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The transcriptional repressor NKAP is required for the development of iNKT cells

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Invariant natural killer T cells have a distinct developmental pathway from conventional $\alpha\beta$ T cells. Here we demonstrate that the transcriptional repressor NKAP is required for invariant natural killer T cell but not conventional T cell development. In CD4-cre NKAP conditional knockout mice, invariant natural killer T cell development is blocked at the double-positive stage. This cell-intrinsic block is not due to decreased survival or failure to rearrange the invariant V α 14-J α 18 T cell receptor- α chain, but is rescued by overexpression of a rec-V α 14-J α 18 transgene at the double-positive stage, thus defining a role for NKAP in selection into the invariant natural killer T cell lineage. Importantly, deletion of the NKAP-associated protein histone deacetylase 3 causes a similar block in the invariant natural killer T cell development, indicating that NKAP and histone deacetylase 3 functionally interact to control invariant natural killer T cell development.

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Invariant natural killer T (iNKT) cells are a distinct, innate-type lineage of $\alpha\beta$ T cells (reviewed in refs 1, 2). In contrast to conventional $\alpha\beta$ T cells that recognize peptide antigen presented on major histocompatibility complex (MHC) molecules, iNKT cells recognize glycolipids, such as α -GalCer and PBS-57, presented on the non-polymorphic MHC-like molecule CD1d. iNKT cells have limited T-cell receptor (TCR) diversity and express an invariant V α 14-J α 18 TCR α -chain combined with limited TCR β -chains. iNKT cells comprise approximately 3% of mature thymocytes and splenic T cells, but account for ~30% of the liver lymphocyte population. Mature iNKT cells can be primed to produce significant quantities of multiple cytokines, including interferon- γ and interleukin-4, within minutes to hours after stimulation.

iNKT cells follow a developmental pathway distinct from conventional T cells. At the CD4⁺CD8⁺ double-positive (DP) stage, rearrangement and expression of the canonical V α 14-J α 18 TCR, and recognition of its cognate ligand, initiate selection into the iNKT cell lineage. At the TCR α locus, rearrangements are biased towards proximal V and J segments³. The J α 18 segment required for the iNKT invariant chain, however, is located distally. Therefore, the canonical V α 14-J α 18 iNKT TCR α usually occurs as a secondary rearrangement. Furthermore, mutations in genes that affect DP T cell survival cause a preferential defect in iNKT as compared with conventional $\alpha\beta$ T cell development, as a shortened life span results in fewer secondary rearrangements^{4–6}. Although conventional $\alpha\beta$ T cells are selected on peptide/MHC expressed on thymic epithelial cells, iNKT cell development depends on positive selecting signals through recognition of glycolipid/CD1d complexes presented on DP T cells. In addition, stronger signals through the TCR are suggested to positively select DP T cells into iNKT cell lineage than conventional CD4 or CD8 T cells⁷.

NKAP associates with DNA by chromatin immunoprecipitation, although this is likely indirect as NKAP lacks any previously characterized DNA-binding domains. NKAP associates with histone deacetylase 3 (Hdac3), and the Hdac3-binding domain is required for repression of transcription. NKAP also associates with CBF1-interacting repressor (CIR), which is part of the Notch corepressor complex, and NKAP has been demonstrated to be a negative regulator of Notch signalling. Conditional deletion of NKAP early in T cell development using Lck-cre led to a severe block in $\alpha\beta$ T cell development at the DN3 to DP transition⁸. Deletion of NKAP at a later stage using CD4-cre, however, did not lead to any defects in the development or selection of conventional T cells in the thymus⁹. Here we show that deletion of NKAP with CD4-cre leads to an ablation of iNKT cell development. Furthermore, we show that deletion of NKAP-associated Hdac3 results in a similar disruption of iNKT cell development, implying functional interplay between these two factors that modulate gene expression.

Results

NKAP is required for iNKT cell development. To determine whether NKAP had a role in iNKT cell development, we examined the thymus, spleen and liver for the presence of iNKT cells using CD1d tetramers loaded with the glycolipid PBS-57, or using unloaded CD1d tetramers as control. In CD4-cre NKAP conditional knockout (cKO) mice, although conventional T-cell development proceeded normally⁹ there was a dramatic reduction of iNKT cells in the thymus (Fig. 1a). Similarly, iNKT cells were also missing from the spleen and liver of CD4-cre NKAP cKO mice (Fig. 1b). Therefore, the loss of NKAP leads to a severe block in iNKT cell development.

iNKT cell development is blocked at the DP stage. As iNKT cells develop, they proceed from the DP stage through four stages

defined by cell-surface expression of CD24, CD44 and NK1.1: cells progress from stage 0 (CD24^{hi}CD44⁻NK1.1⁻) to stage 1 (CD24^{-/lo}CD44^{-/lo}NK1.1⁻), then to stage 2 (CD24⁻CD44⁺NK1.1⁻) and finally to stage 3 (CD24⁻CD44⁺NK1.1⁺), as shown schematically in Fig. 1c. Using flow cytometry, we examined the various stages of iNKT cell development in CD4-cre NKAP cKO mice (Fig. 1c). In contrast to wild-type (WT) mice, the few tetramer⁺ thymocytes in CD4-cre NKAP cKO mice expressed CD24, indicative of stage 0 iNKTs. However, these cells did not express the iNKT canonical V α 14-J α 18 TCR α chain, and thus are not stage 0 iNKT (Fig. 1d)^{10–12}. Therefore, iNKT cell development in CD4-cre NKAP cKO mice is blocked at the DP stage.

The block in iNKT cell development is cell-intrinsic. The development of iNKT cells depends on positive selecting signals through glycolipids presented on DP T cells by the MHC class I-like molecule CD1d, as well as homotypic interactions through the SLAM family receptors Slamf1 (CD150) and Slamf6 (Ly108)¹³. To exclude the possibility that the block in iNKT development was due to lack of expression of any of these proteins in DP T cells, expression of CD1d, Slamf1 and Slamf6 was examined in WT and CD4-cre NKAP cKO mice. As shown in Fig. 2a, CD4 expression was equivalent in WT and CD4-cre NKAP cKO DP T cells. CD1d expression was decreased ~33% in CD4-cre NKAP cKO DP T cells as compared with WT. This change was unlikely to be the cause of the loss of iNKT cell development, as CD1d heterozygous mice express approximately half the amount of CD1d on the cell surface as WT B6 mice, but exhibit normal iNKT cell development¹⁴. Expression of Slamf1 and Slamf6 were slightly elevated (~30%) in CD4-cre NKAP cKO DP T cells as compared with WT. Therefore, the block in iNKT cell development in the absence of NKAP is not due to loss of expression of CD1d, Slamf1 or Slamf6. The failure in iNKT cell development could be due to an inability of NKAP-deficient cells to present endogenous glycolipids in CD1d. To determine if the block in iNKT cell development in CD4-cre NKAP cKO mice was cell-intrinsic, mixed bone marrow stem cell chimeras were generated. Bone marrow from CD4-cre NKAP cKO (CD45.2) mice and congenic B6.SJL (CD45.1) mice was used to reconstitute lethally irradiated B6.SJL recipients. Although thymocytes derived from both types of donor cells were observed, only WT B6.SJL-derived thymocytes, and not CD4-cre NKAP cKO thymocytes, developed into iNKT cells (Fig. 2b). Thus, the early block in iNKT cell development in CD4-cre NKAP cKO mice is cell-intrinsic. Furthermore, these data confirm that NKAP-deficient DP thymocytes cannot be selected into the iNKT cell lineage even in the presence of WT DP T cells. To understand the nature of the developmental defect, DP thymocytes from WT and CD4-cre NKAP cKO mice were examined by quantitative PCR (qPCR) for expression of genes necessary for iNKT cell development, including *SAP*^{15–17}, *Egr2* (ref. 18), *Fyn*^{19,20}, *c-Myc*^{21,22}, *c-Myb*⁴, *Runx1* (ref. 6)/*CBFB*, *T-bet*²³ and *HEB*⁵. However, all of these genes were expressed at similar levels (less than twofold difference in expression) in DP thymocytes from WT and CD4-cre NKAP cKO mice (Fig. 2c). Hence, the loss of NKAP does not alter the expression of many genes previously demonstrated to be required for iNKT cell development.

