (Male et al., 2014). NK cells in conditional knockouts of Nfil3 under the Ncr1 promoter develop comparably to controls, whereas they fail to develop in mice globally deficient in Nfil3, because Nfil3 is required earlier in NK cell development than it is excised in these mice (Firth et al., 2013).

To address whether the requirement for Tbet, like that of Nfil3, occurs at a specific developmental stage, we examined bone marrow leukocytes from Ncr1<sup>iCre</sup> Tbx21<sup>fl/fl</sup> mice for Tbet expression at various stages of NK development. We found significant loss of Tbet in NK-lineage committed progenitors, rNKp, in both conditional knockout and knockout mice relative to their controls, and notably before Ncr1 is detectable at the protein level (Fig. 1h,i). Since the deletion of Tbet occurs earlier than might be expected in conditional knockout mice, this indicates that Ncr1, and consequently Cre-recombinase, is transcribed at an earlier developmental stage than the protein reaches a level that can be detected by flow cytometry. This earlier-than-expected loss of Tbet expression also rules out the possibility that the differences in NK cell accumulation that we observe between the global and conditional knockouts are caused by transient expression of Tbet.
leukocytes (Fig. 2a) (Pereira et al., 2009). As NK cells mature from CD11b+ iNK to CD11b+CD27+ mNK1 and then to CD11b+CD27+ mNK2 in wild type mice, their frequencies decrease in the parenchyma (Fig. 2a,c) and increase in the sinusoids (Fig. 2a,d). This indicates migration out of the bone marrow occurring at all NK cell developmental stages but increasing in more mature cells. We did not observe this pattern in Tbx21−/− NK cells (Fig. 2b), whose frequency instead remained constant between iNK and mNK1 in both parenchyma and sinusoid, indicating a reduced ability to migrate. We also observed a decrease in the frequency of mNK2 in both anatomical compartments, consistent with a defect at the mNK1 to mNK2 transition that has previously been reported (van Helden et al., 2015). In the conditional knockout mouse compared to the control, we observed a failure of developing NK cells to leave the parenchyma and enter the sinusoid (Fig. 2e,f), similar to that in the conventional knockout. Therefore, this more sensitive test of bone marrow egress reveals that conditional knockout NK cells, like their conventional knockout counterparts, do have a defect in leaving the BM, although we had been unable to detect this by a simple examination of NK cell frequencies (Fig. 1c).

**Tbet controls the expression of cell migration mediators in bone marrow NK cells**

Having confirmed that the accumulation in the bone marrow of Tbx21−/− mice is caused by a migration defect, we sought to define the mediators involved. We therefore sorted iNK and mNK1 cells from the bone marrow of wild type and Tbx21−/− mice, and mNK2 (which are not present in Tbx21−/− mice) from wild type mice and analyzed them by RNAseq. Raw RNAseq data and differentially expressed gene lists are available from the National Center for Biotechnology Information Gene Expression Omnibus under accession no. GSE122874. Consistent with previous reports (Jenne et al., 2009), we found S1pr5 mRNA underexpressed by a factor of approximately seven-fold in both iNK (Fig. 3a,c) and mNK1 (Fig. 3b,c) cells in Tbx21−/− mice compared to wild type. More severely affected by the absence of Tbet was Cxcr6, which was underexpressed by a factor of 125-fold in iNK cells (Fig. 3a,c). The expression of Kit, often considered a marker of immature NK cells, was increased in the absence of Tbet (Fig. 3a,b,d) whereas the expression of genes associated with mature NK effector function, such as Ifng, Gzmb and Prf1, were reduced (Fig. 3a,b,e). Consistent with reports that Tbet promotes Zeb2 (van Helden et al., 2015) and antagonizes Eomes (Pikovskaya et al., 2016) transcription, Zeb2 expression was decreased and increased, respectively, in Tbx21−/− NK cells (Fig. 3a,b,f).

We next selected genes identified as differing transcriptionally between wild type and Tbx21−/− NK cells to confirm the differences in their expression at the protein level by flow cytometry (Fig. 4). The genes were selected based on a significance cutoff of p_adj < 0.05 and a two-fold or more differential expression between wild type and Tbx21−/− iNK and mNK1 cells. Sell and Cd69 did not have a two-fold difference between KO and WT cells, but were included in the analysis because we observed differential transcription of these genes in both NK subsets.

