



Main Manuscript for

Nur77 controls tolerance induction, terminal differentiation, and effector functions in semi-invariant natural killer T cells

Amrendra Kumar^{1,2}, Timothy M. Hill^{2,3}, Laura E. Gordy², Naveenchandra Suryadevara², Lan Wu², Andrew I. Flyak^{2,4}, Jelena S. Bezbradica^{2,5}, Luc Van Kaer², and Sebastian Joyce^{1,2,*}

¹Department of Veterans Affairs, Tennessee Valley Healthcare System

²Department of Pathology, Microbiology & Immunology, Vanderbilt University Medical Centre; Nashville, TN 37232

³Department of Chemistry and Life Science, United States Military Academy, West Point, NY, 10996;

⁴Department of Biology, California Institute of Technology and Biological Engineering, Pasadena, CA;

⁵The Kennedy Institute of Rheumatology, University of Oxford, Oxford, UK.

* Corresponding author: Sebastian Joyce

Email: sebastian.joyce@vumc.org

Paste ORCIDs (if any) here. Please note individual authors must also link their ORCID account to their PNAS account at www.pnascentral.org.

Classification

Biological Sciences | Immunology and Inflammation

Keywords

NKT cells/Nur77/tolerance/effector differentiation

Author Contributions

A.K., T.M.H., L.E.G., J.S.B., L.V.K, and S.J. designed research; A.K., T.M.H., L.E.G., N.C.S., L.W., A.I.F., J.S.B. performed research; A.K., T.M.H., L.E.G., J.S.B. analysed data; A.K., J.S.B., L.V.K, and S.J. wrote and edited the manuscript

This PDF file includes:

Main Text
Figures 1 to 7

Abstract

Semi-invariant natural killer T (iNKT) cells are self-reactive lymphocytes, yet how this lineage attains self-tolerance remains unknown. iNKT cells constitutively express high levels of *Nr4a1*-encoded Nur77, a transcription factor that integrates signal strength downstream of the T cell receptor (TCR) within activated thymocytes and peripheral T cells. Nur77 function, however, is unknown in iNKT cells. Here we report that sustained Nur77 overexpression (Nur77^{tg}) in mouse thymocytes abrogates iNKT cell development. Introgression of a rearranged *Vα14-Jα18* TCR α-chain gene into the Nur77^{tg} (Nur77^{tg};Vα14^{tg}) mouse rescued iNKT cell development up to an early precursor stage 0. iNKT cells in bone marrow chimeras that reconstituted thymic cellularity developed beyond stage 0 precursors and yielded interleukin-4-producing NKT2 but not interferon-γ-producing NKT1 cell subset. Nonetheless, developing thymic iNKT cells that emerged in these chimeras expressed the exhaustion marker PD1 and responded poorly to glycolipid agonists. Hence, Nur77 integrates signals emanating from the TCR to control

thymic iNKT cell tolerance induction, terminal differentiation, and effector functions.

Significance Statement

Semi-invariant natural killer T (iNKT) cells are innate-like lymphocytes that control a variety of immune functions. iNKT cells functions are mediated by high-affinity interactions with self-agonist/s displayed by lipid-presenting CD1d molecule. How iNKT cells maintain tolerance to high affinity interactions with self remains unknown, understanding which is of immunological importance as an unbridled iNKT cell-mediated response can cause inflammatory disease. We discovered that Nur77—a transcription factor expressed at high levels in iNKT cells—induced caspase-3 mediated apoptosis and markers of T cell exhaustion such as PD-1 within precursors of iNKT cells that led to iNKT cell hyporesponsiveness to a high affinity lipid agonist. Thus, Nur77 plays a central role self-tolerance induction in iNKT cells.

