HHS Public Access

Author manuscript

J Immunol. Author manuscript; available in PMC 2017 June 15.

Published in final edited form as:

J Immunol. 2016 June 15; 196(12): 4987–4998. doi:10.4049/jimmunol.1501653.

NKAP regulates iNKT cell proliferation and differentiation into ROR-γt expressing NKT17 cells

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Abstract

Invariant Natural Killer T (iNKT) cells are a unique lineage with characteristics of both adaptive and innate lymphocytes, and recognize glycolipid presented by an MHC Class I-like CD1d molecule. During thymic development, iNKT cells also differentiate into NKT1, NKT2 and NKT17 functional subsets that preferentially produce cytokines IFN-γ, IL-4 and IL-17, respectively, upon activation. Newly selected iNKT cells undergo a burst of proliferation, which is defective in mice with a specific deletion of NKAP in the iNKT cell lineage, leading to severe reductions in thymic and peripheral iNKT cell numbers. The decreased cell number is not due to defective homeostasis or increased apoptosis, and is not rescued by Bcl-xL overexpression. NKAP is also required for differentiation into NKT17 cells, but NKT1 and NKT2 cell development and function are unaffected. This failure in NKT17 development is rescued by transgenic expression of PLZF; however, the PLZF transgene does not restore iNKT cell numbers or the block in positive selection into the iNKT cell lineage in CD4-cre NKAP cKO mice. Therefore, NKAP regulates multiple steps in iNKT cell development and differentiation.

Introduction

Invariant Natural Killer T (iNKT) cells are a unique lineage of T cells with characteristics of both adaptive and innate lymphocytes ([1–3]). Similar to conventional T cells, iNKT cells express a rearranged αβTCR, however the TCRα chain and the specificity of the TCR for glycolipids presented by CD1d is fixed. Similar to innate lymphocytes, iNKT cells respond quickly to stimulation, producing effector cytokines within hours. The development of iNKT cells occurs in the thymus, where their development diverges from conventional T cells at the double-positive (DP) thymocyte stage. Upon positive selection into the iNKT cell

[1]This work was supported by an NIH R21 grant to V.S.S., internal funds from the Mayo Clinic to V.S.S., and the Mayo Graduate School to P.T.

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P.T. performed the experiments and wrote the manuscript. P.T. and V.S.S. designed the research, analyzed the data and edited the manuscript, D.S. provided suggestions for experiments, feedback and analysis, and edited the manuscript. M.C., D.M., P.B. and M.C. managed the mouse colony, performed genotyping and confirmed genotypes of all mice used experimentally.

lineage, iNKT cells proceed through distinct stages of development (stages 0–3), characterized by differential expression of surface markers, CD24, CD44, and NK1.1. At Stage 0 ($CD24^+$ $CD44^{lo}$ $NK1.1^-$), iNKT cells express PLZF, the transcription factor characteristic of the iNKT cell lineage, which is required for their innate-like function ([4–8]). At Stage 1 ($CD24^-$ $CD44^{lo}$ $NK1.1^-$), iNKT cells undergo a burst of proliferation, which is dependent on c-myc expression and efficient cellular metabolism ([9–13]). At Stage 2 ($CD24^-$ $CD44^+$ $NK1.1^-$), iNKT cells can egress to the periphery (mainly to the spleen and liver) or remain in the thymus ([14]). iNKT cells can also differentiate into functional subsets (NKT1, NKT2, NKT17), which are analogous to CD4 T helper cell subsets ([15]). The differentiation of iNKT cells is characterized by the preferential expression of the transcription factors T-bet, GATA3, and ROR-γt, respectively; and the cytokines they predominately produce, IFN-γ, IL-4, and IL-17, respectively. The functional definitions for NKT subsets overlap with traditional staging. Stage 3 iNKT ($CD24^-$ $CD44^+$ $NK1.1^+$) are exclusively NKT1, while NKT17 are exclusively found within Stage 2. NKT2 overlap with both Stage 1 and Stage 2. Undifferentiated iNKT cells are found in Stage 0–2 ([15, 32]). Various studies have shown the importance of cytokines such as IL-15 for iNKT homeostasis, and signaling via IL-7 and TGF-β for the development and differentiation of NKT17 cells ([16–20]). Additional NKT subsets such as Bcl-6 expressing NKTfh and IL-10 producing NKT10 cells are induced in peripheral tissues upon glycolipid challenge ([21, 22]).

The transcriptional repressor NKAP is crucial for the development of conventional T cells and iNKT cells ([23–26]). Previously, we demonstrated that the loss of NKAP at the double positive (DP) stage of thymocyte development, using CD4-cre, leads to normal conventional single positive (SP) T cell development (but has a defect in peripheral T cell maturation) but a complete block in the development of all iNKT cells, including Stage 0. Therefore, NKAP is required for the positive selection of cells into the iNKT cell lineage at the DP stage. To determine whether NKAP is required for iNKT cell development and differentiation after selection into the iNKT cell lineage, we generated PLZF-cre NKAP cKO mice. In this model, deletion of NKAP occurs after iNKT lineage selection at Stage 0, which bypasses the requirement for NKAP at the DP stage. Here, we show that NKAP is required for the proliferation burst during iNKT cell development. In addition, NKAP is also required for differentiation into NKT17 cells, which is rescued by an lck-PLZF transgene.

Materials and Methods

Mice

NKAP fl/fl mice ([23]), Rag1-GFP mice ([27]), Lck-PLZF mice ([6]), PLZF-cre mice ([28]), PLZF-GFP ([29]) and CD4-cre NKAP cKO mice ([24]) were previously described. Bcl-xL trangenic mice ([30]) were purchased from the Jackson laboratory. Mice were housed in a barrier facility and experiments were performed at the Mayo Clinic with the approval of the Institutional Animal Care and Use Committee. All mice were analyzed between the ages of 4 to 10 weeks. All genetically modified mice were examined with either littermate or age-matched controls, which may include either NKAP floxed mice, PLZF-cre only, or WT mice. As no difference was observed between NKAP floxed mice or PLZF-cre only mice as compared to WT mice, they are all considered here as “WT” controls.

Generation of radiation chimeras

B6.SJL mice were purchased from NCI Frederick, and radiation chimera generation was performed as previously described ([23], [25])

Flow cytometry

FACS analysis was performed on an LSRII flow cytometer (BD) or Attune NxT (Life Technologies) and all experiments were analyzed using FlowJo (Tree Star). Analysis was done on cells after size gating (FSC-A/SSC-A) and doublet exclusion (both FSC-H/FSC-W and SSC-H/SSC-W). Dead cells were excluded from analysis by staining with DAPI or Fixable Viability Dye (eBioscience or Tonbo Biosciences). For each FACS analysis of iNKT cell population, at least 1 million events was collected for WT thymocyte samples while, at least 3.5 million events were collected for the PLZF-cre NKAP cKO thymocytes. PBS-57 (glycolipid) loaded flurorochrome (PE, BV421, APC) conjugated mCD1d tetramers and unloaded tetramer controls were obtained from the NIH Tetramer Facility. All other reagents for flow cytometry were purchased from BD Bioscience, eBioscience, Biolegend, Cell Signaling Technologies or Tonbo Biosciences.

Isolation of iNKT cells

All sorting was performed with a FACSAria (Becton Dickinson). To isolate iNKT cells, thymocytes were labeled with PE conjugated CD1d-PBS57 loaded tetramer. Cells were positively selected with anti-PE coated magnetic beads (Miltenyi Biotec) and separated through an (LS) MACS separation column. Postively selected cells were then labeled with other antibodies cojugated with different fluorochromes to distinguish iNKT cells at different stages of development. For the sort, gated iNKT cells (PBS57-CD1d^{+} TCRβ^{+}) were distinguished as Stage 1 (CD24^{-}CD44^{lo}NK1.1^{-}), Stage 2 (CD24^{-}CD44^{hi}NK1.1^{-}) and Stage 3 (CD24^{-}CD44^{hi}NK1.1^{+}). Cells were sorted directly into lysis buffer from an RNeasy kit (Qiagen).

Quantitative PCR

mRNA was isolated from sorted populations using an RNeasy kit (Qiagen). cDNA from sorted iNKT cells was generated and amplified using an Ovation PicoSL WTA V2 kit (NuGen). Taqman probes (Applied Biosystems) for NKAP, Rag1 and Gapdh were used. An ABI StepOne Plus System (Applied Biosystems) was used and relative expression was calculated with the 2-ΔΔCT method ([31]). The relative mRNA expression of *NKAP* or *Rag1* from the sorted iNKT cells was analyzed using Q-PCR. Statistics were calculated comparing NKAP expression in PLZF-cre NKAP cKO to WT cells using unpaired student's t test.

Stimulation of iNKT cells

For cytokine analysis, isolated thymocytes were stimulated or left unstimulated overnight in complete culture media (RPMI) with 200nM PMA and 1μM Ionomycin. 3 hours after stimulation, BrefeldinA/Monensin (BD) was added to stop secretion of cytokines for intracellular flow cytometry anaylsis. Cell were harvested the following day and stained for intracellular cytokines.

Intracellular staining

Cytoplasmic and nuclear proteins were examined via intracellular flow cytometry. Thymocytes were labelled with surface markers for iNKT cells before being fixed and permeablized with Foxp3 fix:perm buffer (eBioscience) or cytofix/cytoperm (BD), according to their respective intracellular staining protocols.