The expression of several proteins associated with cell migration differed between wild type and Tbx21−/− NK cells. In particular, CXCR6 was expressed at a lower level by knockout iNK and mNK1, compared to their wild type counterparts (Fig. 4g). Consistent with the RNASeq data, the most signifi-
CD66, which suppresses the expression of S1PR1 in order to retain immune cells in lymph nodes and tissues, is upregulated in both knockout NK subsets (Fig. 4d). CD62L (encoded by Sell), which is involved in leukocyte homing from the blood to tissues, also has elevated expression in knockout NK cells compared to wild type (Fig. 4c). We also observed a decrease in the expression of CD49a in knockout iNK (Fig. 4b).

S1pr5 and Ccr2 displayed relatively large differences at the RNA level (Fig. 3a), but using antibodies validated on appropriate positive control cells (S1pr5-transfected 293T cells and CD11b* Ly6G* monocytes, respectively) we did not see any differences at the protein level (Fig. 4a,i). Neither did we see any significant difference in CX3CR1 expression between wild type and knockout cells, although the expression of this protein did increase in mNK2 compared to previous stages of development (Fig. 4h).

Immature NK cells accumulate in the bone marrow of CXCR6-deficient mice

We have previously reported that ILC progenitors require CXCR6 to leave the bone marrow (Chea et al., 2015), so we were intrigued by the underexpression of CXCR6 in Tbx21−/− NK cells, which are also less able to leave the bone marrow. If CXCR6 is one of the mediators through which Tbet controls NK cell bone marrow egress, we would expect to see an accumulation of NK cells in the bone marrow of CXCR6-deficient mice, similar to our previous observations of ILC progenitors.

To test this hypothesis, we examined NK cells in the bone marrow of Cxcr6−/− mice, in which both Cxcr6 alleles are inactivated and replaced by a reporter cassette encoding GFP, or Cxcr6−/+ controls. We did not observe an accumulation among the general population of CLP, αLP1 or αLP2 in the bone marrow of these mice, although we did observe an accumulation specifically among the Cxcr6−/− population of αLP2 (Fig. 5a). Examining NK cell progenitors, we observed an accumulation of iNK, although this was not significant. However, we did observe a significant accumulation specifically among the Cxcr6−/− population of iNK (Fig. 5b). In contrast to the Tbet knockout, we did not detect an accumulation of cells among either of the mature NK cell subpopulations in the bone marrow of CXCR6-deficient mice, suggesting that any requirement for CXCR6 in bone marrow egress occurs relatively early in NK cell development.

CXCR6 is highly expressed by ILC1 (Fig. 5c) and we have previously shown that it is required for reconstitution of ILC3 progenitors (Chea et al., 2016). Therefore, we investigated the relative contributions of CXCR6 to reconstitution of ILC1 compared to NK cells. For these experiments, we focused on the liver, since a large ILC1 population is present here. At steady state, CXCR6-deficient mice displayed a defect in ILC1 frequency and number (Fig. 5c), consistent with the requirement for CXCR6 in ILC progenitor trafficking during fetal life that we have previously reported (Chea et al., 2016). To determine the requirement for CXCR6 in ILC1 and NK cell reconstitution in adult life, we performed competitive reconstitution experiments (Fig. 5d). Surprisingly, we did not observe any difference in the relative reconstitution of ILC1 in competitive reconstitutions using either CXCR6-sufficient or -deficient progenitors. This suggests that, in contrast to the situation in fetal life, ILC1 in adults do not seem to be dependent on CXCR6 for trafficking. On the other hand, the reconstitution of NK cells was reduced when mice were reconstituted with progenitors that lacked either one or both alleles of Cxcr6, compared to wild type. This points to a role for CXCR6 in NK cell circulation during adult life, with the deficiency in reconstitution observed in heterozygous progenitors suggesting that a threshold of CXCR6 protein expres-
Discussion