Main Text

Introduction

Semi-invariant natural killer T (iNKT) cells, which express an invariant TCR α -chain (mouse V α 14J α 18 or human V α 24-J α 18) paired with a limited number of TCR β -chains, are innate-like T lymphocytes that respond quickly to glycolipid

agonists such as the marine sponge-derived glycosphingolipid α -galactosylceramide (α GC) [1-3]. Activated iNKT cells secrete a variety of proinflammatory cytokines and chemokines with which they steer innate and adaptive immune responses to microbial antigens, autoantigens and alloantigens to promote health or disease [1-3]. iNKT cell functions are controlled by self and non-self-lipid agonists presented by MHC-like CD1d molecules [1-4]. This recognition of self-agonists by iNKT cells especially in the context of sterile inflammation [5] warrants an in-depth understanding of tolerance induction mechanism/s in iNKT cells.

iNKT cell precursors arise in the thymus through a developmental program that, until the CD4⁺8⁺ double-positive (DP) stage, is shared with conventional T cells [2, 3, 6, 7]. From this point on iNKT cell precursors undergo a unique developmental program, specified by a lineage-specific gene regulatory network induced by recognizing agonistic self-lipid ligand/s by the semi-invariant TCR in the presence of growth factors, such as interleukin (IL)-7 and IL-15 [2, 3, 6-9]. These signals result in progressive maturation of stage 0 precursors to stage 1 through stage 3 [2, 3, 6, 7] and differentiation into functional NKT1, NKT2 and NKT17 subsets [3, 10-15]. TCR ligation by agonistic self-lipid ligand/s signals iNKT cell lineage commitment, resulting in the induction of several transcription factors:

e.g., *Nr4a1*-encoded Nur77 and PLZF (pro-myelocytic leukemia zinc finger encoded by *Zbtb16*). Whilst the functions of PLZF and several lineage-specific transcription factors have been studied in detail, the contribution/s of Nur77 to iNKT cell development and function is yet to be investigated [2, 3, 6, 7, 16].

The Nur77 family of orphan nuclear receptor transcription factors consists of three members, viz., Nur77, Nurr1 and Nor1 that are encoded by *Nr4a1*, *Nr4a2* and *Nr4a3*, respectively [17, 18]. These transcription factors are related by high degree of homology in their DNA binding domain [17, 18]. Hence, only mice that lack all three members in T cells succumb to autoimmunity by 14–21 days after birth due to impaired thymic regulatory T cell (Treg) development and overt T cell auto-reactivity[19]. Mechanistically, Nur77 binds to the *Foxp3* gene promotor to induce *Foxp3* expression and, thereby, the generation of Tregs [19]. On the other hand, high Nur77 levels induce T cell apoptosis by converting the pro-survival factor Bcl-2 to a pro-apoptotic agent [20]. Overexpression of *Nr4a1* (Nur77^{tg}) in mice under the control of the proximal *Lck* promoter abrogates conventional CD4⁺ and CD8⁺ T cell development, whilst overexpression of a dominant negative *Nr4a1* mutant, which lacks the DNA binding domain, blocks negative selection of CD4⁺ and CD8⁺ cells. Together these findings support a role for Nur77 in thymic negative selection [17, 20-23]. Beyond thymic development, Nur77 controls

peripheral T cell function as Nur77 induction in peripheral T cells results in T cell exhaustion [24, 25], and the combined ablation of *Nr4a1*, *Nr4a2* and *Nr4a3* expression results in the reversal of exhaustion and enhances T cell responsiveness against tumor cells and virus infections [24, 25]. Thus, in conventional T cells, Nur77 induces tolerance through distinct mechanisms: induction of negative selection of auto-reactive thymocytes, the generation of Tregs, and induction of peripheral T cell exhaustion [17, 18, 20-25]. Despite high Nur77 expression in iNKT cells, its function, if any, has not been investigated.

iNKT cells in NF κ B-signaling deficient IkB Δ N^{tg} mice arrested at stage 0/stage 1 of development were enriched in cells expressing Nur77 [26]. Subsequent studies showed that Nur77 levels in iNKT cells correlated with TCR signal strength [16, 27]. As iNKT cells undergo agonist selection, stage 0 iNKT cell precursors constitutively express high levels of Nur77 [16]. Amongst the terminally differentiated thymic iNKT cell subsets, NKT2 cells have relatively higher Nur77 levels when compared to NKT1 and NKT17 subsets [11, 27, 28]. IL-10-producing fat tissue-derived iNKT cells also express higher *Nr4a1* transcripts than thymic iNKT cells [14]. Hence, these reports warrant an understanding of Nur77 functions in iNKT cells.