Results

NKAP is required for iNKT cell development after Stage 0

To study the role of NKAP in iNKT cell development after positive selection into the lineage, we generated PLZF-cre NKAP conditional knock out (cKO) mice ([28]). Lineage tracing analysis with PLZF-cre demonstrated that PLZF-cre was transiently expressed in a proportion of progenitors to the HSC pool during embryonic development, leading to labeling of a subset of HSCs and all subsequent differentiated hematopoietic cells ([28, 32]). However, NKAP is required for HSC maintenance and survival ([23]). Inducible deletion of NKAP using Mx1-cre leads to hematopoietic failure and loss of the HSC pool within days ([21]). Hence, any HSCs that deleted NKAP due to transient expression of PLZF-cre during embryogenesis will not persist to contribute to hematopoiesis in the adult. Thus, in this model, only NKAP-sufficient HSCs are present in the adult and PLZF-cre will lead to NKAP deletion only in the iNKT cell lineage after induction of PLZF at Stage 0. To confirm this, we examined NKAP mRNA expression in sorted DP thymocytes of WT and PLZF-cre NKAP cKO mice using q-PCR, where one of the primers is located in an exon that would be deleted by cre expression. NKAP mRNA expression was similar in DP thymocytes between WT and PLZF-cre NKAP cKO (Supplemental Figure 1a). NKAP is also required for conventional T cell development at the DN3 to DP transition, and for post-positive selection maturation of SP thymocytes ([23, 25]). No defects in the development or maturation of conventional T cells in the thymus of PLZF-cre NKAP cKO was observed (Supplemental Fig. 1b). Thus, in developing T cells from the PLZF-cre NKAP cKO mice, NKAP is not deleted in conventional T cells. NKAP deletion in PLZF-cre NKAP cKO mice will initiate at iNKT Stage 0, bypassing the block observed from DP thymocytes to iNKT cell lineage in CD4-cre NKAP cKO mice and allowing analysis of the function of NKAP at subsequent stages of iNKT cell development and differentiation ([26]). To confirm that expression of PLZF starts at Stage 0, we examined the expression of PLZF using a PLZF-GFP reporter mouse ([29]). In PLZF-GFP mice, a subset of Stage 0 cells expressed PLZF-GFP. At Stage 1, 80% of iNKT cells are positive for GFP (Supplemental Fig. 1c). Thus, in PLZF-cre NKAP cKO mice during thymocyte development, NKAP deletion initiates at Stage 0 in the iNKT cell lineage.

Development of iNKT cells in the thymus is characterized by stages 0–3: Stage 0 ($CD24^+$ $CD44^{lo}$ $NK1.1^-$), Stage 1 ($CD24^-CD44^{lo}$ $NK1.1^-$), Stage 2 ($CD24^-$ $CD44^{hi}$ $NK1.1^-$) and Stage 3 ($CD24^-$ $CD44^{hi}$ $NK1.1^+$). In PLZF-cre NKAP cKO mice, iNKT cells in the thymus were dramatically reduced in number, and strongly biased towards Stage 0 ($CD24^+$ CD1d-PBS57 $tetramer^+$) (Fig. 1a). Consistent with a block in thymic iNKT cell development, there were also dramatically fewer iNKT cells in the spleen and liver of PLZF-cre NKAP cKO mice (Fig. 1b). Analysis of the absolute number of iNKT cells at each stage in WT and

PLZF-cre NKAP cKO mice demonstrated that while there were equivalent numbers of Stage 0 iNKT cells, there was a severe reduction in absolute numbers at all subsequent iNKT cell stages (Fig. 1c). Since $CD24^+$ PBS57/CD1d $tetramer^+$ Stage 0 cells are newly selected iNKT cells from the DP thymocytes, they express CD69 and Egr2 ([33], [34]). To determine if Stage 0 cells present in PLZF-cre NKAP cKO was similar to WT Stage 0 cells, the expression of CD69 and Egr2 as markers of iNKT positive selection was examined. No significant difference was seen in the expression of CD69 and Egr2 between Stage 0 iNKT cells of WT and PLZF-cre NKAP cKO mice (data not shown). There was a 7.0-fold decrease in the absolute number of Stage 1, a 5.5-fold decrease in the number of Stage 2 and a 23-fold decrease in the absolute number of Stage 3 iNKT cells in PLZF-cre NKAP cKO mice as compared to WT mice. Therefore, there is a block in iNKT cell development starting at Stage 1 in PLZF-cre NKAP cKO mice. One possibility was that the iNKT cells that developed beyond Stage 0 in PLZF-cre NKAP cKO mice escaped cre-mediated deletion. To test this we sorted Stage 1, Stage 2, and Stage 3 iNKT cells from the thymus of WT and PLZF-cre NKAP cKO mice and analyzed mRNA expression of NKAP by Q-PCR, where one primer was specific for a site located in a cre-deleted exon of NKAP ([23]). NKAP was efficiently deleted in all iNKT cells of PLZF-cre NKAP cKO (Fig. 1d). Therefore, the few iNKT cells that develop in PLZF-cre NKAP cKO beyond Stage 0 were not cells that had escaped NKAP deletion. Thus, the block in development and deletion of NKAP in PLZF-cre NKAP cKO mice is specific to the iNKT cell lineage.

Block in iNKT cell development in PLZF-cre NKAP cKO mice is cell intrinsic

To address whether the role of NKAP in iNKT cell development is cell intrinsic, we generated mixed bone marrow chimeras. Bone marrow from PLZF-cre NKAP cKO mice ($CD45.1^-$) or B6 WT ($CD45.1^-$) littermate mice were mixed 1:1 with B6.SJL ($CD45.1^+$) congenic bone marrow and used to reconstitute lethally irradiated B6.SJL mice. iNKT cell development was analyzed 8–10 weeks later. As PLZF-cre will not delete NKAP in conventional T cells, the relative chimerism within thymic conventional T cells (TCR-β^{hi}, CD1d:PBS-57 $tetramer^-$) was used for a baseline comparison (Fig. 2a). In the control B6 WT/B6.SJL mixed chimera, the relative contribution of iNKT cells from B6 WT did not decrease at any stage of iNKT cell development. However, in the PLZF-cre NKAP cKO/ B6.SJL mixed chimeras, the relative contribution of iNKT cells from the PLZF-cre NKAP cKO donor started to decrease at Stage 0–1 and dropped severely at subsequent stages. In particular, almost no Stage 3 iNKT cells were generated from the PLZF-cre NKAP cKO donor in the mixed chimera (Fig. 2a). Quantification of the average relative chimerism of $CD45.1^-$ in the mixed chimeras of the WT/SJL in the WT SJL: B6 PLZF-cre NKAP cKO chimeras showed a significant decrease in representation of PLZF-cre NKAP cKO ($CD45.1^-$) in Stage 0–1, 2, 3 iNKT cells, while there was no reduction in representation by the WT ($CD45.1^-$) iNKT cells in the WT/SJL chimeras (Fig. 2b). Thus, in the mixed chimera, the loss of NKAP leads to a cell intrinsic block in iNKT cell development.

The defect in NKAP deficient iNKT cells is not due to abnormal survival or dysregulated homeostasis

Immune cells require cytokines for their survival and homeostasis. The critical cytokine for iNKT cell homeostasis and survival is IL-15, which controls expression of Bcl-xL ([19], [35]).

We examined the surface expression of the IL-15 receptor, IL-15Rα, IL-15Rβ (CD122), and γc (CD132) in iNKT cells from WT and PLZF-cre NKAP cKO mice. There was no difference in the expression of IL-15Rα, IL-15Rβ, or γc between WT and NKAP-deficient iNKT cells in Stage 1, Stage 2, or Stage 3 (Fig. 3a). In addition, expression of Mcl-1 and Bcl-xL in PLZF-cre NKAP cKO iNKT cells was not decreased as compared to WT Stage 1, Stage 2, and Stage 3 iNKT cells (Fig. 3b). The expression of the pro-apoptotic protein Bim is associated with negative selection. Therefore, to address if decreased iNKT cell number was due to increased negative selection, we examined intracellular Bim expression. However, the expression of Bim in NKAP deficient iNKT cells was also similar to WT iNKT cells in Stage 1, Stage 2, and Stage 3 (Fig. 3b). To determine whether the decreased iNKT cell numbers is due to increased apoptosis in PLZF-cre NKAP cKO mice, we examined binding of Annexin V. There was no significant difference in Annexin V binding in NKAP-deficient iNKT cells compared to WT iNKT cells (Fig. 3c). Therefore the decrease in iNKT cell number is not due to increased apoptosis or decreased cell survival in PLZF-cre NKAP cKO mice. Previously, it was shown that transgenic overexpression of Bcl-xL rescued a defect in iNKT homeostasis and survival in IL-15 receptor knockout mice ([19]). Therefore, we generated Bcl-xL transgenic/PLZF-cre NKAP cKO mice ([30]). However, the defect in iNKT cell numbers in PLZF-cre NKAP cKO mice was not rescued by the transgenic overexpression of Bcl-xL (Fig. 3d). We confirmed that the Bcl-xL transgene led to higher intracellular Bcl-xL expression in Bcl-xL transgenic/PLZF-cre NKAP cKO mice as compared to PLZF-cre NKAP cKO mice (Fig. 3d). Therefore, the defect in iNKT cell development upon the loss of NKAP is not due to altered iNKT cell survival or homeostasis.