In this study, we revisited the role of Tbet in mediating NK cell egress from the bone marrow during development. We observed an accumulation of NK cells in the bone marrow of Tbx21−/− mice, in agreement with previous reports (Townsend et al., 2004; Jenne et al., 2009). The accumulating NK cells in Tbet-deficient mice have previously been described as CD27hi and KLRG1lo (Jenne et al., 2009), but no distinction was made between the two CD27+ subsets, CD27+CD11b− iNK and CD27+CD11b+ mNK1. Here we report that the accumulation occurs specifically within the mNK1 compartment, although this is unsurprising given a more recent report, which we confirm here, that there is a block at the mNK1 to mNK2 transition in the absence of Tbet (van Helden et al., 2015). We confirmed that the requirement for Tbet in NK cell bone marrow egress is cell-intrinsic, demonstrating accumulation of Tbx21−/− NK cells in the bone marrow of mixed bone marrow chimeras more convincingly than in previous studies (Jenne et al., 2009). Potentially, this could be because the earlier studies were carried out using a Tbet-deficient strain called Duane, in which Tbet protein levels were reduced by approximately four-fold, whereas Tbx21−/− NK cells completely lack Tbet.

Fig. 5. Accumulation of Cxcr6+ iNK in the bone marrow of CXCR6-deficient mice. (a) Representative flow cytometry gating strategy used to identify CLP, αLP1, αLP2 progenitors (Lin-IL7Rα+) in the Cxcr6gfp+/− and Cxcr6gfp/gfp bone marrow (Yu et al., 2014). αLP2 cells were identified as Flt3−α4β7+ cells and contour plots indicate the fraction of Cxcr6-gfp+ cells among αLP2 from both Cxcr6gfp+/− and Cxcr6gfp/gfp mice. Frequencies of the CLP, αLP1 and αLP2 are not significantly different among total bone marrow progenitors. When progenitors are selected as Cxcr6-expressing cells, accumulation of αLP2 fraction in the bone marrow is significant. n = 10 mice per group, means and SD are shown. (b) NK progenitor subsets among Lin-IL7Rα+ or the Lin-IL7Rα− subsets are identified using CD122 and NK1.1 expression as shown. rNKP and iNK are identified among the IL7Rα+ Flt3−α4β7+ fraction as CD122+NK1.1− and CD122+NK1.1+ subsets, respectively. Among IL7Rα− cells, iNK, mNK1 and mNK2 are identified as shown in Figure 1. In CXCR6-deficient mice, iNK frequency presents a tendency to increase with a significant increase for the Cxcr6-gfp+ IL7Rα− iNK fraction. n = 10 mice per group, means and SD are shown. Significance was determined using two sample, one-tailed t tests. * p < 0.05, ** p < 0.01, *** p < 0.001. (c) CXCR6 expression in liver NK cells and ILC1. Frequency as a percentage of parent population (CD45+NK1.1+NKp46+CD49b+ for NK cells; CD45+NK1.1+NKp46−CD49b− for ILC1) and absolute number of Cxcr6-gfp+ expressing ILC1 are significantly decreased in the liver of CXCR6-deficient mice. n = 20 mice per group, means and SD are shown. (d) CXCR6 deficiency impacts hepatic NK cell reconstitution. Schematic representation of experimental procedure for competitive reconstitution of congenic lymphopoeic Rag2−/− double KO mice. Frequencies of CD45.1 (WT reconstitution control) and CD45.2 (WT, Cxcr6gfp+/− or Cxcr6gfp/gfp experimental) cells among reconstituted hepatic NK cells and ILC1 are shown.
deficient mice, together with the cells’ underexpression of S1pr5 mRNA and the defect in bone marrow egress displayed by S1pr5−/− NK cells led to the suggestion that Tbet mediates NK cell bone marrow egress via S1PR5 (Jenne et al, 2009; Walzer et al, 2007; Mayol et al, 2001). However, no previous study had formally addressed the question of whether the accumulation of NK cells in the absence of Tbet occurs due to a migration defect. We used in vivo staining of sinusoidal NK cells to show that Tbet-deficient iNK and mNK1 cells are less able to move from the parenchyma to the sinusoid of the bone marrow, supporting the idea that Tbet promotes NK cell bone marrow egress. The in vivo bone marrow egress staining further revealed that Tbet knockout iNK cells and conditional knockout iNK and mNK1 cells all have a migration defect that was not apparent on simple examination of cell frequencies at steady state, suggesting that this test detects migration defects with greater sensitivity. We also noted a slight increase in the frequency of NK cells in the parenchyma of Tbx21−/− mice, compared to wild type, although the experiments were not designed such that these two could be statistically compared. This slight accumulation of parenchymal NK cells in the control animals may have contributed to the reduced difference between the conditional knockouts and controls on examination of cell frequencies at steady state, despite the defect in NK cell migration still being present.