The block in development is not due to decreased DP survival. The rearrangement of the canonical V α 14-J α 18 TCR chain is usually a secondary rearrangement, as the J α 18 gene segment is located distally. Mice with mutations that effect DP T cell survival have a preferential defect in iNKT as compared with conventional $\alpha\beta$ T cell development, as a shortened life span results in fewer

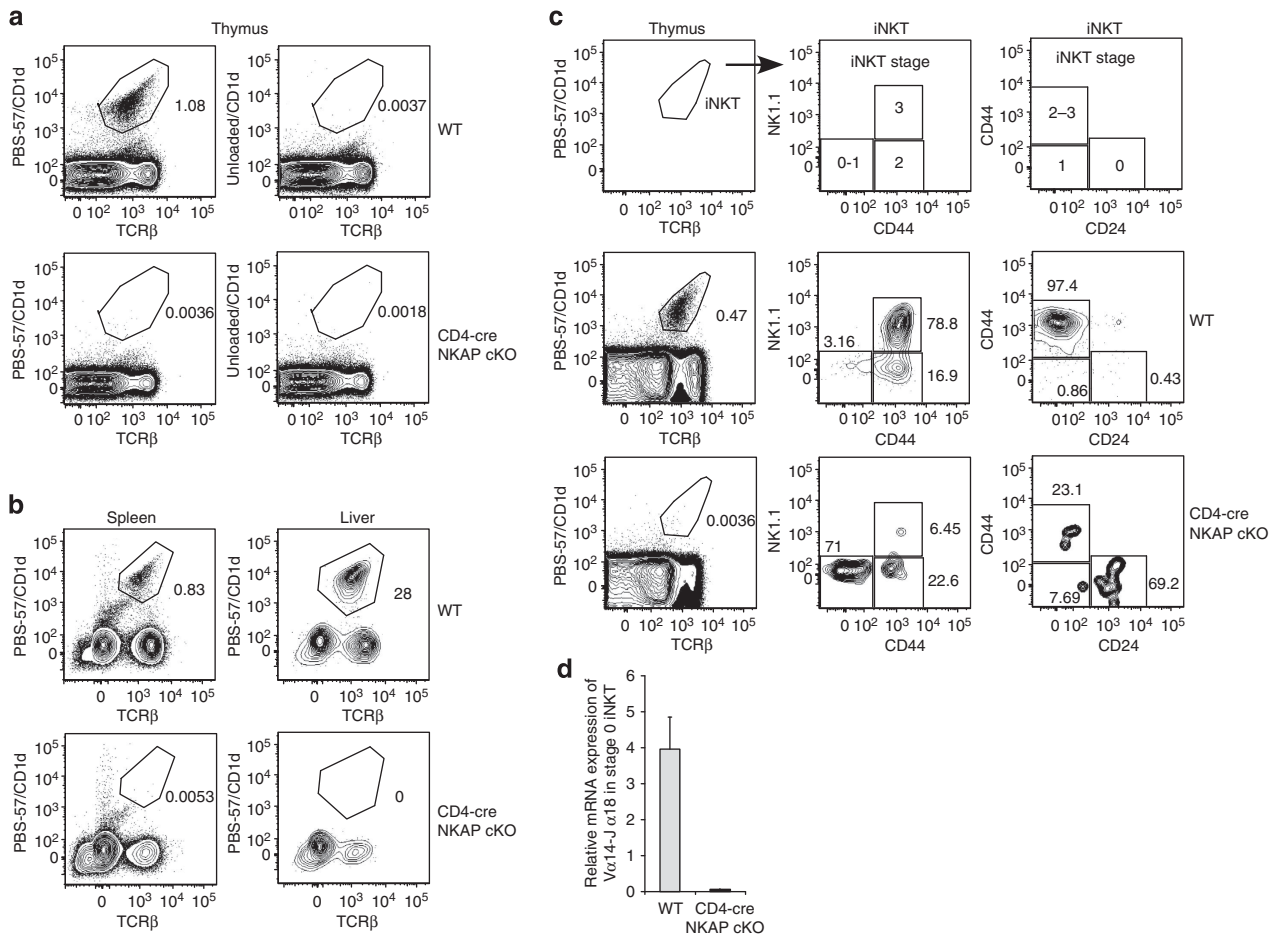


Figure 1 | NKAP is required for the development of iNKT cells. Lymphocytes from thymus (**a**), spleen and liver (**b**) were analysed for the presence of iNKT cells by flow cytometry using antibodies to TCRβ- and PBS-57-loaded CD1d tetramer to detect the presence of the canonical invariant TCR or empty CD1d tetramer alone without any associated glycolipid as a negative control. Data are representative of at least ten independent experiments. (**c**) Analysis of iNKT cell development from the earliest stage 0 (PBS-57/CD1d tetramer⁺ TCRβ⁺ CD24^{hi} CD44⁻) to the latest stage 3 (PBS-57/CD1d tetramer⁺ TCRβ⁺ CD44⁺ NK1.1⁺). Shown on the top is the gating strategy used in the analysis of iNKT cell development from WT thymocytes (middle row) and CD4-cre NKAP cKO thymocytes (bottom row). Data are representative of at least ten independent experiments. (**d**) Relative mRNA expression of the invariant Vα14-Jα18 TCR in stage 0 iNKT cells from WT and CD4-cre NKAP cKO mice. Stage 0 iNKT were isolated from CD4-cre NKAP cKO and WT littermates, by positive selection using PBS-57-loaded CD1d tetramer-PE/anti-PE-coated microbeads by MACS magnetic separation, followed by fluorescence-activated cell sorting for PBS-57/CD1d tetramer⁺ TCRβ⁺ CD24⁺ NK1.1⁻ stage 0 iNKT cells. cDNA was generated and examined for relative mRNA expression of the invariant Vα14-Jα18 TCR by qPCR. The data shown are the average expression of the invariant Vα14-Jα18 TCR from three WT and three CD4-cre NKAP cKO mice from three independent sorts. Data were normalized (=1) to the expression of the invariant Vα14-Jα18 TCR in one of the three WT mice. Error bars represent the s.e.m.

secondary rearrangements. iNKT cell development can be partially rescued in cases of decreased DP survival by overexpression of Bcl-2 or Bcl-x⁴⁻⁶. Thus, we examined whether decreased survival of DP thymocytes was the cause for the lack of iNKT cells in CD4-cre NKAP cKO mice. Using flow cytometry, we observed similar expression of Bcl-2, Nur77 and Annexin V in DP thymocytes from WT and CD4-cre NKAP cKO mice (Fig. 3a). By qPCR, mRNA transcript levels of Bcl-2, Bcl-x, Mcl-1 and Nur77 were similar in DP thymocytes of WT and CD4-cre NKAP cKO mice (Fig. 3b). We also generated Bcl-xL transgenic/CD4-cre NKAP cKO and Bcl-2 transgenic/CD4-cre NKAP cKO mice. However, overexpression of either Bcl-xL or Bcl-2 did not rescue iNKT cell development in these mice, as iNKT cells were not detected in the thymus (Fig. 3c), spleen and liver (Supplementary Fig. S1). Furthermore, if loss of NKAP led to decreased survival we would expect to have seen a decrease in relative chimerism in DP and semimature CD4 single-positive (SP) thymocytes derived from CD4-cre NKAP cKO progenitors in mixed radiation

chimeras (Fig. 2b). However, there was no difference in relative chimerism of DP and semimature CD4 SP population of CD4-cre NKAP-deficient thymocytes as compared with WT controls (Fig. 3d). Therefore, the early block in iNKT cell development in CD4-cre NKAP cKO mice is not due to decreased survival of DP thymocytes.