Decreased proliferation in NKAP-deficient iNKT cells

As iNKT cells develop, they undergo a burst of proliferation at Stage 1 ([13], [34], [35]). To understand if the reduction in iNKT cell numbers was due to decreased proliferation in PLZF-cre NKAP cKO mice, we examined intercellular expression of the proliferation capacity marker Ki-67. Expression of Ki-67 was similar between WT and NKAP deficient iNKT cells across all developmental stages (Fig. 4a). This indicated that NKAP deficient iNKT cells have the same potential to proliferate as WT iNKT cells. However, Ki-67 expression does not address whether there is a difference in the rate of proliferation. To examine differences in the rate of proliferation, we crossed PLZF-cre NKAP cKO mice with a Rag1-GFP reporter mouse to generate Rag1-GFP/ PLZF-cre NKAP cKO mice. Here, GFP is knocked into one allele of the *Rag1* locus ([27]). During T cell development, transcription of *Rag1* is turned off at the DP thymocyte stage after the TCRα gene has successfully rearranged. However, GFP protein is very stable with a long half-life ([36]). This reporter is often used to mark recent thymic emigrants in the periphery, as GFP expression persists in conventional T cells (which do not proliferate after the DP stage) for approximately 3 weeks ([37]). Thus, as iNKT cells proliferate early during development, dilution of Rag1-GFP reporter may be used to indicate the extent of proliferation. This method would be analogous to CFSE labeling, but would allow for measurement of iNKT cell proliferation during development in vivo. In WT mice, the expression of Rag1-GFP is highest at Stage 0, as cells have just entered iNKT cell lineage from the DP stage. The expression of GFP decreases as iNKT cells proliferate at Stage 1, and by Stage 2, most are GFP^{low}. There is almost no GFP expression in Stage 3 iNKT cells, in part due to preceding dilution during proliferation but

also due to most Stage 3 iNKT cells in the thymus being long-lived resident iNKT cells. In Rag1-GFP/ PLZF-cre NKAP cKO mice while Rag1-GFP reporter expression at Stage 0 was similar to (Rag1-GFP) WT mice, significant changes were observed at Stage 1 and 2 (Fig. 4b). There was dramatically higher GFP expression in Stage 1 and Stage 2 iNKT cells from Rag1-GFP/PLZF-cre NKAP cKO mice as compared to Rag1-GFP WT mice (Fig. 4b). To quantify the changes in proliferation, the MFI of GFP expression in Stage 0 and the lowest 30% of cells of Stage 1 iNKT cells was examined (Fig. 4c). While there is no significant difference in Rag1-GFP MFI at Stage 0 between WT and PLZF-cre NKAP cKO mice, there is a 23-fold difference in GFP expression between the lowest 30% (representing those cells that have proliferated the most at that stage) in NKAP-deficient as compared to WT Stage 1 NKT cells. The higher GFP expression indicates NKAP-deficient Stage 1 NKT underwent significantly fewer rounds of cell divisions compared to WT. This demonstrates that efficient proliferation of developing iNKT cells is dependent on NKAP, and that the significant reduction of absolute iNKT cell numbers in PLZF-cre NKAP cKO mice is primarily due to decreased proliferation at Stage 1. To confirm that increased GFP expression was not due to abnormal re-expression of Rag1 in NKAP-deficient iNKT cells, sorted iNKT cells were examined for Rag1 mRNA transcripts using QPCR. Rag1 mRNA was not expressed in either WT or NKAP deficient iNKT cells at Stage 1, Stage 2, or Stage 3 (data not shown). Therefore, higher GFP expression was not due to aberrant *Rag1* re-expression in NKAP deficient iNKT cells and instead solely reflects a decreased rate of proliferation. The expression of c-myc, which is important for intra-thymic iNKT cell proliferation ([9], [12]), was also examined in WT and PLZF-cre NKAP cKO mice. Similar expression of c-myc in WT and NKAP deficient iNKT cells at stages 1 and 2 were observed (Fig. 4d). Hence, the decreased rate of proliferation in NKAP deficient iNKT cell is not due to a defect in c-myc expression. Proliferating cells have higher metabolism and require GLUT1, transferrin receptor (CD71) and L-amino acid transporter (CD98) ([10]). The expression of GLUT1, CD98, and CD71 was similar between WT and NKAP deficient iNKT cells at Stages 1 and 2 (Fig. 4d), indicating that expression of critical proteins required for cellular metabolism during proliferation are not the cause of decreased proliferation in NKAP deficient iNKT cells.

NKAP is required for the generation of NKT17 cells

During development iNKT cells differentiate into functional subsets and produce cytokines similar to CD4 T helper subsets ([15]). Differentiation into NKT1, NKT2, and NKT17 is characterized by the preferential expression of transcription factors (T-bet, GATA3, and ROR-γt, respectively) and production of cytokines (IFN-γ, IL-4, and IL-17, respectively) upon stimulation. To determine whether NKAP has a role in the functional differentiation of iNKT cells, we analyzed the proportions of these populations using T-bet and PLZF as was originally described ([15]) to identify NKT1, NKT2, and NKT17 cells, as well as specifically examining each lineage by their transcription factor (T-bet in NKT1 cells, $GATA3^{hi}/PLZF^{hi}$ in NKT2 cells, and ROR-γt in NKT17 cells) within the iNKT cell pool (Fig. 5a). Expression of Gata3 is not restricted to NKT2 exclusively but is expressed in all NKT subsets although at a higher level in NKT2 cells. Therefore, NKT2 cells were identified by their higher expression of both Gata3 and PLZF as compared to the rest of the NKT cell pool. To affirm that our gating strategy for NKT2 subset using $Gata3^{hi}/PLZF^{hi}$ was specific, we examined

the surface expression of IL-17Rβ which is preferentially expressed on NKT2 cells ([15]) on the Gata3^{hi}/PLZF^{hi} population as compared to the rest of the iNKT cell pool after gating out Stage 0 (CD24^{+}) iNKT cells. As expected Gata3^{hi}/PLZF^{hi} cells expressed higher levels of IL-17Rβ as compared to non-NKT2 Gata3^{lo}/PLZF^{lo} NKT cell population (Supplemental Fig. 2a), confirming the use of Gata3 and PLZF to identify these cells. In PLZF-cre NKAP cKO mice, there was a similar percentage of GATA3^{hi}/PLZF^{hi} expressing NKT2 cells within the iNKT cell pool; however there were fewer T-bet expressing NKT1 cells. Interestingly, in PLZF-cre NKAP cKO mice, almost no ROR-γt expressing NKT17 cells were found (Fig. 5a), demonstrating dependence on NKAP in the generation of these cells. Although NKT1 and NKT2 cells are present in the thymus of PLZF-cre NKAP cKO mice, their numbers are significantly reduced as compared to WT mice (Supplemental Fig. 2b). Expression of Bcl-6 and CXCR5, indicative of NKT_{FH} ([21]), was examined in the PLZF^{lo} NK1.1^{-} 'NKT17' gate to determine if there was skewing of NKT_{FH} precursors in the thymus of PLZF-cre NKAP cKO mice. However, neither Bcl-6 nor CXCR5 were increased in NKAP-deficient compared to WT PLZF^{lo} NK1.1^{-} iNKT cells (data not shown). We also examined whether NKAP was required for the effector functions of different NKT subsets. The production of cytokines was examined in all three functional subsets using PLZF and NK1.1 to distinguish each population: NKT1 (PLZF^{+} NK1.1^{+}), NKT2 (PLZF^{hi} NK1.1^{-}) and NKT17 (PLZF^{lo} NK1.1^{-}). In the original description of the NKT subsets ([15]), PLZF and Tbet was used to differentiate the cells, but as only NKT1 cells express NK1.1, it was used as a substitute for Tbet in our stimulations to better separate the populations (Fig. 5b). Using either Tbet or NK1.1 in conjunction with PLZF in PLZF-cre NKAP cKO mice showed enlargement in the proportion of cells that are low for all three markers (Fig. 5a and 5b), which were originally identified as NKT17 cells ([15]). However, as PLZF-cre NKAP cKO mice have disproportionately more Stage 0 iNKT cells, these undifferentiated CD24^{+} CD1d/PBS57 tetramer^{+} cells fell into this gate (data not shown) giving the appearance of enlargement of the NKT17 pool, but specific staining with RORγt demonstrates that NKT17 cells are not present in the absence of NKAP.

To examine NKT cell function, thymocytes were stimulated and examined for production of IFN-γ, IL-4 and IL-17. Although decreased in frequency and number, most NKAP deficient NKT1 cells produced similar levels of IFN-γ as compared to WT NKT1 cells. IL-4 production by NKAP deficient NKT2 cells was also similar to WT NKT2 cells (Fig. 5b). The generation of innate 'memory-like' CD8 T cells is dependent on IL-4 production by NKT2 cells in the thymus ([38], [39]). To determine if the decreased number of NKT2 cells in the PLZF-cre NKAP cKO mice affected the generation of eomes expressing innate CD8 T cells, we examined thymocytes for presence of CD8^{+}CD24^{-} Eomes^{+} CXCR3^{+} innate CD8 T cells. In the PLZF-cre NKAP cKO mice, there was similar generation of eomes expressing innate CD8 T cells present compared to WT (Supplemental Fig 2c). Although NKT cells are the predominant producers of IL-4, other innate cells such as PLZF^{hi} γδ T cells secrete IL-4 as well ([38], [40]). The absolute number of innate PLZF^{hi} $V_{\gamma}1.1V_{\delta}6.3^{+}$ γδ T cells was examined, to determine whether this population was enhanced and therefore could compensate for the decreased number of NKT2 cells in PLZF-cre NKAP cKO mice. However, there was a significant reduction in the absolute cell number of PLZF^{hi} $V_{\gamma}1.1V_{\delta}6.3^{+}$ γδ T cells in the thymus in PLZF-cre NKAP cKO mice as compared to WT

mice (Supplementary Fig. 2d and e). Therefore, although decreased in number, NKAP-deficient NKT2 produce enough IL-4 to support the generation of innate eomes expressing CD8 T cells. Consistent with the loss of ROR-γt expressing NKT17 cells; NKAP deficient NKT cells did not produce IL-17 (Fig. 5b). An analysis of cytokine production across all subsets demonstrates that none of the subsets from the PLZF-cre NKAP cKO mice can produce IL-17 as compared to WT demonstrating a global inability to make IL-17 (Supplemental Figure 3). Therefore, NKAP is absolutely required for the generation of and IL-17 production by NKT17 cells.