It is generally assumed that the iNKT cell TCR repertoire is solely generated by positive selection resulting from the interactions of the precursors with CD1d-agonistic ligand complex and SLAM (signaling lymphocyte activation molecule) receptors on DP thymocytes [5, 29-36]. Nonetheless, indirect evidence supports a role for negative selection in sculpting a functional iNKT cell TCR repertoire [37-41], potentially weeding out high affinity self-reactive iNKT cells. The two processes, however, do not explain why Nur77 expression persists at a higher level in iNKT cells when compared to conventional T cells. Moreover, TCR signal strength controls positive and negative selection of conventional T cells, and also the functional differentiation of both conventional T and iNKT cells [17, 19, 27]. As Nur77 levels proxy for TCR signal strength, and because the role of Nur77 in iNKT cells remains unknown, we investigated iNKT cell development and function in Nur77^{tg} mice, which overexpress *Nr4a1* selectively in thymocytes. This model was used because iNKT cells develop normally in *Nr4a1*-deficient and *Nr4a1*, *Nr4a2* and *Nr4a3* triple-deficient (TKO) mice [19, 42]. The TKO mice survive only for ~18–21 days and succumb to severe autoimmune disorders [19]. Hence, the TKO mice do not allow a detailed study of iNKT cell biology [43, 44].

Herein we report studies of iNKT cell development in mice that express high Nur77 within thymocytes. The data revealed that Nur77 integrates signals

emanating from the TCR to control thymic iNKT cell tolerance induction, terminal differentiation, and effector functions.

Results

iNKT cells fail to develop in mice overexpressing Nur77 in thymocytes

Mice lacking either Nur77 or all three Nur77 members (Nur77, Nor1 and Nurr1, TKO) develop iNKT cells [19, 42]. As the TKO mice survive only for ~18–21 days and succumb to severe autoimmune disorders [19], it is not feasible to perform detailed analyses of iNKT cell biology in the TKO mice [43, 44]. Therefore, we analyzed mice in which a *Nr4a1* transgene (Nur77^{tg}) was expressed in a thymocyte-specific manner under the control of the proximal *Lck* promoter [17]. Consistent with previous reports [17, 23], we found that conventional T cell development and peripheral T cell frequency were severely reduced in Nur77^{tg} mice when compared to C57BL/6 (B6) mice and non-transgenic littermates (**SI Appendix Fig. S1A,B**). As reported before [21], we found that CD4⁺CD25⁺Foxp3⁺ Tregs were enriched in spleen and lymph nodes of Nur77^{tg} mice, which is consistent with high constitutive Nur77 expression in Tregs [16] and Treg resistance to Nur77-induced apoptosis [21]. As iNKT cells—akin to Tregs that undergo thymic agonist selection—have high constitutive Nur77 expression [16], we predicted iNKT cells

to be resistant to Nur77-induced apoptosis. Contrary to this prediction, we found that Nur77^{tg} mice had no iNKT cells in the thymus and periphery (**SI Appendix Fig. S1C,D**). Thus, akin to conventional T cells, high and sustained Nur77 levels in thymocytes abrogate iNKT cell development.

Introgression of a rearranged V α 14-J α 18 α -chain gene into Nur77^{tg} mice partially rescues iNKT cell development

Owing to overt apoptosis, DP thymocyte numbers are severely reduced in mice constitutively overexpressing Nur77 in thymocytes [17, 45]. We and others have shown that iNKT cells failed to develop in mice in which DP thymocyte survival window was shortened, as this prevents distal V α 14 to distal J α 18 rearrangement to encode the α -chain of the NKT cell TCR [3, 45-48]. Therefore, we introgressed a rearranged V α 14-J α 18 α -chain gene into Nur77^{tg} mice and evaluated iNKT cell development and function in the resulting Nur77^{tg};V α 14^{tg} mice. We found that iNKT cells developed in Nur77^{tg};V α 14^{tg} mice. Nonetheless, total thymic and splenic iNKT cell numbers remained significantly lower in Nur77^{tg};V α 14^{tg} mice in comparison to V α 14^{tg} and B6 mice (**Fig. 1A,B**). Further analysis revealed that iNKT cell development was arrested at stage 0 (CD24^{HI}CD44^{NEG}NK1.1^{NEG}; **Fig. 1C,D**). Consistent with this early arrest, Nur77^{tg};V α 14^{tg} mice, when compared to V α 14^{tg}

mice, had fewer thymic CCR7^{HI}PD1^{LO} iNKT cells, which are downstream precursors that succeed stage 0 cells [12] (**SI Appendix Fig. S2A,B**).