Recombination of iNKT TCRα is not altered by loss of NKAP.

Next, we examined whether loss of NKAP altered the recombination and expression of the canonical Vα14-Jα18 TCRα chain. One of the direct targets of NKAP is Skp2, a component of the E3 ubiquitin ligase Skp2-SCF⁸. Skp2-SCF regulates Rag2 protein expression during cell cycle, binding phosphorylated Rag2 and targeting it for degradation to prevent Rag-mediated recombination outside of the G1 cell cycle phase²⁴. Skp2 deficiency is associated with persistence of Rag activity throughout the cell cycle²⁴. As the loss of NKAP at the DP stage may lead

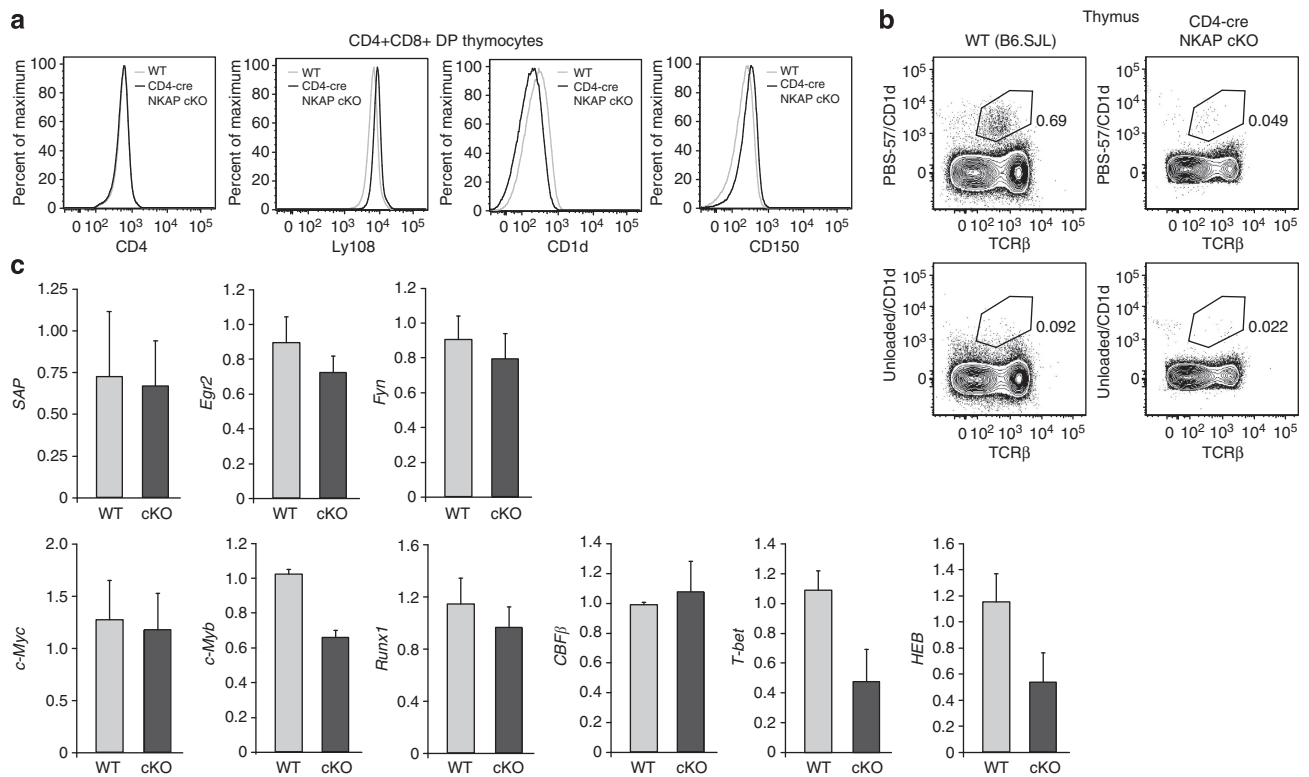


Figure 2 | Cell autonomous requirement for NKAP in iNKT cell development. (a) Expression of CD4, Ly108, CD1d and CD150 in DP thymocytes from CD4-cre NKAP cKO mice and WT littermate controls was examined using flow cytometry. The grey lines represent expression in WT DP thymocytes and the black lines represent expression in CD4-cre NKAP cKO DP thymocytes as denoted in the figure. Data are representative of at least two independent experiments. (b) Mixed stem cell chimeras were generated using equal proportions of bone marrow from WT B6.SJL congenic mice (CD45.1⁺) and CD4-cre NKAP cKO mice (CD45.2⁺), to reconstitute lethally irradiated B6.SJL recipients. Ten weeks later, thymocytes were examined for the development of iNKT cells (as in Fig. 1) using the CD45.1 allelic marker to distinguish the contribution from each genotype. Results shown are representative of three mixed stem cell chimeras. (c) Expression of genes required for iNKT cell development. cDNA from fluorescence-activated cell-sorted DP thymocytes from CD4-cre NKAP cKO and WT littermates was examined for expression of *SAP*, *Egr2*, *Fyn*, *c-Myc*, *c-Myb*, *Runx1*, *CBFβ*, *T-bet* and *HEB*. Data shown are the average from two WT and two CD4-cre NKAP cKO mice from independent sorts. Data are normalized to the relative expression in one of the WT mice (= 1). Error bars represent s.e.m.

to overexpression of *Skp2*, we considered the possibility that this could suppress distal rearrangements, including $V\alpha 14\text{-}J\alpha 18$, through enhanced degradation of *Rag2*. To test this, we crossed our CD4-cre NKAP cKO mice with *Rag2*-T490A knock-in mice, which have a mutation in the phosphorylation site required for the periodic destruction mediated by *Skp2*-SCF²⁴. However, iNKT cell development remained blocked in CD4-cre NKAP cKO/*Rag2*-T490A mice (Fig. 4a), indicating that an increase in *Skp2*-SCF-mediated *Rag2* ubiquitination is not responsible for the failure to generate iNKT cells.