The defect in NKT17 differentiation in PLZF-cre NKAP cKO mice is not due to defects in TGF-β, mTOR or IL-7 signaling

During iNKT cell development, TGF-β is required for the generation of ROR-γt expressing NKT17 cells ([16], [17]). Therefore, we examined if the loss of NKAP led to altered TGF-β receptor expression or signaling. Surface expression of TGF-βRII and the intracellular expression of TGF-β targets: Egr1 and Egr2 ([41], [42]) were similar between WT and NKAP-deficient iNKT cells (Fig. 6a). The transcription factor ThPOK is a negative regulator of NKT17 differentiation ([43]) and therefore we examined whether increased ThPOK may have caused the loss of NKT17 cells in PLZF-cre NKAP cKO mice. However, ThPOK expression was also similar in NKAP deficient iNKT cells as compared to WT iNKT cells (Fig. 6b). Signaling through the mTOR pathway is also critical for development and functional differentiation of iNKT cells ([44]–[46]). Loss of mTORC2 signaling leads to severe decreases in ROR-γt expressing NKT17 cells ([46]). We examined the expression of phosphorylated AKT (pS473) and phospho-S6 kinase as readouts of mTOR signaling ([47]). The intracellular expression of pAKT (S473) and phospho-S6 kinase were similar between WT and NKAP deficient iNKT cells across all stages of development (Fig. 6c). The cytokine IL-7 has also been shown to be required exclusively for the survival of ROR-γt expressing NKT17 cells ([18], [20]), and signaling through the IL-7 receptor leads to the expression of the pro-survival molecule Bcl-2 ([48], [49]). We examined the surface expression of IL-7Rα and intracellular expression of Bcl-2 in iNKT cells. The expression of both IL-7Rα was similar between WT and NKAP deficient iNKT cells, and expression of Bcl-2 was actually increased in Stage 1 and Stage 2 NKAP-deficient iNKT cells (Fig. 6d). Hence, lack of IL-7R expression or signaling is also not the cause for the defect in NKT17 differentiation.

The block in NKT17 generation and function in PLZF-cre NKAP cKO mice is rescued by transgenic expression of PLZF

The expression of PLZF is important for iNKT cell development and is essential for rapid cytokine production ([4], [5]). Transgenic expression of PLZF during T cell development led to an innate-type phenotype and increased IL-17 production by conventional T cells ([6], [8]). To address if defects in NKT17 cell generation in PLZF-cre NKAP cKO mice could be overcome by ectopic expression of PLZF, we generated lck-PLZF transgenic/PLZF-cre NKAP cKO to constitutively express PLZF in T cells, including iNKT cells. Due to the PLZF transgene, expression of PLZF cannot be used to delineate the functional NKT subsets using PLZF and Tbet or NK1.1 ([15]) and Fig. 5a). However, NKT17 cells are exclusively found in the $CD44^{+}NK1.1^{-}$ Stage 2 NKT population, and thus ROR-γt expression and IL-17 production were examined in Stage 2 NKT cells of lck-PLZF tg and lck-PLZF tg/ PLZF-cre

NKAP cKO mice. Transgenic expression of PLZF in NKAP deficient iNKT cells did not rescue the defect in absolute cell number or normalize the proportion iNKT cells across all stages in PLZF-cre NKAP cKO mice (Fig. 7a). However, expression of the PLZF transgene rescued the generation of ROR-γt expressing NKT17 in PLZF-cre NKAP cKO, although the absolute number of NKT17 cells was not restored to normal levels (Fig. 7c). Furthermore, upon stimulation of PLZF tg/PLZF-cre NKAP cKO iNKT cells, NKAP deficient Stage 2 iNKT cells produced IL-17 (representative FACS shown in Fig. 7b and quantified in Fig. 7d). While PLZF transgene rescued the generation of NKT17 cells in the absence of NKAP, it did not rescue the block in positive selection of iNKT cells in CD4-cre NKAP cKO mice (Supplemental Fig 4). Therefore, the defect in differentiation and function of ROR-γt expressing NKT17 cells resulting from lack of NKAP can be complemented by transgenic expression of PLZF.

Discussion

Here, we show that NKAP has additional roles in iNKT cell development beyond positive selection into the iNKT cell lineage at the DP stage ([26]). NKAP is also required for iNKT cell proliferation during development, and for the generation of ROR-γt expressing NKT17 cells. The block in iNKT cell development in PLZF-cre NKAP cKO mice is cell intrinsic and cannot be rescued by the ectopic expression of Bcl-xL, indicating that a defect in survival and homeostasis are not responsible. Altered TGF-β signaling, mTOR signaling, and IL-7R signaling are also not causes for the lack of NKT17 cells in PLZF-cre NKAP cKO mice. Constitutive expression of PLZF using a transgene ([6]) bypassed this block and allowed for the differentiation of ROR-γt expressing NKT17 cells and production of IL-17 by NKAP deficient iNKT cells. However, PLZF expression did not restore iNKT cell numbers demonstrating that proliferation was still impaired. In addition, the PLZF-transgene did not rescue the block in positive selection of DP thymocytes into the iNKT cell lineage in CD4-cre NKAP cKO mice, demonstrating that a failure to induce PLZF is not the cause for the failure in the development of iNKT cells in this model.

The expression of the transcription factor c-myc at Stage 1 of iNKT cell development has been shown to be important for intra-thymic proliferation of iNKT cells ([9], [12]), however NKAP deficient iNKT cells displayed normal c-myc expression. Other mice with mutations leading to abnormal metabolic activity, such as Fnip KO mice ([11]) which have reduced ATP uptake and increased ROS in NKT cells, also have defects in iNKT cell proliferation. NKT cells from PDK1 KO mice have a reduction in CD71 and CD98 surface expression ([10]), leading to a decreased absolute number of iNKT cells. However, NKAP deficient iNKT cells do not exhibit increased levels of ROS (data not shown) or CD71 and CD98 surface expression. Therefore, NKAP must control mechanisms currently unknown, which are critical for NKT proliferation. How NKAP regulates iNKT cell proliferation is currently under investigation.

Previously, transient expression of histone H2B-labelled GFP (H2Be-GFP) in DP thymocytes was used to analyze proliferation in conventional T cells, Treg and iNKT cells ([13]). Using this model, H2B-eGFP expression is observed in Stage 0 iNKT, but is gone in Stage 1 iNKTs. However, the range of expression of H2B-eGFP is relatively small,

approximately 10-fold between Stage 0 in H2B-eGFP reporter mice as compared to WT mice lacking this reporter. Here, the use of Rag1-GFP has a greater dynamic range of expression during iNKT cell development of approximately 3 logs. By examining the lowest 30% of Rag1-GFP expression in Stage 1 as compared to the uniform high level of expression of Rag1-GFP at Stage 0 (fig. 4c), the change in MFI is approximately 400-fold (Fig. 4b). If we assume that the change in MFI in Stage 1 is solely due to proliferation, then a 400-fold change in expression would correspond to approximately 8–9 cell divisions having occurred by the end of Stage 1 in WT iNKT cells. By comparison, in the PLZF-cre NKAP cKO mice, there is only a 23-fold decrease in the MFI of Rag1-GFP expression from Stage 0 to the lowest 30% of Rag1-GFP^{+} Stage 1 iNKT cells. The MFI of Stage 0 iNKT from WT and PLZF-cre NKAP cKO mice is identical (Figure 4c), and this increase in Rag1-GFP expression in PLZF-cre NKAP cKO mice is not due to re-expression of the *Rag1* gene as demonstrated by Q-PCR (data not shown). Thus, the 23-fold change in MFI by the end of Stage 1 in the absence of NKAP would correspond to approximately 4–5 cell divisions, as compared to 8–9 cell divisions in WT mice. If NKAP-deficient iNKT cells undergo 4 fewer cell divisions, it would lead to a 16-fold change in total iNKT cell numbers, which is close to the differences in absolute cell numbers observed (Fig. 1) and thus provides additional evidence that the primary cause for decreased iNKT cell numbers is due to proliferation, and not homeostasis, survival or apoptosis.

NKAP deficient NKT cells have substantial over-representation in the proportion of the undifferentiated CD24^{+} Stage 0 iNKT cells and Stage 1 iNKT cells, due to decreased proliferation in the absence of NKAP. The increased proportion of Stages 0–1 cells alters the proportion of cells in each gate when examining functional NKT subsets (NKT1, NKT2, NKT17) using PLZF and either T-bet or NK1.1. Using specific transcription factors to identify each population, we observed the near absence of ROR-γt NKT17 cells and decreased number of Tbet^{+} NKT1, Gata3^{hi} PLZF^{hi} NKT2 cells in PLZF-cre NKAP cKO mice. Although decreased in number, NKAP deficient NKT2 cells produce enough IL-4 to support the generation of innate eomes-expressing CD8 T cells.

mTOR signaling pathways have been implicated in different iNKT cell developmental stages and differentiation. Deletion of raptor to inhibit mTORC1 signaling diminishes the ability of iNKT cells to proliferate at Stage 1, while overactive mTORC1 signaling (via deletion of TSC1) leads to increased NKT17 differentiation ([44], [45]). Furthermore, signaling via mTORC2 is important for differentiation of ROR-γt expressing NKT17 cells and their effector function ([47]). However, it is unlikely that the decreased number of iNKT cells in PLZF-cre NKAP cKO mice is due to altered mTORC1 signaling as expression of phospho-S6 kinase is similar between WT and NKAP deficient iNKT cells. Similarly, the block in NKT17 generation in PLZF-cre NKAP cKO mice is not due to altered mTORC2 pathways, as expression of pAKT (S473) was similar between WT and NKAP deficient iNKT cells.