Further analyses showed Nur77 expression was higher within all stages of iNKT cell development in Nur77^{tg};Vα14^{tg} mice when compared to Vα14^{tg} mice (**Fig. 1E,F**). Conversely, PLZF expression was significantly lower within thymic stage 0+1 iNKT cells in Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (**Fig. 1G,H**). So also, compared to Vα14^{tg} mice, Nur77^{tg};Vα14^{tg} mice had lower percent of PLZF⁺ iNKT cells within thymic stage 0+1 (**Fig. 1I**). Based on this result, we analyzed the three iNKT cell subsets (NKT1, NKT2 and NKT17) identifiable by differential PLZF, T-bet, and RORγt expression [10]. Consistent with reduced PLZF expression, Nur77^{tg};Vα14^{tg} mice lacked thymic NKT1 (PLZF^{INT}T-bet^{HI}), NKT2 (PLZF^{HI}T-bet^{NEG}RORγt^{NEG}), and NKT17 (PLZF^{INT-HI}RORγt⁺) subsets (**SI Appendix Fig. S2A,B**), all of which expressed higher Nur77 in Nur77^{tg};Vα14^{tg} mice when compared to those in Vα14^{tg} mice (**SI Appendix Fig. S2C,D**). Collectively, these results suggest that Nur77 overexpression within thymocytes arrests iNKT cell development at a very early stage.

Peripheral iNKT cell development is altered in Nur77^{tg};Vα14^{tg} mice

After development, thymic iNKT cells emigrate and complete maturation at peripheral sites. To investigate the effect of Nur77 overexpression, we tracked iNKT cells in the spleen, liver, lung, lymph node and adipose tissue. Peripheral iNKT cell frequency was reduced in Nur77^{tg};Vα14^{tg} mice when compared to Vα14^{tg} mice (**Fig. 2A,B**). A proportion of peripheral iNKT cells matured to stages 2 and 3, but, due to significantly low T cell numbers, the absolute number of mature iNKT cells in Nur77^{tg};Vα14^{tg} mice remained significantly lower in all organs in comparison to Vα14^{tg} mice (**Fig. 2A**). The presence of few stage 2 and 3 peripheral iNKT cells in Nur77^{tg};Vα14^{tg} mice led us to investigate iNKT cell subsets. Similar to thymic iNKT cells, the majority of peripheral iNKT cells in Nur77^{tg};Vα14^{tg} mice had either PLZF^{LO} or PLZF^{INT}, which contrasted with Vα14^{tg} mice where the majority of peripheral iNKT cells were PLZF^{HI}. Further, peripheral NKT1 and NKT2 cell numbers were consistently low in Nur77^{tg};Vα14^{tg} mice (**Fig 2C; SI Appendix Fig. S2E**). Interestingly, the peripheral NKT17 subset was enriched in spleen, liver and lungs of Nur77^{tg};Vα14^{tg} mice to comparatively similar numbers to those in Vα14^{tg} mice (**Fig 2c; SI Appendix Fig. S2E**). Thus, akin to thymic iNKT cells, Nur77 overexpression altered peripheral iNKT cell development and subset differentiation.

CD1d and SLAM levels in DP thymocytes and endogenous antigen-presenting function are intact in Nur77^{tg} mice

ROR γ t expression by DP thymocytes is required for DP thymocyte survival and *V α 14* to *J α 18* gene rearrangement. ROR γ t expression was assessment showed higher expression in DP thymocytes of Nur77^{tg};V α 14^{tg} mouse when compared to those in V α 14^{tg} mouse (**SI Appendix Fig. S2F,G**). Unlike conventional T cells that are positively selected by MHC on thymic epithelial cells, iNKT cell positive selection depends on interactions of the precursors with CD1d and SLAM receptors on DP thymocytes [1, 3-6, 29-36]. We found that Nur77^{tg};V α 14^{tg} and V α 14^{tg} mouse thymocytes expressed similar levels of CD1d; SLAM (SLAMF1) expression was twice as much on stage 0 iNKT cells of Nur77^{tg};V α 14^{tg} mouse when compare dto those in V α 14^{tg} mouse (**SI Appendix Fig. S2H—M**). Hence, we predict that CD1d+self agonist-TCR and SLAM-SLAM interactions are intact in Nur77^{tg};V α 14^{tg} mouse and is consistent with developmental arrest after positive selection.