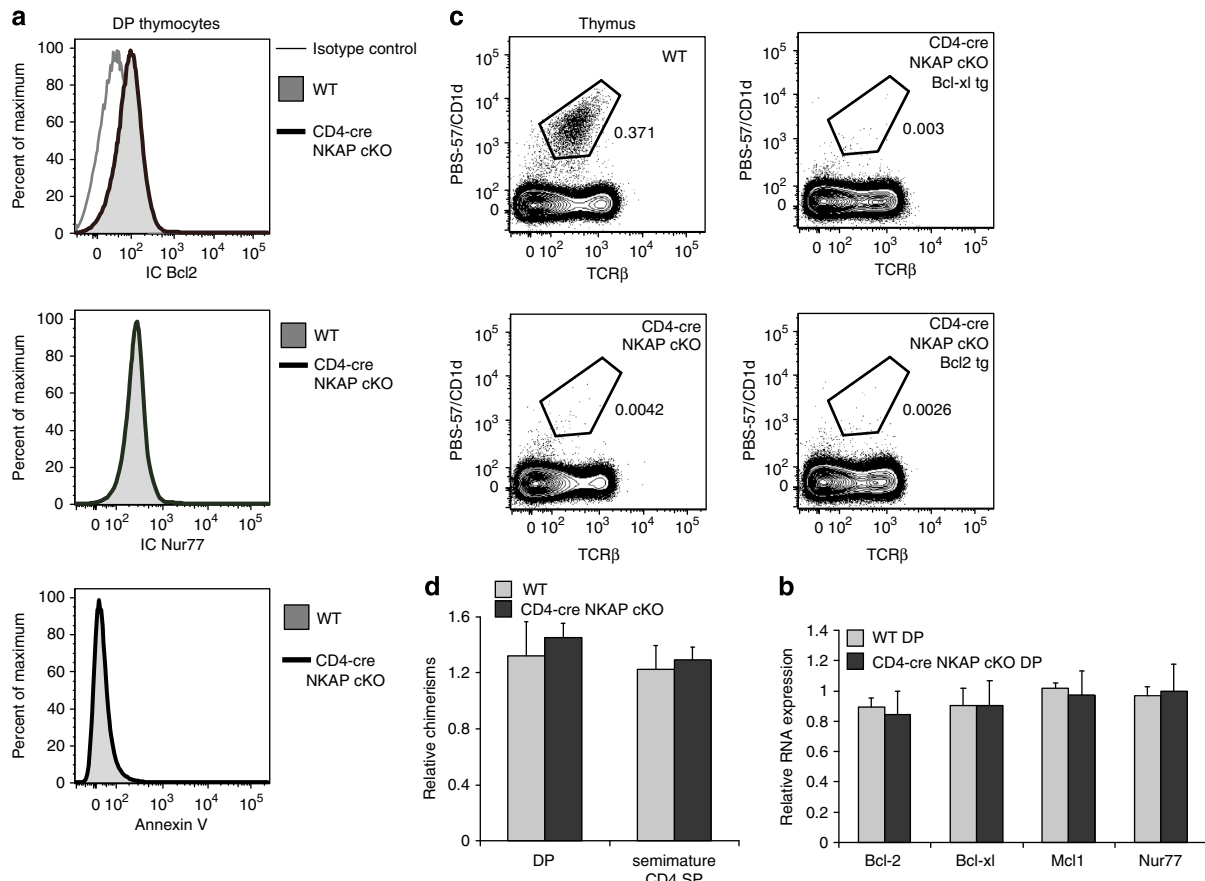
To directly examine recombination of the canonical $V\alpha 14\text{-}J\alpha 18$ TCR α chain, DP thymocytes from CD4-cre NKAP cKO and WT mice were sorted. DNA rearrangement of $V\alpha 14\text{-}J\alpha 18$ was examined by semi-qPCR (Fig. 4b). Rearrangement of the invariant $V\alpha 14\text{-}J\alpha 18$ chain was similar between NKAP-deficient and WT thymocytes, demonstrating that the loss of NKAP does not result in a failure to recombine $V\alpha 14$ with $J\alpha 18$, to generate the canonical iNKT TCR α chain. Hence, the lack of iNKT cells in CD4-cre NKAP cKO mice is not due to a failure in rearrangement of the canonical $V\alpha 14\text{-}J\alpha 18$ TCR α chain.

The loss of NKAP in DP thymocytes could also affect the expression level of the iNKT cell receptor; hence, we assessed the level of $V\alpha 14\text{-}J\alpha 18$ TCR expression in mature iNKT cell after loss of NKAP. Using ER-cre NKAP cKO mice, we induced NKAP deletion *in vivo* by 4-hydroxy-tamoxifen (4-OHT) and analysed the mean fluorescence intensity of iNKT TCR by tetramer

staining with a PBS-57-loaded CD1d tetramer. As shown in Fig. 4c, there was no change in the expression of $V\alpha 14\text{-}J\alpha 18$ TCR in 4-OHT-treated ER-cre NKAP cKO as compared with 4-OHT-treated WT mice. Thus, deletion of NKAP does not alter expression of the TCR on iNKT cells.

Overexpression of $V\alpha 14\text{-}J\alpha 18$ TCR rescues iNKT development.

As NKAP deficiency did not alter DP survival, $V\alpha 14\text{-}J\alpha 18$ rearrangement or iNKT TCR expression, another possibility is that NKAP functions in positive selection of DP thymocytes into the iNKT cell lineage. Stronger TCR signals are needed to positively select DP thymocytes into the iNKT lineage than conventional T cells⁷. Therefore, transgenic overexpression of $V\alpha 14\text{-}J\alpha 18$ may rescue iNKT cell development in CD4-cre NKAP cKO mice. However, although TCR expression normally does not appear at the cell surface until the DP stage, many TCR transgenes are expressed at the double-negative (DN) stage²⁵. This is a significant complication, as premature expression of a rearranged TCR may allow for selection before conditional deletion at the DP stage using CD4-cre. Therefore, a new transgenic line in which expression of $V\alpha 14\text{-}J\alpha 18$ was delayed until the DP stage was needed. To restrict the expression of the transgene, the $V\alpha 14\text{-}J\alpha 18$ TCR chain was inserted into a modified pT α Cass transgene vector with a reconstituted complete TCR α locus control region (LCR), as was previously described for the



generation of 'rec-HY' transgenics²⁶. This vector contains recombination signal sequences (RSS) and a 5-kb stuffer fragment inserted into the coding sequence after the initiating methionine, thus preventing expression of the transgene until the stuffer fragment is removed by RAG-mediated recombination. Using this system to express the HY TCR α chain in rec-HY mice, the timing and regulation of expression of the HY TCR α chain mirrors that of endogenous TCR α ²⁶. This system was used here to generate 'rec-V α 14' mice. Flow cytometry confirmed that the rec-V α 14 transgene is not expressed earlier than the endogenous iNKT TCR (Supplementary Fig. S2).

As shown in Fig. 5a, when crossed to CD4-cre NKAP cKO mice, expression of rec-V α 14 rescued development of iNKT cells in the thymus, and restored iNKT cells in the periphery (Fig. 5a). We examined the expression of the invariant V α 14-J α 18 TCR in WT, rec-V α 14 transgenic mice and rec-V α 14/CD4-cre NKAP cKO mice by measuring the mean fluorescence intensity of a CD1d-PBS-57-loaded tetramer stain using flow cytometry. There was ~20% increase in the expression of rec-V α 14-J α 18 TCR α chain in rec-V α 14 transgenic and rec-V α 14/CD4-cre NKAP cKO mice than that in the endogenous V α 14-J α 18 TCR of WT mice (*P*-value < 0.01 and *P*-value < 0.001, respectively; Fig. 5b).

We also examined the absolute number of various stages of iNKT cells in the WT, rec-V α 14 and rec-V α 14/CD4-cre NKAP cKO mice. We observed a statistically significant increase in the number of immature stage 0, 1 and 2 (NK1.1⁻) iNKT cells in the rec-V α 14 and rec-V α 14/CD4-cre NKAP cKO mice as compared with WT mice (Fig. 5c), as may be expected due to increased expression of this transgene. However, there was no statistically significant change in the absolute number of mature stage-3 iNKT cells in the WT, rec-V α 14 and rec-V α 14/CD4-cre NKAP cKO mice. Thus, ectopic overexpression of the V α 14-J α 18 TCR α chain rescues iNKT cell development in the absence of NKAP.

The block in iNKT development is not due to Notch activation.