Apart from the developmental defect, NKAP is also required for the generation of NKT17 cells, which exclusively requires IL-7 signaling ([18]). Nevertheless, the expression of IL-7Rα and Bcl-2 were not decreased between WT and NKAP-deficient NKT cells. TGF-β signaling is also important for iNKT cell development and the differentiation of NKT17 cells. However in NKAP-deficient NKT cells there was similar expression of TGF-βRII and its

targets Egr1 and Egr2 ([16], [17], [41], [42]). Hence, NKAP regulates the generation of NKT17 cells independent of mTOR, IL-7, and TGF-β signaling pathways.

Interestingly, constitutive expression of PLZF rescued the generation and function of NKT17 cells in PLZF-cre NKAP cKO mice, indicating that NKAP may contribute to the ability of PLZF to induce ROR-γt expression. PLZF can bind and regulate ROR-γt expression ([50]). It is not simply that NKAP regulates PLZF expression as PLZF expression is similar in Stage 2 iNKT cells from WT and PLZF-cre NKAP cKO mice (data not shown). However, PLZF transgene did not rescue the development of iNKT cells in CD4-cre NKAP cKO mice, nor does it rescue proliferation/ iNKT cell numbers in PLZF-cre NKAP cKO mice. Rather, constitutive expression of PLZF in NKAP deficient NKT cells complements the defect leading to NKT17 generation. However, it is unclear at this moment how NKAP may mediate the regulation of ROR-γt expression by PLZF, which is under investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge the NIH Tetramer Core Facility (contract HHSN272201300006C) for provision of PBS-57 loaded and unloaded mCD1d tetramers. We thank Dr. Nobuo Sakaguchi for the Rag1-GFP knock-in mice. We would like to thank Christopher Parks, Barsha Dash, Fan-Chi Hsu and Michael Shapiro for critical reading of the manuscript.

Abbreviation

cKO	conditional knock-out
DP	double positive
Egr	early growth response
iNKT	invariant Natural Killer T
MFI	mean fluorescent intensity
PLZF	promyelocytic leukemia zinc finger
SP	single positive
Tg	transgenic,

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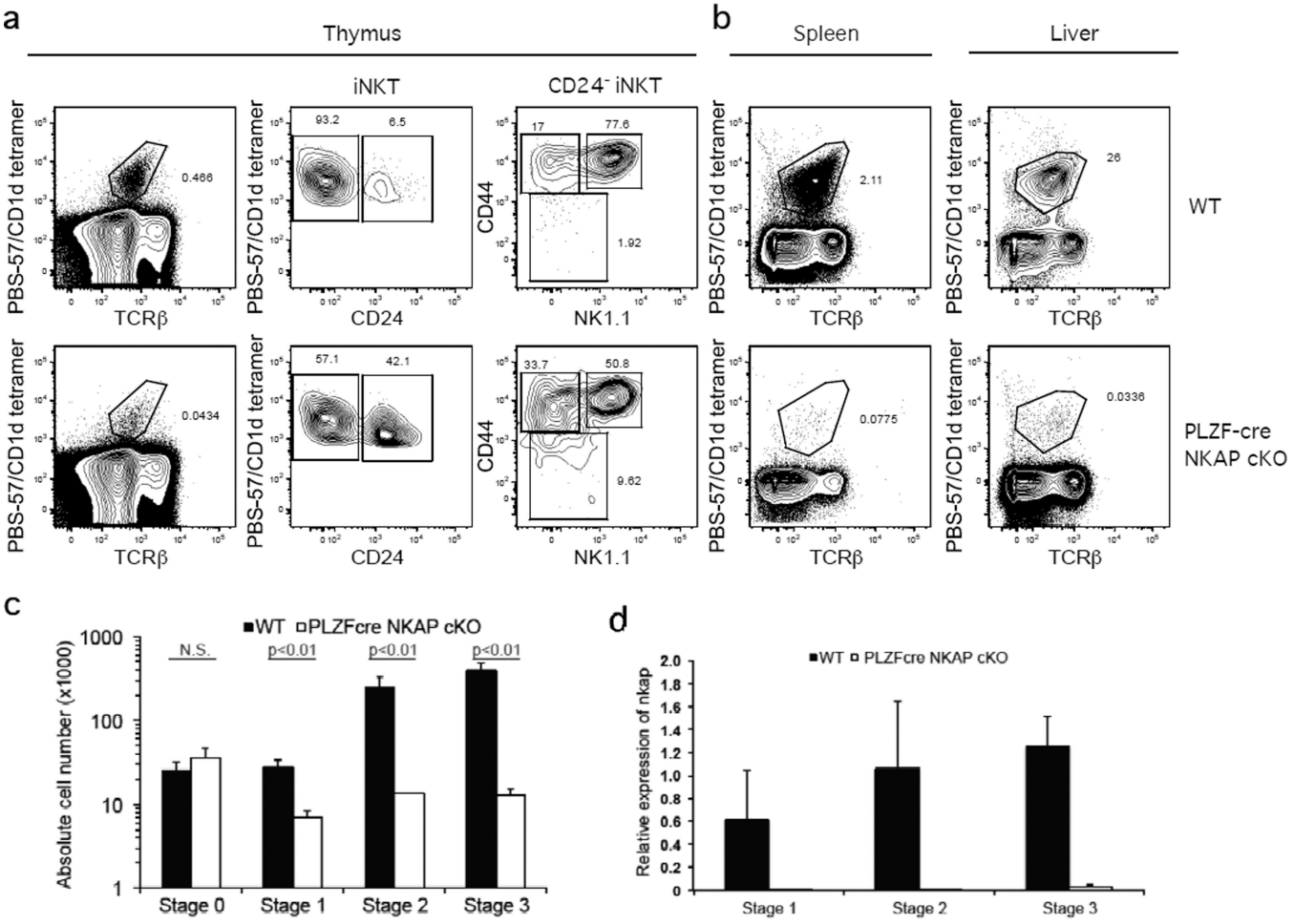

Figure 1. NKAP is required for iNKT cell development at Stage 1 onwards

FACS analysis of iNKT cell development in WT and PLZF-cre NKAP cKO mice. (**a**) Thymocytes were stained with fluorochrome-conjugated antibodies to TCRβ, PBS-57 loaded CD1d tetramer, NK1.1, CD44, and CD24. Thymocytes were first gated for TCRβ and PBS-57 loaded CD1d tetramer to identify iNKT cells. iNKT were then analyzed to distinguish different stages of development: Stage 0 ($CD24^+$ CD1d:PBS-57 $tetramer^+$), Stage 1–3 ($CD24^-$ CD1d $tetramer^+$): Stage 1 ($CD24^-$ $CD44^{lo}NK1.1^-$) Stage 2 ($CD24^-CD44^{hi}NK1.1^-$) Stage 3 ($CD24^-CD44^{hi}NK1.1^+$). Data is representative of at least fifteen independent experiments. (**b**) Lymphocytes from spleen and liver of WT and PLZF-cre NKAP cKO mice were examined for the presence of iNKT cells. Data is representative of at least five independent experiments. (**c**) The number of iNKT cells in the thymus of WT (black bars) and PLZF-cre NKAP cKO (white bars) mice was examined for all stages. Statistical significance was calculated using a student's t test. Data was calculated from fifteen mice from at least fifteen independent experiments. Please note that cell numbers are presented on a logarithmic scale. (**d**) Relative mRNA expression of *NKAP* in Stage 1, 2, and 3 of iNKT cells from WT and PLZF-cre NKAP cKO mice was examined. The data was normalized to expression of NKAP in WT Stage 3 iNKT cells (n=1). The data shown are the

average expression of *NKAP* in six WT and six PLZF-cre NKAP cKO from at least three independent sorts. Error bars represent the SEM.