After we confirmed that the NK cell accumulation in Tbet-deficient bone marrow was caused by defective migration, we explored which mediators were involved, identifying a transcriptional signature associated with Tbet expression in developing NK cells. We confirmed previous reports that S1pr5 is reduced in Tbet-deficient NK cells at the transcriptional level (Jenne et al, 2009), but we were unable to corroborate this at the protein level. We were also surprised not to see significant differences in CCR2 and CX3CR1 protein expression, since both differed at the transcript level and both CCR2-deficient and CX3CR1-deficient mice display an accumulation of NK cells in their bone marrow (Ponzetta et al, 2013; Fujimara et al, 2015). On the other hand, a number of molecules involved in cell migration did differ between wild type and Tbx21−/− NK cells, with increased expression of CD69 and CD62L, which would be expected to retain cells in the parenchyma, as we observed (Cibirán and Sánchez-Madrid, 2017) and decreased expression of CD49a and CXCR6.

We have previously shown that CXCR6 is important for ILC progenitors leaving the bone marrow (Chea et al, 2016) so we went on to investigate the role of CXCR6 in NK cell bone marrow egress. In the bone marrow of CXCR6-deficient mice, we found an accumulation of iNK, although this was only significant among Cxrc6-gfp−/− cells. This accumulation among iNK was consistent with our observation that CXCR6 expression was both highest and most differentially expressed within this population. We did not observe an accumulation among the CD11b+ mNK populations. Therefore, the NK cell accumulation we observed in the absence of CXCR6 was less pronounced than that in the absence of Tbet, and was only observed in early developmental stages. This suggests that although CXCR6 may be one of the molecules through which Tbet mediates NK cell bone marrow egress, its role is likely to be relatively minor, with other targets of Tbet, such as S1pr5 and Cd69, playing a greater role at later stages of NK cell development.

One key question which could affect the interpretation of these results is the extent to which the CXCR6+ cells which we found within the iNK subset could represent Tbet-dependent ILC1, significant numbers of which have been reported by some studies (Klose et al, 2014), although others have found smaller or negligible numbers (Turchinovich et al, 2018; Wang et al, 2018). The variation in these reports may result from different ages of animals being used in the experiments, since ILC1 are more dominant in younger animals (Constantinides et al, 2015), or even from as-yet undefined colony-specific factors. In the bone marrow of the animals used in this study, we were unable to find a significant Eomes+ CD49a+ ILC1 population. Indeed, the CD49a+ cells were identified in this study were also positive for CD49b, similar to a previous report, which found these double-positive cells to account for approximately 10% of NK cells in the bone marrow (Wang et al, 2018). This supports the idea that the cells we are characterizing in the bone marrow in this study are, indeed, NK cells. Our observations of CXCR6-deficient mice further support the idea that this is an NK cell and not an ILC1 phenomenon, since in adult life CXCR6-deficient bone marrow progenitors are somewhat less able to reconstitute the NK cell compartment than their CXCR6-sufficient counterparts, whereas they are equally able to reconstitute the adult ILC1 compartment. Indeed if, as some recent studies have suggested, NK cells and ILC1 do not form separate lineages, it will not be possible to define phenomena as pertaining to NK cells separately from ILC1 (Gao et al, 2017; Cortez et al, 2017, Park et al, 2019; Xu et al, 2019).

Overall, we have shown that Tbet is required for NK cells to leave the bone marrow in a cell-intrinsic manner. We further define a profile of gene expression coordinated by Tbet which promotes NK cell bone marrow egress. Among this group of genes which mediate localization of NK cells within the bone marrow, is Cxcr6. Bone marrow egress of iNK cells is somewhat defective in the absence of CXCR6, but the phenotype occurs both at an earlier developmental stage and is less pronounced than that in the absence of Tbet, suggesting that other targets of Tbet are likely to have a more significant role in the more mature NK cells that are the primary population leaving the bone marrow. As we move into an era where the cancer-fighting potential of NK cells is being harnessed to develop immunotherapies, it will become increasingly important to understand NK egress in order to maximize the full potential of those therapies (Souza-Fonseca-Guimaraes et al, 2019) and it will be interesting in the future to better define the roles of some of the other genes that we identified in NK cell bone marrow egress.
Materials and Methods