Previously, we demonstrated that NKAP is a transcriptional repressor and a component of the Notch corepressor complex. NKAP deletion during early T-cell development using *Lck-cre* led to an increase of 8- to 20-fold in the expression of the Notch target genes *Deltex*, *CD25* and *Hes1* in DP T cells⁸. Interestingly, recent evidence suggests that suppression of Notch signalling may be required for iNKT development to proceed. Embryonic stem cells can differentiate in culture into early CD24^{hi}CD44^{lo} iNKT

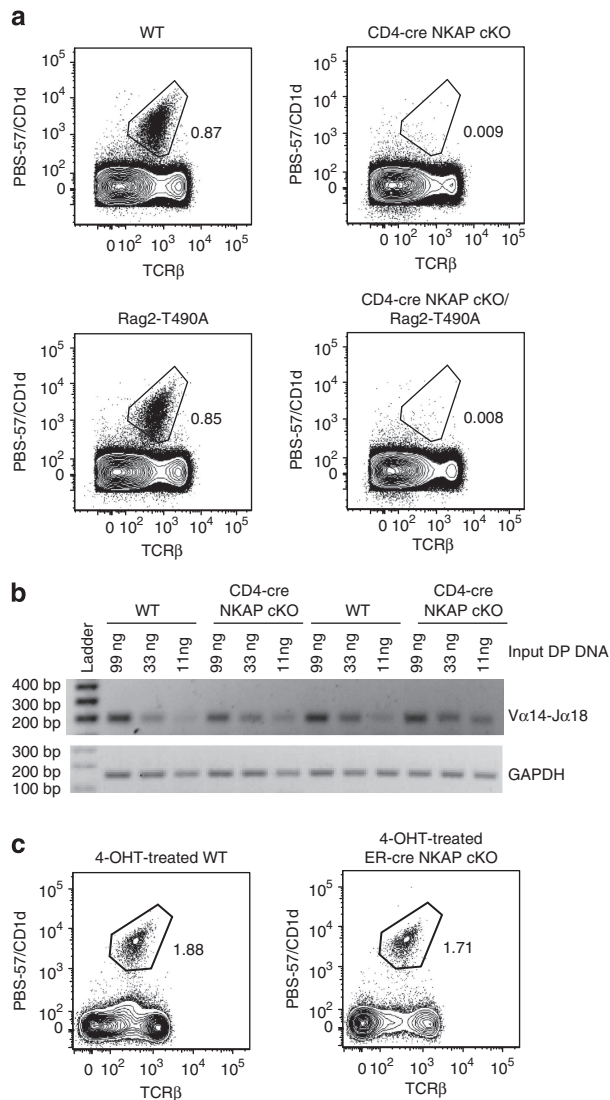


Figure 4 | The block in development is not because of failure to rearrange the V α 14-J α 18 TCR α chain. (a) The presence of iNKT cells in WT, CD4-cre NKAP cKO, Rag2(T490A) and CD4-cre NKAP cKO/Rag2(T490A) mice was examined using flow cytometry. Analysis was done as described in Fig. 1. Data are representative of three independent experiments. (b) V α 14-J α 18 recombination was examined by semi-qPCR by serial dilution (99, 33 or 11 ng per PCR reaction) of genomic DNA from DP thymocytes from two WT and two CD4-cre NKAP cKO mice from independent sorts. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was examined as a control. (c) The expression of invariant V α 14-J α 18 TCR of iNKT cells in 4-OHT-treated WT and ER-cre NKAP cKO mice was measured by the presence of PBS-57-CD1d-loaded tetramer stain using flow cytometry. Data are representative from four mice in each group.

Figure 5 | Transgenic expression of invariant rec-V α 14-J α 18 TCR α chain rescues iNKT cell development. (a) The presence of iNKT cells in the thymus, liver and spleen from WT, CD4-cre NKAP cKO, recV α 14-J α 18 TCR transgenic and CD4-cre NKAP cKO/recV α 14-J α 18 TCR mice was examined as in Fig. 1. Data are representative of at least four mice per group from three independent experiments. (b) The expression of invariant V α 14-J α 18 iNKT TCR from WT to recV α 14 transgenic and WT to CD4-cre NKAP cKO/recV α 14-J α 18 TCR transgenic mice was measured by the mean fluorescence intensity (MFI) of PBS-57-CD1d-loaded tetramer using flow cytometry. The difference in relative MFI is statistically significant using Student's *t*-test ($P < 0.01$ and $P < 0.001$, respectively). The relative MFI is mean from seven WT, nine recV α 14-J α 18 TCR transgenic and six CD4-cre NKAP cKO/recV α 14-J α 18 TCR transgenics mice from six independent experiments. (c) Absolute number of stages 0, 1, 2 and 3 iNKT cells in WT, recV α 14-J α 18 TCR transgenic and CD4-cre NKAP cKO/recV α 14-J α 18 TCR transgenics was calculated. The absolute number is the mean of four WT, six recV α 14-J α 18 TCR transgenic and six CD4-cre NKAP cKO/recV α 14-J α 18 TCR transgenic mice from three independent experiments. The differences in absolute numbers of immature stage 0, 1 and 2 iNKT cells from WT, recV α 14-J α 18 TCR transgenic and CD4-cre NKAP cKO/recV α 14-J α 18 TCR transgenics is statistically significant, using Student's *t*-test as denoted in the figure, whereas there are no differences in the absolute number of mature stage 3 iNKT cells (N.S., not significant).

cells²⁷, when cultured on the well-characterized OP9/Dll1 system²⁸. However, for iNKT maturation to proceed, early (stage 0) CD24^{hi}CD44^{lo} iNKT cells had to be transferred to OP9 stromal cells that lacked Dll1. One interpretation of these data are that Notch signalling must be downregulated for iNKT development to proceed²⁷. As NKAP is a negative regulator of Notch signalling, one possibility was that the lack of iNKT cells in the CD4-cre NKAP cKO mouse results from constitutive activation of the Notch target genes. To test this, we examined iNKT cell development in CD4-cre NKAP cKO mice that also lacked RBP-J κ ²⁹, the transcription factor through which cleaved intracellular Notch activates transcription. As shown in Fig. 6, iNKT development was normal in CD4-cre RBP-J κ cKO mice, demonstrating that Notch signalling is not necessary for the development of iNKT cells. However, iNKT cell development was still abrogated in CD4-cre NKAP cKO/RBP-J κ cKO mice, demonstrating that the block in iNKT cell development in CD4-cre NKAP cKO mice is independent of the role of NKAP as a negative regulator of Notch signalling.