To ascertain whether CD1d expressed on DP thymocytes was functional and presented the endogenous lipid agonist/s, FACS-sorted DP thymocytes from Nur77^{tg};V α 14^{tg} and V α 14^{tg} mice were co-cultivated in vitro with V α 14⁺ NKT cell hybridomas as described previously [49, 50]. This co-culture yielded similar levels

of IL-2 by V α 14⁺ NKT cell hybridomas only when stimulated by DP thymocytes from both Nur77^{tg};V α 14^{tg} and V α 14^{tg} mice (**SI Appendix Fig. S2N**). Taken together, DP thymocytes express functional CD1d and can engage in SLAM-SLAM interactions even when these cells overexpress Nur77.

iNKT cells in Nur77^{tg};V α 14^{tg} mice recognize α GC but are functionally impaired

Intact TCR signaling is critical for iNKT cell intrathymic expansion and developmental progression [27, 51-54]. Nur77 is an immediate early gene induced downstream of TCR signaling [16, 17, 19, 22]. To assess whether iNKT cell TCR signaling was impaired, the Nur77^{GFP} reporter transgene [16] was introgressed into Nur77^{tg};V α 14^{tg} (Nur77^{tg};V α 14^{tg};Nur77^{GFP}) and V α 14^{tg} (V α 14^{tg};Nur77^{GFP}) mice, and constitutive and inducible Nur77^{GFP} expression investigated. Higher constitutive Nur77^{GFP} was expressed in thymic NKT cells of Nur77^{tg};V α 14^{tg};Nur77^{GFP} mice than V α 14^{tg};Nur77^{GFP} mice, with similar Nur77^{GFP} expression in stage 0 iNKT cells, but higher in stages 1 through 3 iNKT cells (**Fig. 3A,B**). Although there was a trend toward higher constitutive Nur77^{GFP} expression in splenic iNKT cells from Nur77^{tg};V α 14^{tg};Nur77^{GFP} mice, it was not statistically significant (**Fig. 3C,D**). Importantly, there was no difference in inducible Nur77^{GFP} expression upon α GC stimulation in vivo (**Fig. 3C,D**). Hence, high Nur77 level does not alter agonistic glycolipid-induced proximal TCR signaling in iNKT cells.

iNKT cells in Nur77^{tg};V α 14^{tg} mice poorly upregulated CD69 in response to in vivo α GC stimulation when compared with V α 14^{tg} mice (**Fig. 3E,F**). Consistent with the low CD69 upregulation, secreted cytokines, such as interferon (IFN)- γ and IL-4, were also low in response to in vivo stimulation of Nur77^{tg};V α 14^{tg} mouse iNKT cells with α GC (**Fig. 3G**). Lower iNKT cell numbers in Nur77^{tg};V α 14^{tg} mice could have resulted in lower serum cytokine response. However, compared to iNKT cells in V α 14^{tg} mice, those in Nur77^{tg};V α 14^{tg} mice had lower frequency of IFN- γ ⁺, IL-4⁺, and IFN- γ ⁺IL-4⁺ cells in response to in vivo α GC stimulation (**SI Appendix Fig. S3A,B**). Taken together we conclude that, whilst TCR proximal signaling events remain intact, functional responsiveness to α GC stimulation was impaired when iNKT cells expressed high Nur77 levels.

Nur77 levels control PD1 expression in CD8⁺ tumor infiltrating lymphocytes [24]. Consistent with this report, a significantly high PD1 expression by NKT cells was observed in Nur77^{tg};V α 14^{tg} mice compared to V α 14^{tg} mice. PD1 expression was high within all stages and subsets of Nur77^{tg};V α 14^{tg} mouse iNKT cells (**Fig. 3H,I; SI Appendix Fig. S3C–F**). Collectively, the foregoing results suggest that Nur77 plays a critical role in iNKT cell development, differentiation and function.