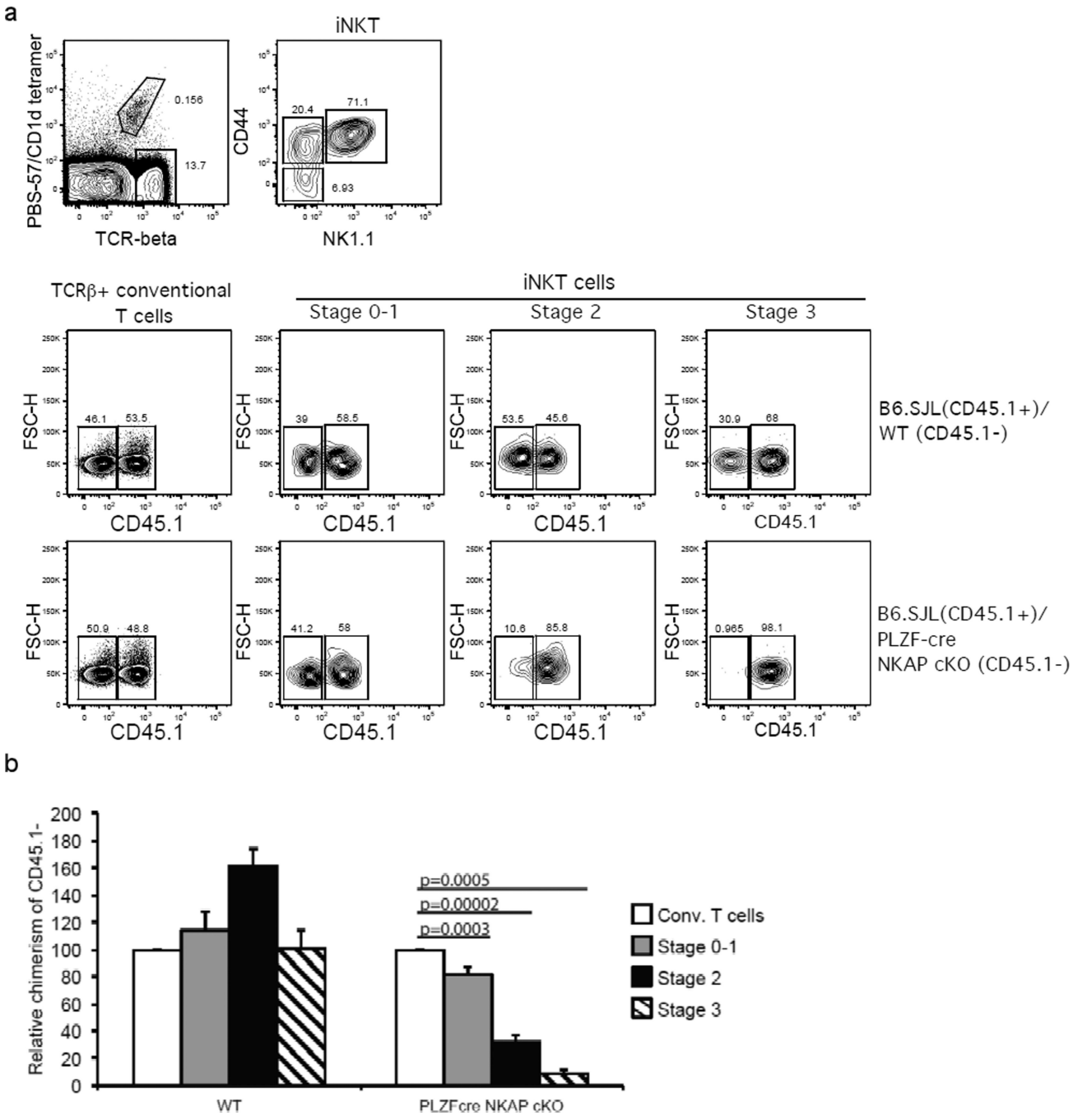

Figure 2. Requirement for NKAP in iNKT cell development is cell autonomous

(**a**) Mixed stem cell chimeras using bone marrow of congenic B6 WT mice (CD45.1^{-}) or B6 PLZF-cre NKAP cKO mice (CD45.1^{-}) mixed 1:1 with B6.SJL WT (CD45.1^{+}) marrow were generated. Using the congenic marker CD45.1, the relative contribution from each donor to different iNKT stages or conventional T cell proportions was determined. Chimeras were analyzed 8–10 weeks post generation. Analysis was done using flow cytometry as described for Figure 1. Data shown are representative of at least four mixed stem cell chimeras per genotype. (**b**) Quantification of relative chimerism represented by CD45.1^{-} cells in Stage 0–

3 iNKT cells from five chimeras of WT SJL:WT B6.SJL and four WT SJL: B6 PLZF-cre NKAP cKO chimeras. Error bars represent the SEM. Statistical significance was calculated using a student's t test.

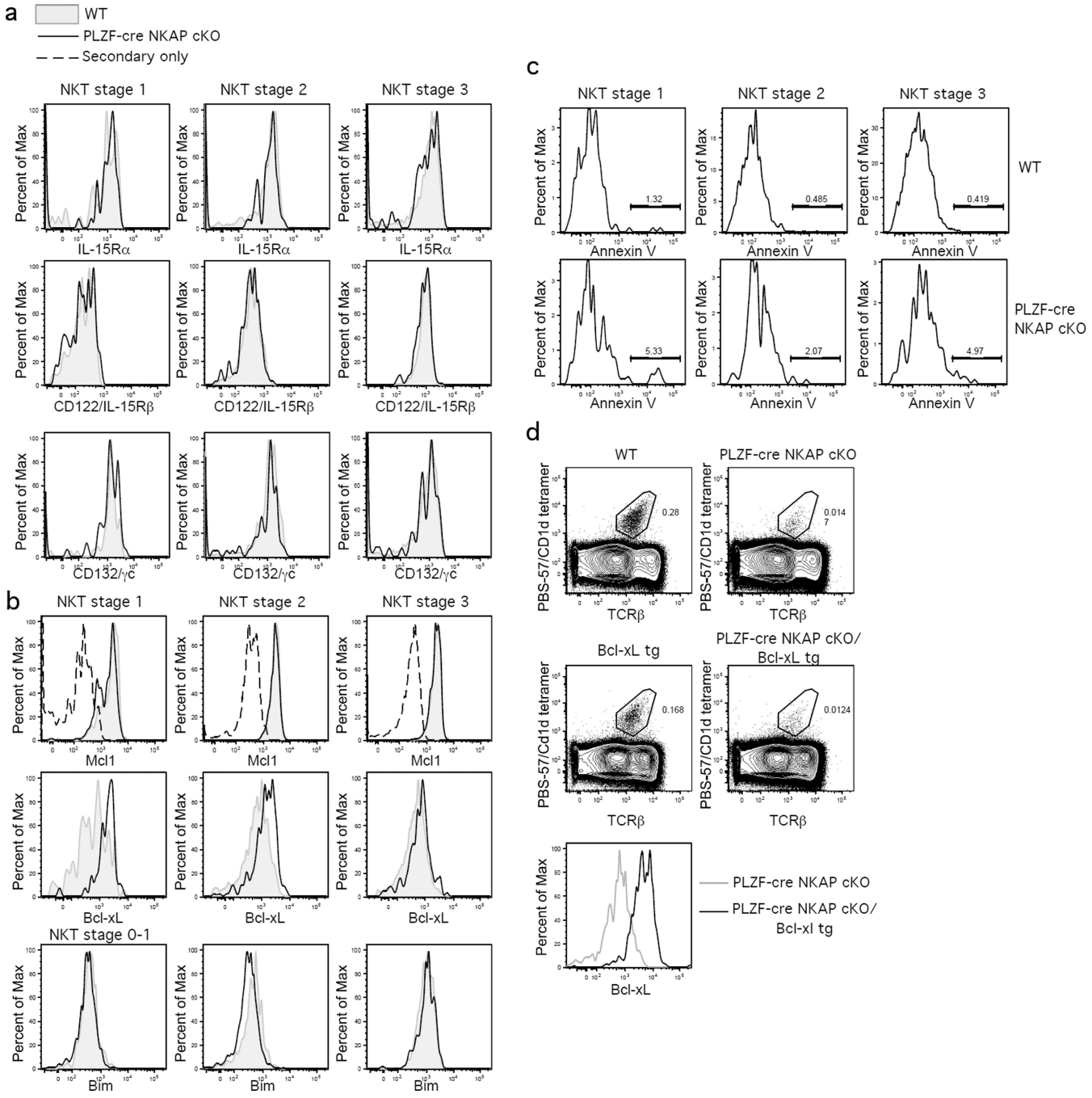

Figure 3. The defect due to loss of NKAP is not due to a failure in iNKT cell homeostasis

Stage 1, 2, and 3 iNKT cells from WT (grey filled) and PLZF-cre NKAP cKO (black line) mice were examined for expression of (**a**) IL-15Rα, IL-15Rβ (CD122), and γc (CD132); or (**b**) intracellular expression of anti-apoptotic or pro-apoptotic molecules Mcl-1, Bcl-xL, and Bim. Secondary antibody staining alone without primary antibody control (dashed) is shown for Mcl-1. (**c**) Annexin V binding was also examined. (**d**) iNKT cell development in the thymus of WT, PLZF-cre NKAP cKO, Bcl-xL Tg, and Bcl-xL Tg/PLZF-cre NKAP cKO mice was examined as described in Figure 1. Bcl-xL expression in iNKT cells of PLZF-cre

NKAP cKO mice and Bcl-xL transgene/PLZF-cre NKAP cKO mice is also shown. Each FACS plot is representative of at least three mice from three independent experiments.