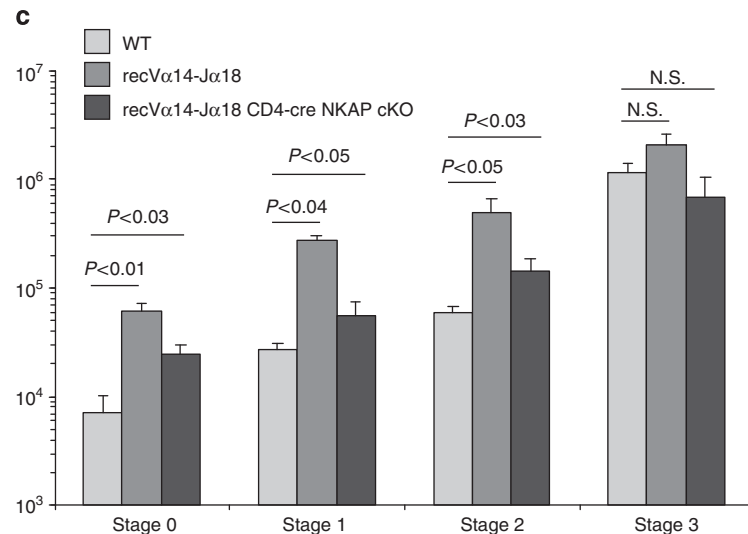
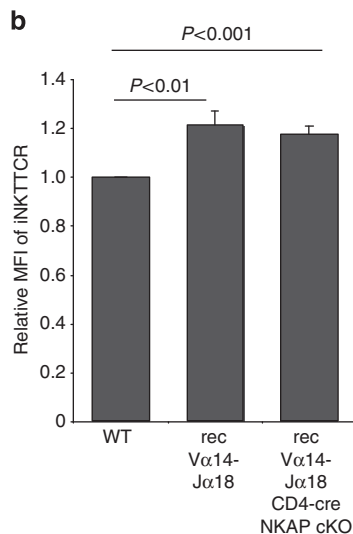
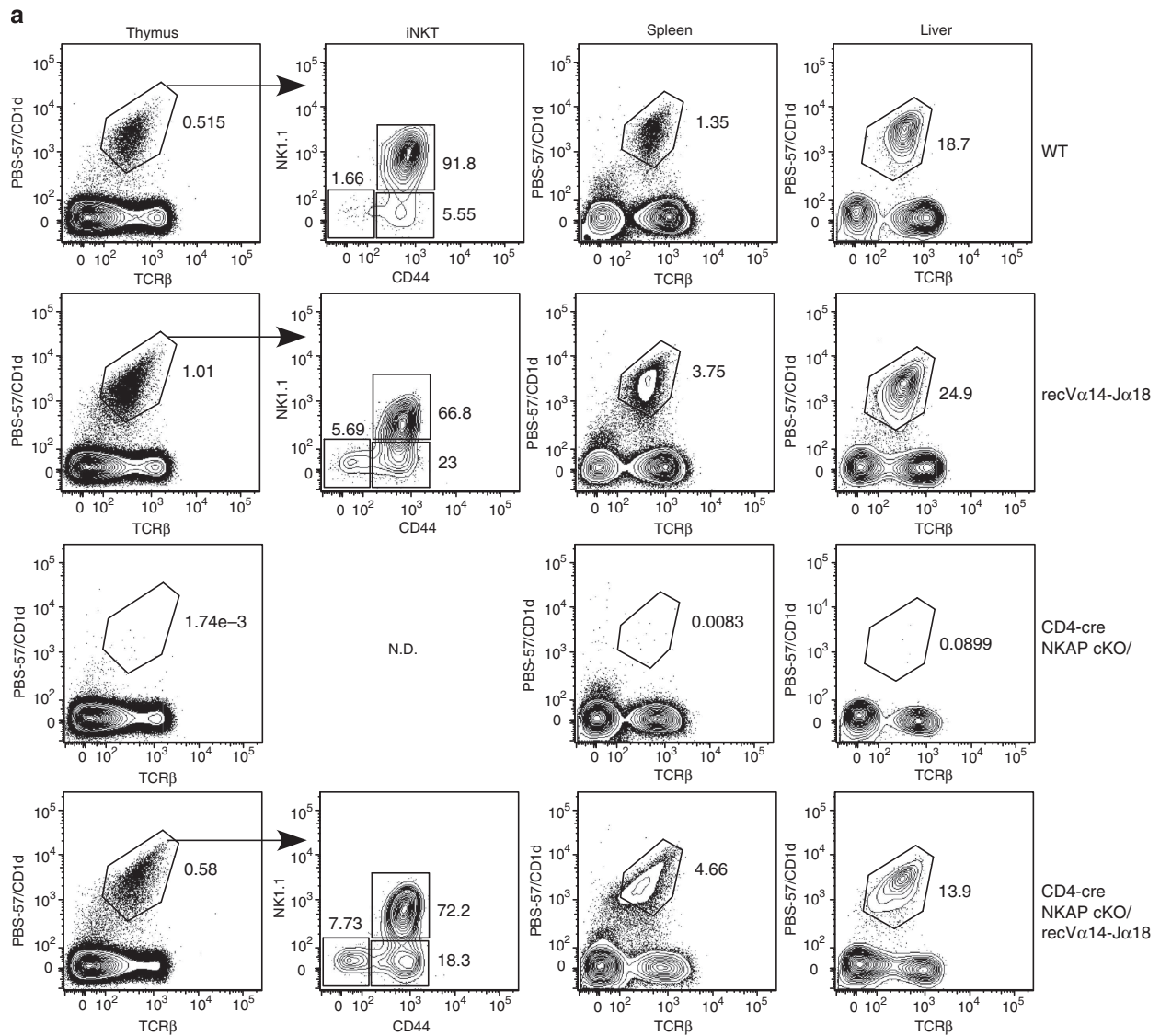
iNKT cell development requires Hdac3. If the regulation of iNKT cell development by NKAP is independent of Notch activation, then NKAP must regulate additional pathways. We previously demonstrated that NKAP associates with Hdac3, and that transcriptional repression by NKAP requires its C-terminal DUF926 domain (domain of unknown function 926) that mediates binding to Hdac3 (ref. 8). If NKAP works together with Hdac3 to regulate iNKT cell development, then we would predict that thymocytes deficient in Hdac3 would phenocopy NKAP-deficient thymocytes and also exhibit a severe block in iNKT cell development. Although there was no gross alteration in conventional T-cell development in CD4-cre Hdac3 cKO mice, they also failed to generate iNKT cells, similar to CD4-cre NKAP cKO mice (Fig. 7). Although Hdacs broadly regulate gene expression, the requirement of Hdac3 for iNKT but not conventional T cell development demonstrates that Hdac3 has unique functions in iNKT cells that cannot be compensated for by any other Hdac family member. The specificity of the defect for the iNKT cell lineage in the absence of either NKAP or Hdac3 indicates that these two associated proteins work together to regulate genes critical for this lineage.

Discussion

We have demonstrated the absolute requirement for NKAP in the development of iNKT cells. iNKT cell development is blocked at the DP stage in CD4-cre NKAP cKO mice. Although there were small differences in expression of CD1d and SLAM family receptors in NKAP-deficient DP thymocytes compared with WT, the developmental defect in iNKT cells in CD4-cre NKAP cKO mice was cell-intrinsic as demonstrated by mixed bone marrow chimeras. The absence of iNKT cells in CD4-cre NKAP cKO mice was not due to decreased survival of thymocytes, as

overexpression of a Bcl-xL or Bcl-2 transgene did not rescue iNKT cell development. There was no difference in the mRNA expression of anti-apoptotic Bcl-2 family genes *Bcl-2*, *Bcl-xL* and *Mcl-1*. Similar levels of V α 14-J α 18 rearrangement in genomic

DNA from NKAP-deficient and WT DP thymocytes demonstrate that the defect in iNKT cell development is not due to an inability to rearrange the invariant TCR α chain. However, ectopic overexpression of the canonical V α 14-J α 18 TCR did restore