Nur77 has cell intrinsic and extrinsic effects on iNKT cell development

To restore thymic cellularity and ascertain whether Nur77 has a cell intrinsic, extrinsic or both roles in iNKT cell development and function, we generated radiation BM chimeras in iNKT cell-deficient $J\alpha 18^{-/-}$ mice. Donor BM from Nur77^{tg};V α 14^{tg} mice either singly or mixed at 1:1 ratio with BM from $J\alpha 18^{-/-}$ mice was transferred into irradiated $J\alpha 18^{-/-}$ mice. BM chimeras from donor B6, $J\alpha 18^{-/-}$, Nur77^{tg} or V α 14^{tg}, transferred alone or mixed with $J\alpha 18^{-/-}$ BM, were controls. As expected, no thymic iNKT cells developed in single and mixed BM chimeras made with Nur77^{tg} BM cells and were not detected in the periphery (**Fig. 4A,B**). As well, chimeras made with Nur77^{tg};V α 14^{tg} BM cells alone developed iNKT cells that were arrested at stage 0 (**Fig. 4A**). Surprisingly, however, Nur77^{tg};V α 14^{tg}/ $J\alpha 18^{-/-}$ mixed chimeras developed thymic iNKT cells that progressed beyond stage 0 and populated the periphery, but progression to stage 3 remained incomplete (**Fig. 4A—d**). iNKT cell numbers, however, remained consistently and significantly lower than those in control V α 14^{tg}/ $J\alpha 18^{-/-}$ mixed chimeras (**Fig. 4B**). Further, in comparison to V α 14^{tg}/ $J\alpha 18^{-/-}$ mixed chimeras, splenic NK1.1^{NEG} and NK1.1⁺ iNKT cell numbers were reduced in Nur77^{tg};V α 14^{tg}/ $J\alpha 18^{-/-}$ mixed chimeras (**Fig. 4A,D**). Consistent with iNKT cell developmental progression beyond stage 0, PLZF expression was restored to normal levels (**SI Appendix Fig. S2A**).

Restoration of PLZF expression prompted a close study of thymic and splenic iNKT cell subsets. Due to low total thymic and splenic iNKT cell numbers, the

absolute numbers of the three iNKT cell subsets were reduced in Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras when compared to Vα14^{tg}/Jα18^{-/-} mixed chimeras. Notably, of the three subsets, NKT1 cells were most severely reduced and, consistent with this arrest, iNKT cells expressed low CD122 levels (**SI Appendix Fig. S4A,B,D**). Progression of iNKT cells beyond stage 0 in mixed chimeras suggests an iNKT cell extrinsic role of Nur77 in blocking iNKT cell development. Nonetheless, incomplete terminal maturation and differentiation to NKT1 cells suggest an additional, cell intrinsic role for Nur77 in iNKT cell development and differentiation.

Use of congenically marked B6 donor BM instead of Jα18^{-/-} BM generated identical results. Additionally, irrespective of the numbers of Jα18^{-/-} BM cells used to generate mixed chimeras, NKT cell maturation was consistently impaired (**SI Appendix Fig. S4C—F**). This result argues against faulty encounters of iNKT cell precursors with positively selecting DP thymocytes as an explanation for impaired iNKT cell development. Taken together, the results from BM chimeras indicate that Nur77 plays cell intrinsic and extrinsic roles in iNKT cell development and differentiation.