Mice

B6.129S6-Tbx21<tm1Glm>/J (RRID:IMSR_JAX:004648; “Tbx21<cre>”) and B6.129-Tbx21tm2Snv/J mice (RRID:IMSR_JAX:022741; “Tbx21<fl/fl>”) were purchased from the Jackson Laboratory. B6(C5-)Ncr1<tm1.1(icre)VivoD2 mice (Narni-Mancinelli et al., 2011) (RRID: MGI:539017; “Ncr1<cre>”) were acquired from the European Mutant Mouse Archive as frozen embryos and rederived at the Royal Free Hospital, London. Ncr1<Cre mice were crossed onto Tbx21<fl/fl> to produce Ncr1<Cre Tbx21<fl/fl> conditional knockouts and Ncr1<WT Tbx21<fl/fl> littermate controls, as previously described [22]. Tbx21<±> mice were crossed onto C57BL/6J mice, bred at the Royal Free Hospital, and the resultant heterozygotes were crossed to produce Tbx21<±> homozygote knockouts and Tbx21<±±> homozygote wild type littermate controls. Mice were sacrificed between 6 and 12 weeks of age with direct cervical dislocation. Death was confirmed by cessation of circulation. The hind leg bones (tibia and femurs) and spleen were dissected to isolate leukocytes. Animal husbandry and experimental procedures were performed according to UK Home Office regulations and institute guidelines under project licence 70/8530.

Ccr7<0/><g/> and Ccr7<0/><g/> mice (Geissman et al., 2005) were bred in the animal facilities at Pasteur Institute, Paris. Mice were bred in accordance with Pasteur Institute guidelines, in compliance with European welfare regulations, and all animal studies were approved by Pasteur Institute Safety Committee in accordance with French and European guidelines. Mice were sacrificed between 8 and 12 weeks of age with carbon dioxide pump. Death was confirmed by cessation of circulation. The hind leg bones (tibia and femurs) were dissected to isolate leukocytes. Animal husbandry and experimental procedures were performed according to UK Home Office regulations and institute guidelines under project licence 70/8530.

Bone marrow transplantation

Recipient mice were given myeloablative irradiation before syngeneic bone marrow transplantation at 10 weeks of age. Mice received a total of 11 Gy lethal irradiation, 5.5 Gy each on day -2 and day 0. On day 0, mice were reconstituted 4 hrs post-irradiation with 5 million donor bone marrow cells in 200 µl Hank’s Balanced Salt Solution (HBSS; Lonza, UK), 10% fetal calf serum (FCS; Sigma Aldrich), 100 units/ml penicillin-streptomycin, 25 mM HEPES buffer and 50 µM 2-Mercaptoethanol (all from Life Technologies).

For cell sorting on a FACS Aria (BD Biosciences, Oxford, UK), isolated bone marrow cells were ACK lysed by incubation in ACK lysing buffer (Life Technologies) for 5 minutes at room temperature to remove excess red blood cells and to enrich for leukocytes before cell sorting. The leukocytes were further enriched for NK cells by immunomagnetic depletion of lineage-associated cells (CD3, CD8, CD19, Gr-1) using the relevant FITC-conjugated antibodies and anti-FITC MicroBeads (Miltenyi, Woking, UK). The sorting buffer consisted of PBS, 0.5% bovine serum albumin (BSA; Sigma Aldrich, Hammerhill, UK) and 2 mM EDTA (Sigma Aldrich).

Spleens were passed through a 40 µm cell strainer. Red blood cells were lysed by incubation in ACK lysing buffer for 5 minutes at room temperature.