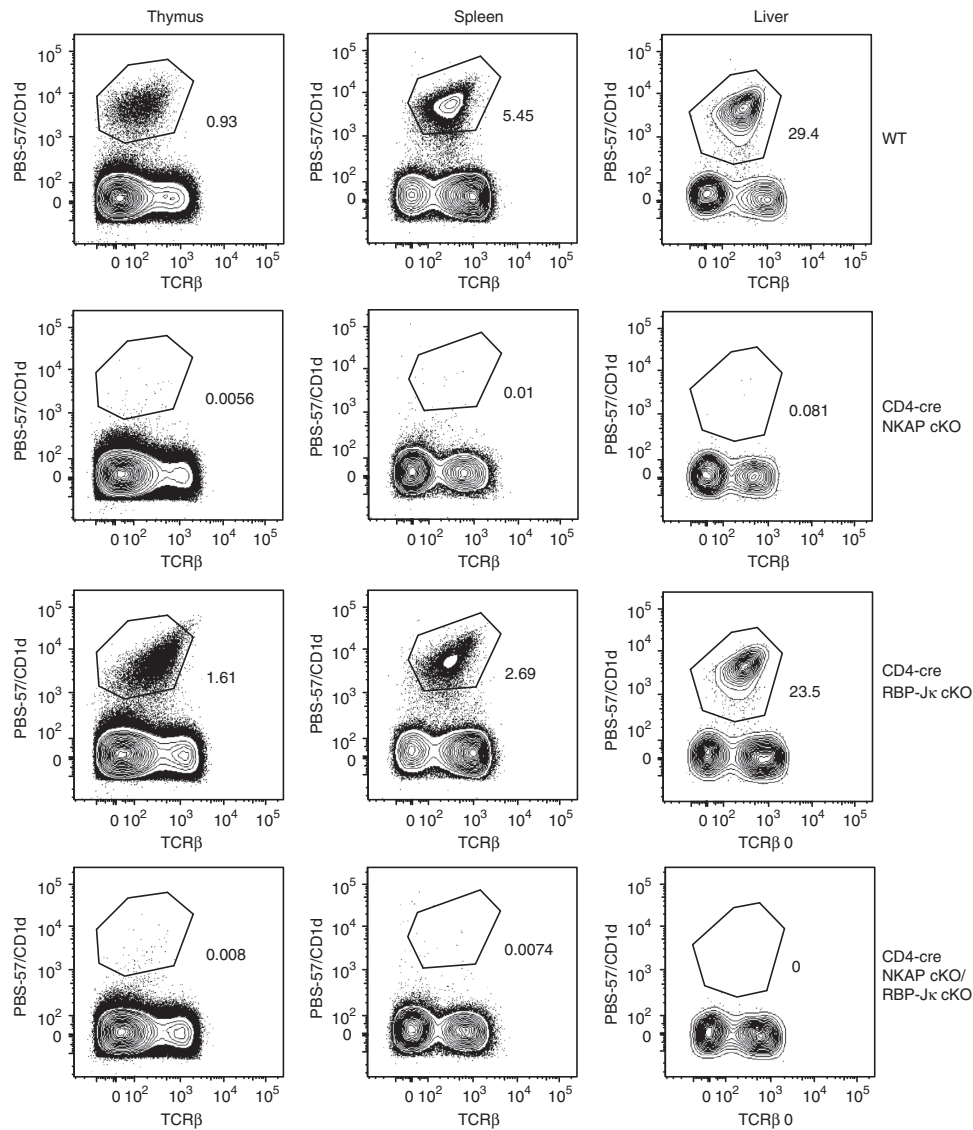


Figure 6 | The block in iNKT cell development due to loss of NKAP is independent of Notch activation. The presence of iNKT cells in the thymus, liver and spleen from WT, CD4-cre NKAP cKO, CD4-cre RBP-Jκ cKO and CD4-cre NKAP cKO/RBP-Jκ cKO mice were examined as in Fig. 1. Data are representative of four independent experiments.

iNKT cell development in CD4-cre NKAP cKO mice. Examination of genes that are required for iNKT cell development did not show changes in the expression of these genes resulting from NKAP deficiency, and the defect was also independent of Notch signalling. However, loss of the NKAP-associated protein histone deacetylase Hdac3 resulted in a similar lack of iNKT cells in the thymus, spleen and liver, suggesting that NKAP works with Hdac3 at an early stage in iNKT cell development. Therefore, NKAP controls entry of thymocytes into the iNKT cell lineage.

To determine whether iNKT cell development in CD4-cre NKAP cKO mice could be rescued by a $V\alpha 14$ - $J\alpha 18$ TCR transgene, a new line of mice had to be generated. Typically, TCR transgenes, even under the control of CD4 regulatory elements, are expressed prematurely at the DN stage²⁵. This is a significant caveat to any examination of iNKT cell development using CD4-cre cKO mice, as the expression of the invariant TCR at the DN stage may permit positive selection into the iNKT cell lineage before cre-mediated deletion. Deleting NKAP earlier in development was not possible for these studies, as Lck-cre NKAP cKO mice have a severe defect in $\alpha\beta$ T-cell development at the DN3 to DP transition, resulting in a 20-fold decrease in absolute

DP thymocyte numbers⁸. Therefore, a new line of $V\alpha 14$ - $J\alpha 18$ transgenic mice was generated, 'rec- $V\alpha 14$ - $J\alpha 18$ ' in which $V\alpha 14$ - $J\alpha 18$ is regulated by the endogenous TCR α LCR, using a modified pT α Cass transgene vector in which expression is dependent on RAG-mediated recombination, to replicate the endogenous pattern of expression for TCR α and prevent premature expression of the transgene²⁶. However, this transgene is expressed at higher levels than the endogenous iNKT TCR, which may be due to use of $V\alpha 10$ promoter in the transgenic construct rather than the endogenous $V\alpha 14$ promoter. The increased expression of the transgenic rec- $V\alpha 14$ - $J\alpha 18$ TCR compensated for a defect in positive selection into lineage in NKAP-deficient DP thymocytes. However, we cannot exclude the possibility that the rec- $V\alpha 14$ - $J\alpha 18$ is expressed earlier in DP cell development, although it is not expressed at DN stage, than endogenously rearranged $V\alpha 14$ - $J\alpha 18$ TCR, as the transgene uses optimized RSS sequences and is separated by only 5 kb, which may account for increased proportion of early-stage iNKT in rec- $V\alpha 14$ - $J\alpha 18$ mice.

The role of NKAP in iNKT cell development is independent of its role as a negative regulator of Notch signalling, as abrogation

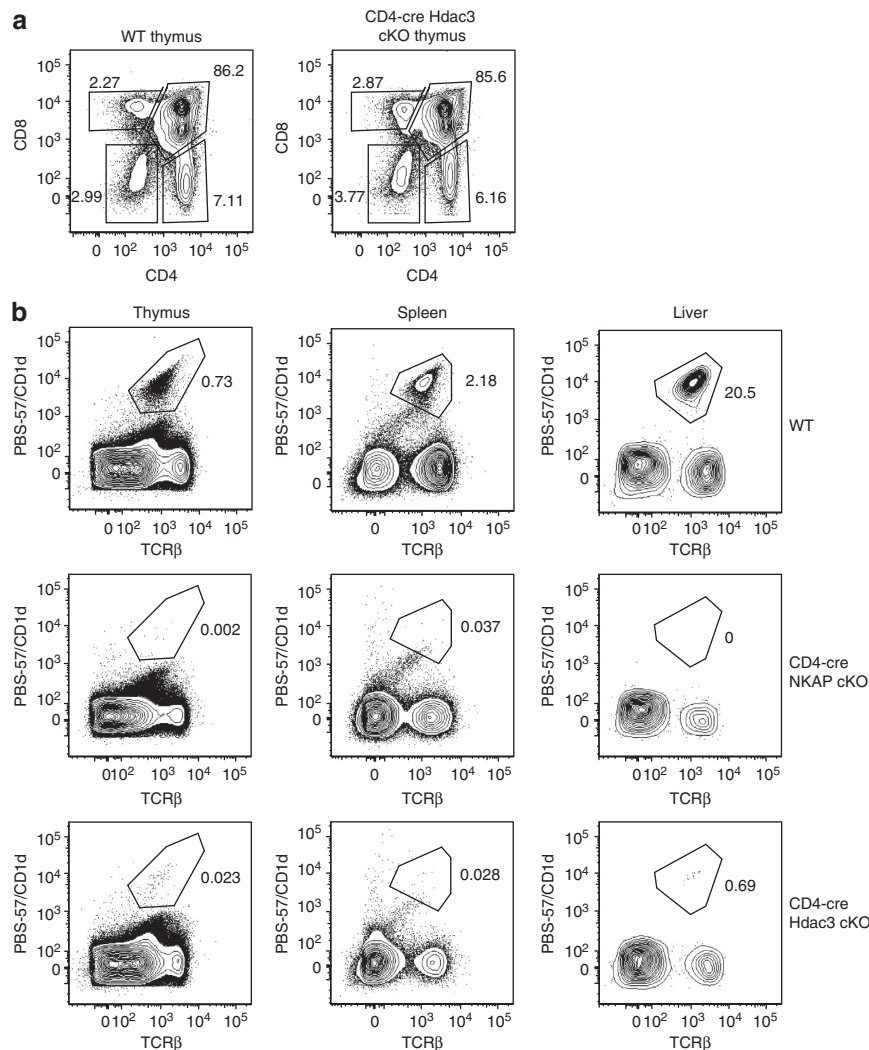


Figure 7 | Conditional deletion of Hdac3 also leads to a block in iNKT cell development. (a) DN, DP and SP thymocytes in WT and CD4-cre Hdac3 cKO mice were examined. Data shown are representative of four independent experiments. (b) The presence of iNKT cells in the thymus, liver and spleen from WT, CD4-cre NKAP cKO and CD4-cre Hdac3 cKO mice was examined as in Fig. 1. Data are representative of four independent experiments.