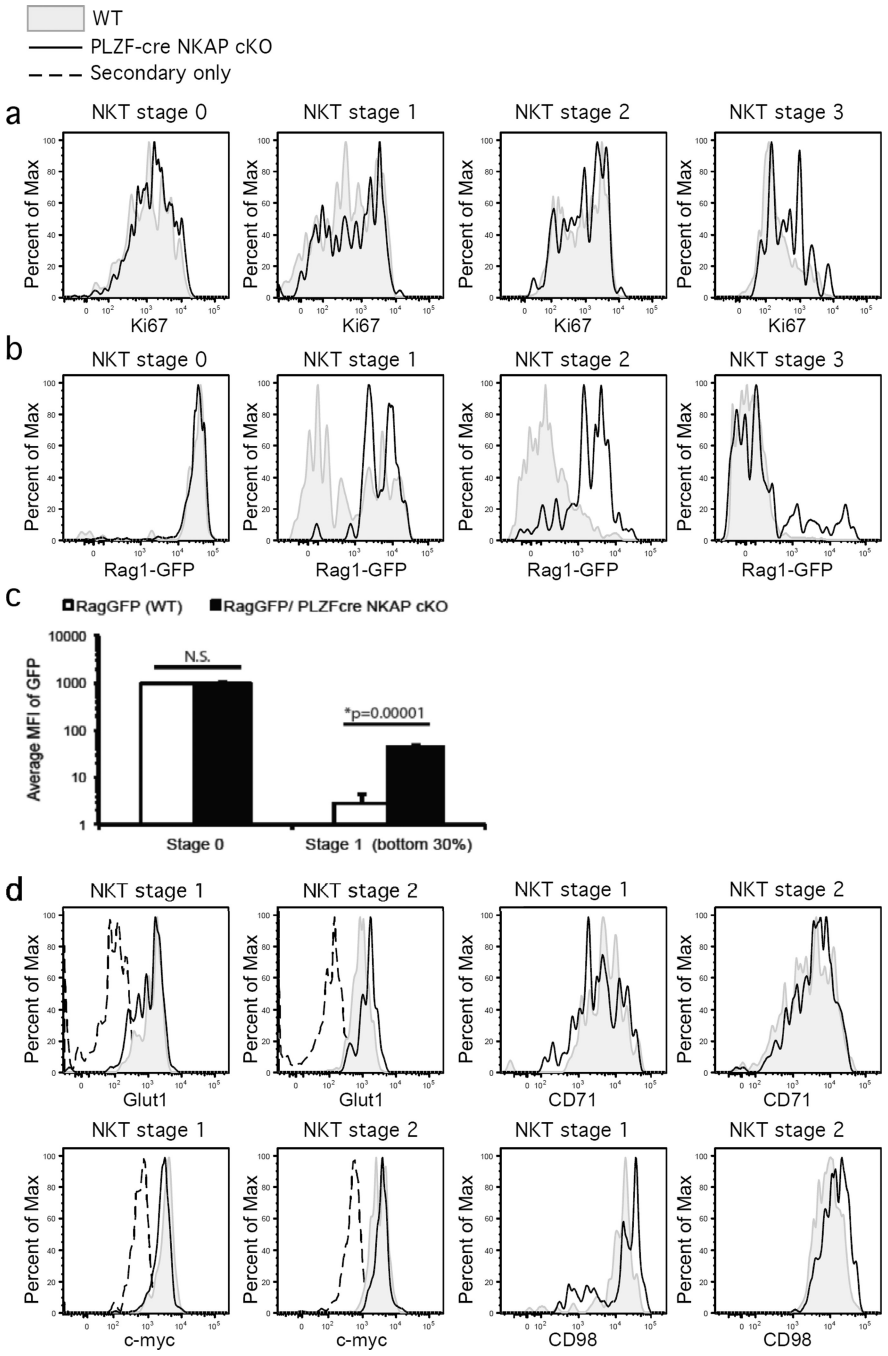

Figure 4. NKAP is required for iNKT cell proliferation

(**a**) Intracellular expression of the proliferation capacity marker Ki-67 in iNKT cells of WT (grey filled) and PLZF-cre NKAP cKO (black line) mice was examined using flow cytometry. Thymocytes were stained with iNKT cell markers to delineate Stage 0–3 as described in Figure 1 and then intracellularly stained for Ki-67. Data is representative of at least five independent experiments. (**b**) GFP expression in iNKT cells of Rag1-GFP (grey filled) and Rag1-GFP/PLZF-cre NKAP cKO (black line). Data is representative of at least four independent experiments. (**c**) Quantification of GFP MFI of Rag1-GFP in Stage 0 and

Stage 1 (bottom 30%) iNKT cells from WT (black line) and PLZF-cre NKAP cKO (grey filled). MFI data is shown on a logarithmic plot. Data is calculated from at least four mice per genotype. Error bars represent the SEM. Statistical significance was calculated using a student's t test. (**d**) Intracellular expression of GLUT1 and c-myc, and surface expression of CD71 and CD98 in Stage 1 and Stage 2 of iNKT cells in WT (grey filled), PLZF-cre NKAP cKO (black line) was examined. Secondary antibody alone without primary antibody control (dashed) is shown for GLUT1 and c-myc. Staining for each iNKT cell stage was performed as described in Figure 1. Data is representative of at least three mice from three independent experiments.

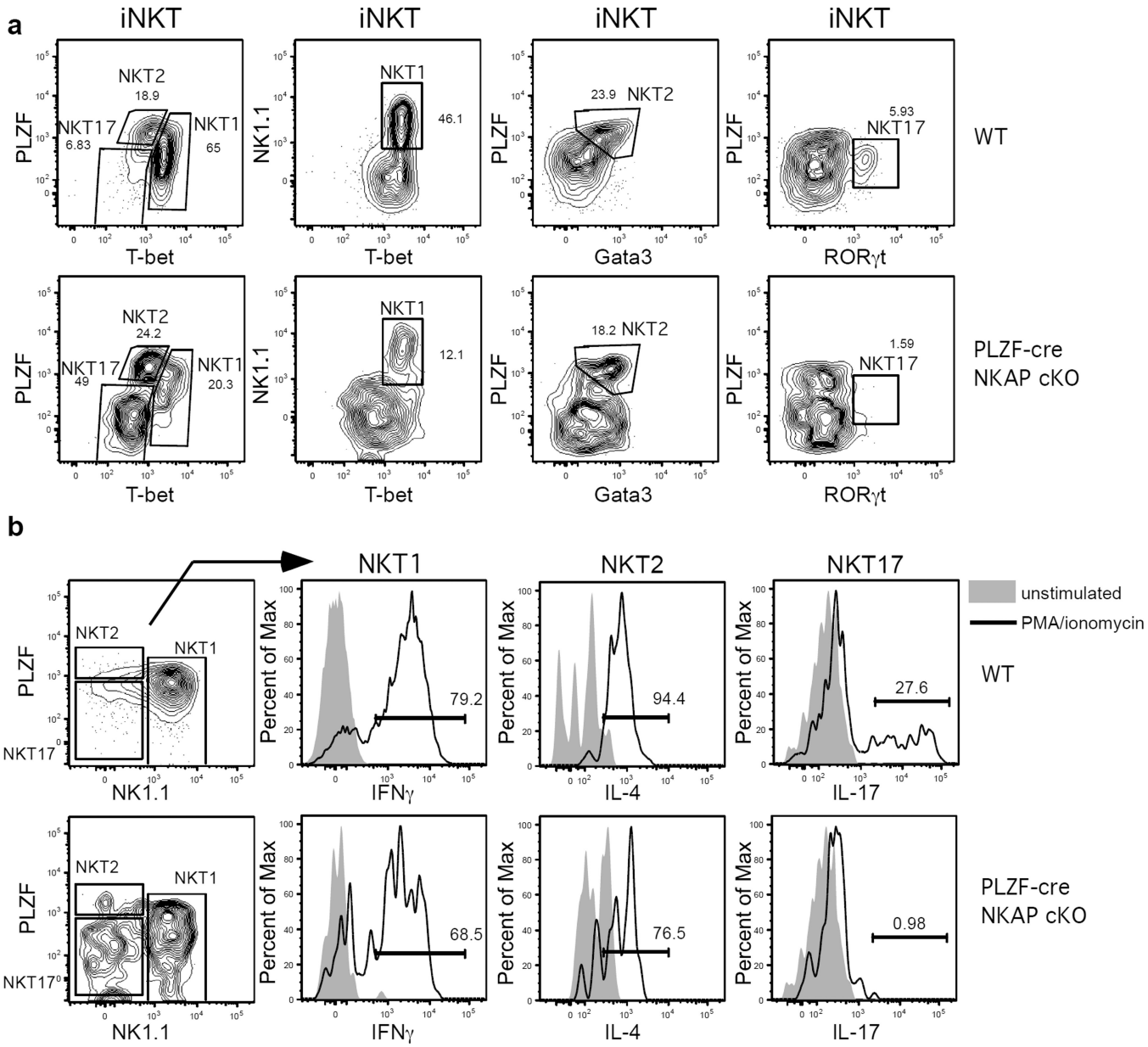

Figure 5. NKAP is required for generation of NKT17 cells, ROR-γt expression, and production of IL-17

(**a**) NKT cells were divided into functional subsets (NKT1, NKT2, and NKT17) in WT (top) and PLZF-cre NKAP cKO (bottom). To identify different subsets, iNKT cells ($CD24^{-}$, TCR-β^{+}, PBS-57 loaded CD1d $tetramer^{+}$) were examined for expression of T-bet, GATA3, ROR-γt, and PLZF to identify: NKT1 ($PLZF^{lo}$ T-bet^{+}), NKT2 ($PLZF^{hi}$ $GATA3^{hi}$), and NKT17 ($PLZF^{int}$ ROR-γt^{+}) according to ([15]). Data is representative of at least ten independent experiments. (**b**) Intracellular production of cytokines IFN-γ, IL-4, and IL-17 in NKT subsets in WT (top) and PLZF-cre NKAP cKO mice (bottom). Thymocytes were stimulated or left unstimulated overnight in PMA/ionomycin and the cytokine production by the NKT subsets was examined using flow cytometry. After surface staining for iNKT cell

antigens, thymocytes were fixed and permeablized to stain for intracellular cytokines (IFN-γ, IL-4, and IL-17) and PLZF. Data is representative of at least five independent experiments.