Flow cytometry

The following anti-mouse antibodies were used: anti-CCR2-PE-Cyanine7 (clone SA203G11, Biologend, London, UK), anti-CD3-FITC (17A2, Biologend), anti-CD3-biotin (clone 145-2C11, Sony Biotechnology, Surrey, UK), anti-CD4-FITC (clone 30-F11, Biolegend), anti-CX3CR1-BV510 (clone 2B8, Biolegend), anti-CD127 (IL-7Rα)-APC (clone TM-β1, Biolegend), anti-CD117 (ckit)-APC-Cyanine5.5 (clone H1.2F3, Biolegend), anti-CD117(cKit)-BV510 (clone HM2, BD Optibuild), anti-CD62L-PerCP-Cyanine5.5 (clone Ha31/8, BD Bioscience), anti-CD49b-FITC (clone M2B4 (B6)458.1, Biolegend), anti-CD45-BV510 (clone 30-F11, Biolegend), anti-CD27-APCeFluor780 (clone LG.7F9, eBioscience, San Diego, CA), anti-CD27-PE-Dazzle594 (clone LG.3A10, Biologend), anti-CD27-APC (clone LG.7F9, eBioscience), anti-CD45-BV510 (clone 30-F11, Biologend), anti-CD45-APC-Cyanine7 (clone 30-F11, BD Pharmingen), anti-CD45.2 PE-eFluor610 (clone 104, eBioscience), anti-CD45.1-PE-Cyanine7 (clone A20, Biologend), anti-CD49a-BUVR395 (clone Ha31/8, BD Bioscience), anti-CD49b-BV510 (clone HM2, BD Optibuild), anti-CD62L-PerCP-Cyanine5.5 (clone MEL-14, Biologend), anti-CD69-PerCP-Cyanine5.5 (clone H1.2F3, Biologend), anti-CD117(cKit)-BV510 (clone 2B8, Biologend), anti-CD117 (ckit)-APC-Cyanine7 (clone ACK2, Sony), anti-CD122 (IL-2β)-eFluor450 (clone TM-β1, eBioscience), anti-CD122 (IL-2β)-APC (clone TM-β1, Biologend), anti-CD127 (IL-7Rα)-PE (clone A7R34, eBioscience), anti-CD127 (IL-7Rα)-PE (clone A7R34, Sony), anti-CD135 (Flt3)-PerCP-eFluor710 (clone A2F10, eBioscience), anti-CD135 (Flt3)-PE (clone A2F10, Sony), anti-CD244-PE-Cyanine7 (clone m2B4 (B6)458.1, Biologend), anti-CX3CR1-BV510 (clone SA011F11, Biologend), anti-CXCRL6-PE-Cyanine7 (clone SA051D1, Biologend), anti-Gr-1-FITC (clone RB6-8C5, Biologend), anti-Gr-1-APC (clone RB6-8C5, Biologend).
Differential expression analysis was carried out using SARTools (Varet et al., 2016) filtering at padj < 0.05.

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References

Chea, S. et al. CXCR6 Expression Is Important for Retention and Circulation of ILC Precursors. Mediators Inflamm. 2015, 368427 10.1155/2015/368427 (2015).

Chiossone, L., Chaix, J., Fuseri, N., Roth, C., Vivier, E., and Walzer, T. Maturation of mouse NK cells is a 4-stage developmental program. Blood 113, 5488 (2009).

Cibrán, D. and Sánchez-Madrid, F. CD69: from activation marker to metabolic gatekeeper. Eur J Immunol. 47, 946-953 (2017).

Constantinides, M.G. et al. PLZF expression maps the early stages of ILC1 lineage development. Proc Natl Acad Sci U S A 112, 5123 (2015).

Cortez, V.S. et al. SMAD4 impedes the conversion of NK cells into ILC1-like cells by curtailing non-canonical TGF-β signaling. Nat. Immunol. 18; 995 (2017).

Cuff, A.O. and Male, V. Conventional NK cells and ILC1 are partially ablated in the livers of Ncr1 iCreTbx21 fl/fl mice. Wellcome Open Res. 2, 39 10.12688/wellcomeopenres.11741.2 (2017).

Daussy, C. 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, 563-77 (2014).

Fathman, J.W. et al. Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. Blood 118, 5439-47 (2011).

Firth, M.A. et al. Nfil3-independent lineage maintenance and antiviral response of natural killer cells. J. Exp. Med. 210, 2981-90 (2013).

Fujimura, N. et al. CCR2 inhibition sequesters multiple subsets of leukocytes in the bone marrow. Sci. Rep. 5, 11664 10.1038/srep11664 (2015).

Gao, Y. et al. Tumor immunoevasion by the conversion of effector NK cells into type 1 innate lymphoid cells. Nat. Immunol. 18; 1004 (2017).