of Notch-mediated activation through deletion of the transcription factor RBP-J κ did not alleviate the block in iNKT cell development upon NKAP deletion. However, CD4-cre-mediated deletion of Hdac3, which associates with NKAP, resulted in the same phenotype as NKAP deletion, that is, loss of iNKT but not CD4 or CD8 SP cells. Although Hdacs broadly regulate gene expression, the specific requirement for Hdac3 for entry into the iNKT cell lineage demonstrates that Hdac3 has unique functions in iNKT cells that cannot be compensated for by any other Hdac family member. The specificity of the defect for the iNKT cell lineage in the absence of either NKAP or Hdac3 indicates that these two associated proteins work together to regulate genes critical for entry into the iNKT cell lineage. Interestingly, there is a complete block in iNKT cell development in CD4-cre Runx1 cKO mice, even though V α 14-J α 18 rearranges normally and is expressed equivalently at the mRNA level in Runx1-deficient DP thymocytes⁶. Runx1 has also been shown to associate with Hdac3 (ref. 30). Whether Runx1, Hdac3 and NKAP work together to regulate iNKT cell development is currently under investigation.

Methods

Mice. The generation of CD4-cre NKAP cKO mice was previously described⁹. RBP-J κ floxed mice were generated and provided by T. Honjo (Kyoto University,

Kyoto, Japan²⁹). Human Bcl-2 transgenic mice were generated by S. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA³¹) and provided by A. Singer (National Institutes of Health, Bethesda, MD). Bcl-xl³² and ER-cre³³ transgenic mice were purchased from the Jackson Laboratories. CD4-cre mice³⁴ were purchased from Taconic through the National Institute of Allergy and Infectious Disease emerging models programme. Rag2 (T490A) mice have been previously described²³. Hdac3 *fl/fl* mice have been previously described³⁵. Construction of the Rec-V α 14 transgene mirrors that previously described for the generation of Rec-HY mice²⁶. Briefly, the rearranged iNKT TCR α chain V α 14-J α 18 was cloned into the pT α Cass transgenic vector with a complete TCR α LCR. The start codon from V α 14 was separated from the rest of the coding sequence by a 5-kb 'stuffer' fragment containing RSS recombination sequences, thus requiring Rag-mediated recombination for expression of the transgenic TCR. Rec-V α 14-J α 18 mice were generated by the transgenic facility at the Memorial Sloan-Kettering Cancer Center.

Mice were housed in a barrier facility, and all experiments were performed under the guidelines and with the approval of the Mayo Clinic or Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee. All animals were analysed between 8 and 16 weeks of age. All genetically altered mice were examined with either littermate or age-matched controls.

Generation of radiation chimeras. B6.SJL donors and recipients were purchased from National Cancer Institute Frederick, and radiation chimera generation was performed as previously described⁹.

Flow cytometry. Fluorescence-activated cell sorting analysis was performed on an LSRII flow cytometer (BD) and all experiments were analysed using Flow Jo (Tree Star). After size gating on FSC-A/SSC-A, doublet exclusion was done using

both FSC-H/FSC-W and SSC-H/SSC-W. Dead cells were excluded from analysis by staining with 4',6-diamidino-2-phenylindole (DAPI). The following tetramers were obtained through the NIH Tetramer Facility: PE-conjugated CD1d tetramer, and PE-conjugated CD1d tetramer loaded with the glycolipid PBS-57 that is recognized by iNKT TCRs. All other reagents for flow cytometry were purchased from Becton Dickinson, eBioscience or Biologend.

Intracellular staining. Thymocytes were stained for surface CD4 and CD8 before being fixed and permeabilized using Foxp3 fix/perm buffer kit (eBioscience) as per the manufacturer's instructions. Cells were then stained for intracellular Bcl-2 and Nur77.

Isolation of stage 0 iNKT cells. All sorting was performed with a FACSAria (Becton Dickinson). To isolate stage 0 iNKT cells, thymocytes were stained with PE-labelled PBS-57-loaded CD1d tetramer. Cells were positively selected with anti-PE magnetic beads (Miltenyi Biotec) and ran through a separation column. Stage 0 iNKT cells were sorted based on PBS-57-CD1d⁺ TCRβ⁺ CD24⁺ NK1.1⁻ from positively selected cells. DP cells were sorted from thymocytes stained and gated with antibodies to CD4 and CD8.

Quantitative PCR. mRNA was isolated from sorted populations using an RNeasy kit (Qiagen). Complementary DNA from stage 0 iNKT cells was generated and amplified using an Ovation PicoSL WTA kit (NuGen). cDNA from DP thymocytes was generated using Superscript III (Invitrogen). Taqman probes (Applied Biosystems) for c-Myc, c-Myb, Runx1, Bcl-xL, Bcl-2, Nur77, Mcl-1 and 18S were used. All other targets were examined using SybrGreen, using previously published primers for Cβ, SAP³⁶, Egr2 (ref. 37), Fyn³⁸, TCR Cα³⁹, CBFβ⁴⁰, T-bet⁴¹, HEB⁴¹, HPRT⁴¹ and glyceraldehyde 3-phosphate dehydrogenase⁴². For the canonical Vα14-Jα18, the following primers were used:

Vα14: 5'-TCAAACAGGACACAGGCAAAGG-3'
Jα18: 5'-TCCTAAGGCTGAACCTCTATC-3'

An ABI RT-PCR StepOne Plus System (Applied Biosystems) was used and relative expression was calculated with the 2-ΔΔCT method⁴³.

Genomic analysis of TCR rearrangement. Genomic DNA was isolated from sorted DP thymocytes (Qiagen). DNA concentration was measured using a Nanodrop (Thermo Scientific). DNA was diluted so that either 99, 33 or 11 ng were used in each PCR reaction. The primers used to measure Vα14-Jα18 were the same as described above for use in qPCR. Glyceraldehyde 3-phosphate dehydrogenase primers were previously described⁴⁴. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

4-OHT induction of ER-cre NKAP cKO mice. WT and ER-cre NKAP cKO mice were injected intraperitoneally with 250 μl of 20 mg ml⁻¹ 4-OHT (Sigma) dissolved in corn oil (Sigma) daily for 5 consecutive days. Mice were allowed to rest 1 day before analysis.

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Author contributions

P.T., R.S., M.N.H. and D.Mc.W. performed flow cytometry; J.D. and D.B.S. generated the rec-V α 14-J α 18 mice; M.S. performed the analysis of V α 14-J α 18 DNA rearrangement; P.T. and D.Mc.W. performed the qPCR analysis; R.S., M.N.H., S.T. and J.A. genotyped our colony and re-confirmed the genetic alterations in all mice used experimentally; S.D. generated Rag2-T490A knock-in mice; V.S.S. and M.N.H. generated the mixed stem cell chimeras; S.H. generated Hdac3 floxed mice; P.T., D.B.S. and V.S.S. designed and conceived of the overall research and analysed the data; P.T. wrote the manuscript and V.S.S. edited the manuscript, which was subsequently reviewed and approved by all authors.

Additional information

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