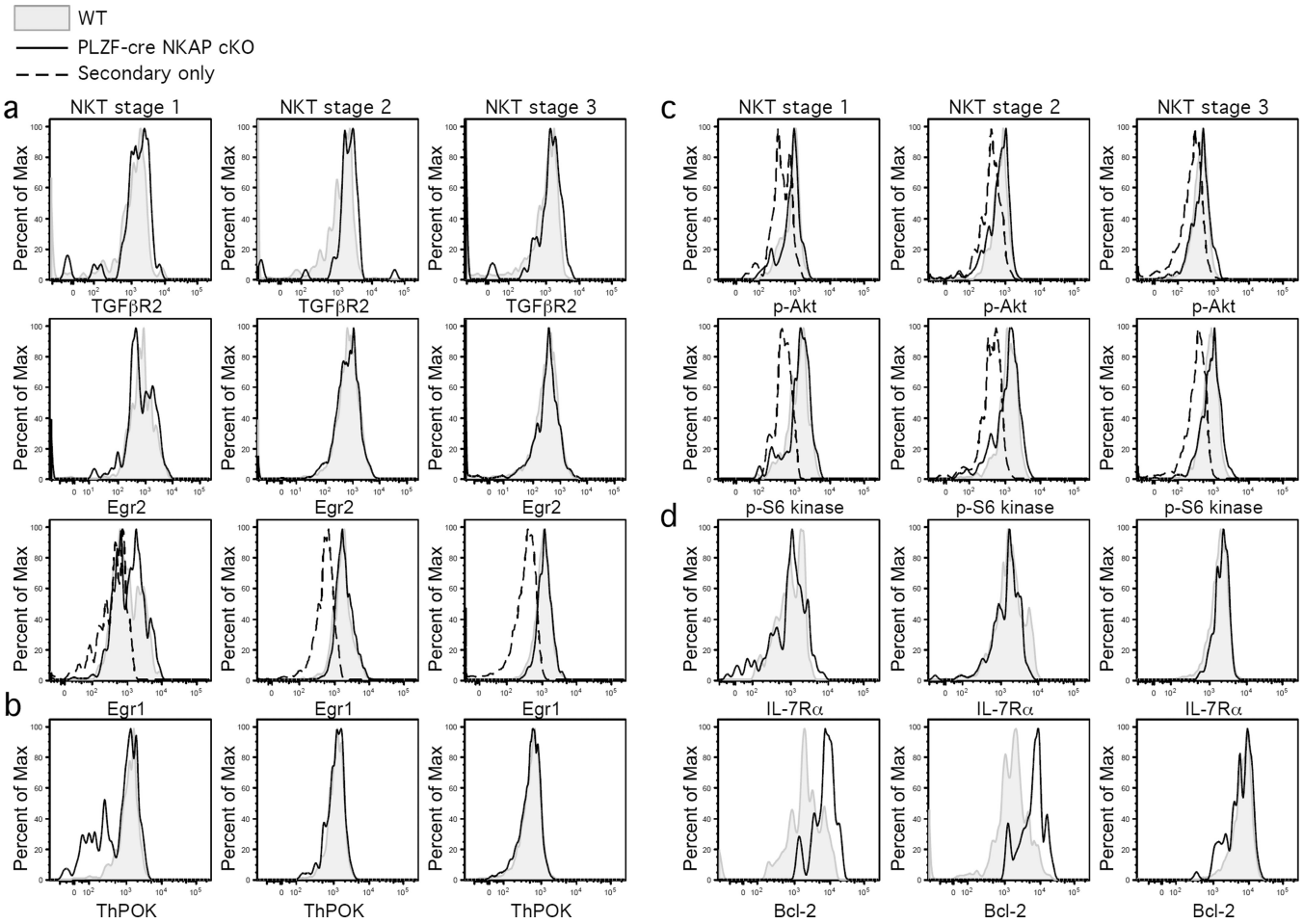

Figure 6. The defect in NKT17 generation in PLZF-cre NKAP cKO mice is not due to changes in TGF-β signaling, mTOR signaling, IL-7Rα signaling, or ThPOK expression

Expression of surface (**a**) TGF-βRII and intracellular Egr1, Egr2; and (**b**) ThPOK, (**c**) p-S6 kinase, and pAKT S473; and surface expression of (**d**) IL-7Rα and intracellular expression of Bcl-2 was examined in Stage 1, 2, and 3 iNKT cells from WT (filled grey), and PLZF-cre NKAP cKO (black line). Secondary antibody alone without primary antibody control (dashed) is shown for Egr1, p-AKT, and p-S6 kinase. iNKT cell stages were identified as in Figure 1. Data is representative of at least three mice from three independent experiments for each FACS plot.

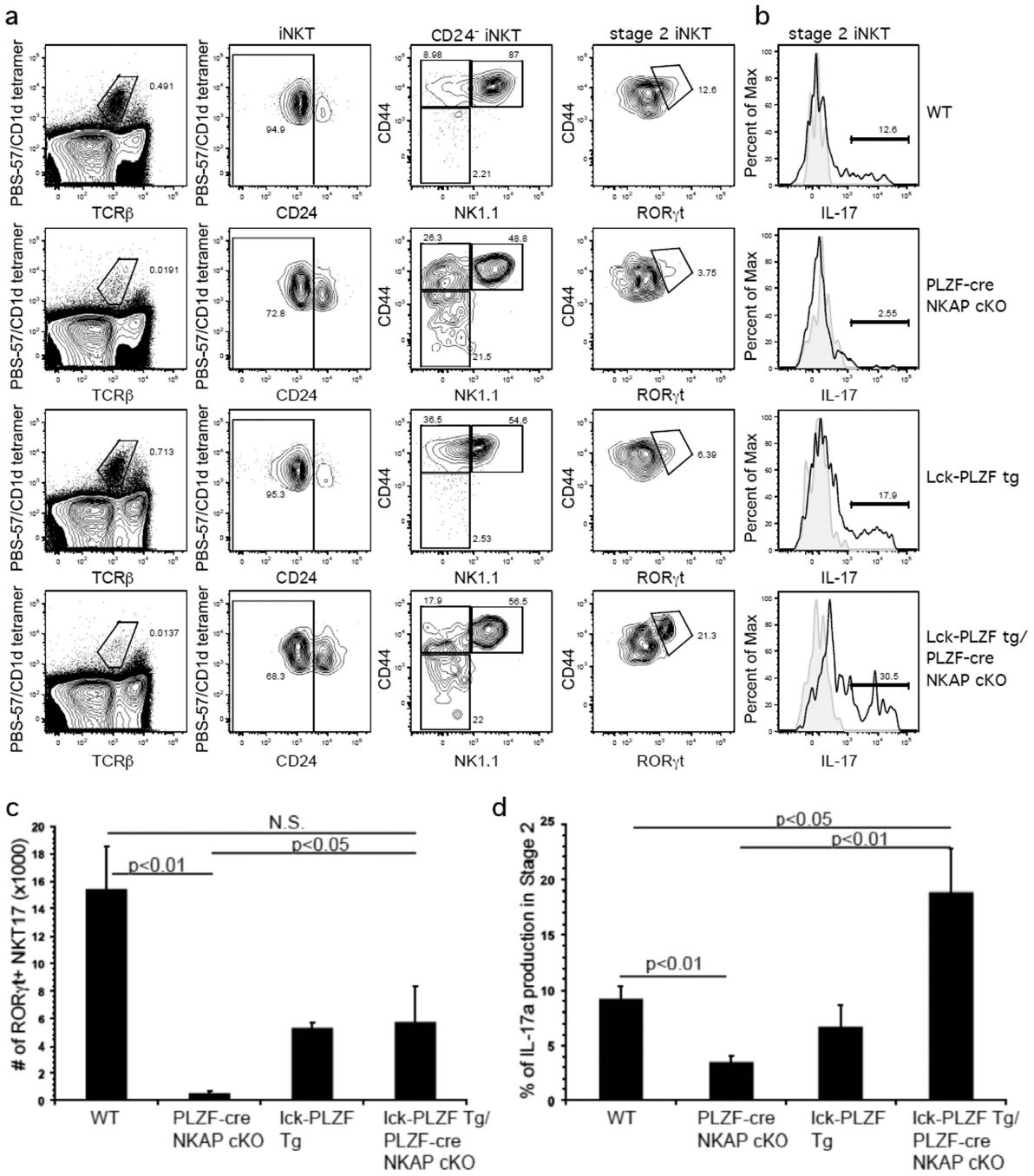

Figure 7. NKT17 development and function is rescued in PLZF-cre NKAP cKO mice by transgenic expression of PLZF

(**a**) FACS analysis of iNKT cell development (Stage 0–3) in the thymus of WT, PLZF-cre NKAP cKO, lck-PLZF Tg, and lck-PLZF Tg/PLZF-cre NKAP cKO mice. Examination with iNKT gating and staging was performed as described in Figure 1. Stage 2 iNKT cells were then examined for expression of ROR-γt to measure generation of NKT17 cells in each mouse. Data is representative of at least five independent experiments. (**b**) The intracellular production of cytokine IL-17 was examined using flow cytometry from thymic iNKT cells of WT, PLZF-cre NKAP cKO, lck-PLZF Tg, and lck-PLZF Tg/PLZF-cre NKAP cKO.

Thymocyte stimulation was performed as described in Fig. 5b, and IL-17 cytokine production was examined in Stage 2 iNKT cells. Data is representative of at least five independent experiments. Quantification of (**c**) absolute number of ROR-γt expressing NKT17 per thymus and (**d**) production of IL-17 in Stage 2 of WT, PLZF-cre NKAP cKO, lck-PLZF tg, and lck-PLZF tg/PLZF-cre NKAP cKO mice. Error bars represent SEM and statistical significance was calculated using the student's t-test. Data was averaged from at least five mice in each genotype.