Goh W. and Huntington, N.D. Regulation of Murine Natural Killer Cell Development Front. Immunol. 8, 130 (2017).

Geissmann, F. et al. Intravascular immune surveillance by CXCR6+ NKT cells patrolling liver sinusoids. PLoS Biol. 3, e113 10.1371/journal.pbio.0030113 (2005).

Jenne, C.N. et al. T-bet-dependent S1P5 expression in NK cells promotes egress from lymph nodes and bone marrow. J. Exp. Med. 206, 2469-81 (2009).

Kallies, A., and Good-Jacobson, K.L. Transcription Factor T-bet Orchestrates Lineage Development and Function in the Immune System. Trends Immunol. 38, 287-297 (2017).

Kim, S. et al. In vivo developmental stages in murine natural killer cell maturation. Nat. Immunol. 3, 523 (2002).

Klose C.S.N. et al. Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. Cell 157, 340 (2014).

Male, V. et al. The transcription factor E4bp4/Nfil3 controls commitment to the NK lineage and directly regulates Eomes and Id2 expression. J. Exp. Med. 211, 635-42 (2014).

Mayol, K., Biajoux, V., Marvel, J., Balabanian, K., Walzer, T. Sequential desensitization of CXCR4 and S1P5 controls natural killer cell trafficking. Blood 118, 4863-4871 (2011).
Narni-Mancinelli, E. et al. Fate mapping analysis of lymphoid cells expressing the NKp46 cell surface receptor. *Proc Natl Acad Sci USA* 108, 18324-9 (2011).

Park, E. et al. Toxoplasma gondii infection drives conversion of NK cells into ILC1-like cells. *Elife* 8, 8 (2019).

Pereira, J.P., An, J., Xu, Y., Huang, Y., and Cyster, J.G. Cannabinoid receptor 2 mediates the retention of immature B cells in bone marrow sinusoids. *Nat. Immunol.* 10, 403-11 (2009).

Pikovskaya, O. et al. Cutting Edge: Eomesodermin Is Sufficient To Direct Type 1 Innate Lymphocyte Development into the Conventional NK Lineage. *J. Immunol.* 196, 1449-54 (2016).

Ponzetta, A. et al. CX3CR1 regulates the maintenance of KLRG1+ NK cells into the bone marrow by promoting their entry into circulation. *J. Immunol.* 191, 5684-94 (2013).

Sojka, D.K. et al. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *eLife* 3, e01659 10.7554/eLife.01659 (2014).

Souza-Fonseca-Guimaraes, F., Cursons, J., and Huntington, N.D. The Emergence of Natural Killer Cells as a Major Target in Cancer Immunotherapy. *Trends Immunol.* 40, 142-158 (2019).

Spits, H. et al. Innate lymphoid cells—a proposal for uniform nomenclature. *Nat. Rev. Immunol.* 13, 145-9 (2013).

Szabo, S.J. et al. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100, 655-69 (2000).

Townsend, M.J. et al. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. *Immunity* 20, 477-94 (2004).

Turchinovich, G., Ganter, S., Bärenwaldt, A., and Finke, D. NKp46 Calibrates Tumoricidal Potential of Type 1 Innate Lymphocytes by Regulating TRAIL Expression. *J. Immunol.* 200, 3762 (2018).

Varet, H., Brillet-Guéguen, L., Coppée, J.Y. and Dillies, M.A. SARTools: A DESeq2- and EdgeR-Based R Pipeline for Comprehensive Differential Analysis of RNA-Seq Data. *PLoS One* 11, e0157022 (2016).

van Helden, M.J. 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, 2015-25 (2015).

Vivier, E. et al. Innate Lymphoid Cells: 10 Years On. *Cell* 174, 1054-1066 (2018).

Walzer, T. et al. Natural killer cell trafficking in vivo requires a dedicated sphingosine 1-phosphate receptor. *Nat. Immunol.* 8, 1337-1344 (2007).

Wang, Y., Dong, W., Zhang, Y., Caligiuri, M.A., and Yu, J. Dependence of innate lymphoid cell 1 development on NKp46. *PLoS Biol.* 16, e2004867 (2018).

Xu, W. et al. An Id2RFP-Reporter Mouse Redefines Innate Lymphoid Cell Precursor Potentials. *Immunity* 50; 1054 (2019).