Sustained Nur77 expression impairs iNKT cell survival but not proliferation

High Nur77 expression induces apoptosis in conventional T cells [17]. Consistent with that report, we found enhanced caspase 3 activation stages 1—3 thymic and

stages 1 and 2 splenic iNKT cell development within Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed BM chimeras. whilst caspase 3 activation was statistically significant at stages 2 and 3 thymic and stages 1 and 2 splenic iNKT cells, the trend toward high caspase 3 was also observed in stage 1 thymic iNKT cells (**Fig. 5A; SI Appendix Fig. S5A**). In additional experiments, as Nur77 also inhibits T cell proliferation [55, 56], EdU (5-ethynyl-2-deoxyuridine) incorporation by iNKT cells was measured in the various chimeric mice but no difference was observed (**Fig. 5B; SI Appendix Fig. S5AB**). Hence, Nur77 alters iNKT cell homeostasis by enhancing apoptosis at all of development without impacting proliferative capacity.

iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras are exhausted

Recent studies have identified Nur77 as a mediator of T cell exhaustion [24, 25]. As iNKT cells in Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras developed beyond stage 0, we tested whether these cells gained functional capacity. Serum and intracellular IFN-γ and IL-4 production by iNKT cells in response to in vivo αGC stimulation was low in Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras compared with Vα14^{tg}/Jα18^{-/-} mixed chimeras at all time points tested (**Fig. 6A—C; SI Appendix Fig. 6A**). Thus, despite partial developmental rescue, sustained Nur77 level in iNKT cells impairs its function in a cell intrinsic manner.

The functional unresponsiveness described above is reminiscent of iNKT cell anergy [13, 50, 57-65] and T cell exhaustion [19, 24, 25, 66]. Upon further examination, we found that similar to Nur77^{tg};Vα14^{tg} mice, PD1 expression was significantly upregulated on iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras, and this was consistently higher than Vα14^{tg}/Jα18^{-/-} mixed BM chimeras. PD1 expression was high on PLZF^{HI} NKT2, PLZF^{INT} NKT1 and PLZF^{LO} iNKT cell subsets (**Fig. 6C—F**). The PLZF^{HI} population consists of CCR7^{HI}PD1^{LO} cells, a precursor to all iNKT cell subsets, and CCR7^{LO}PD1^{HI} NKT2 cells (**ref/s**). PD1 was induced as early as the CCR7^{HI}PD1^{LO} thymic and splenic iNKT cells (**Fig. 6C—F**). Furthermore, markers of IL-10-producing Tregs and NKT10 cells, FR4 and NRP1 as well as Ly108 and CD244.2 [13], were also upregulated on thymic and splenic iNKT cells from Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras compared with Vα14^{tg}/Jα18^{-/-} mixed chimeras (**SI Appendix Fig. 6B—D**). Thus, sustained Nur77 levels induce an exhausted phenotype in iNKT cells.

Sustained Nur77 expression induces Foxp3 expression in a subset of iNKT cells

Consistent with the report that Nur77 family members drive *Foxp3* gene expression and Treg development [19], we found that basal Foxp3 and CD25 levels were increased in iNKT cells from Nur77^{tg};Vα14^{tg} mice compared with Vα14^{tg} mice (**Fig. 7A—D**). Thus, thymic and splenic CD25^{NEG}Foxp3⁺ and CD25⁺Foxp3^{NEG} but

not CD25⁺Foxp3⁺ NKT cells had emerged in Nur77^{tg};Vα14^{tg} mice that were absent in B6 mice but present in very minute proportion in Vα14^{tg} mice (**Fig. 7A,B,E,F**). This result was in agreement with higher proportions of thymic CD4⁺CD25⁺Foxp3^{NEG} and splenic CD4⁺CD25^{NEG}Foxp3⁺ conventional T cells in Nur77^{tg};Vα14^{tg} mice (**Fig. 7**). Importantly, splenic Foxp3^{NEG} and Foxp3⁺ iNKT cells upregulated Nur77^{GFP} and CD25 in response to in vivo αGC stimulation (**Fig. 7E,G—I**). It is possible that the Foxp3⁺ iNKT cells arose in Nur77^{tg};Vα14^{tg} mice because these mice lack NKT1 and NKT2 subsets. This is unlikely because the frequencies of Foxp3⁺ iNKT cells were similar in both Nur77^{tg};Vα14^{tg}/B6 and Nur77^{tg};Vα14^{tg}/Jα18^{-/-} mixed chimeras (**SI Appendix Fig. 7A—C**). Hence, sustained Nur77 drives Foxp3 expression in a subpopulation of iNKT cells in a cell intrinsic manner.

Discussion

This study investigated how iNKT cells attain tolerance to self-lipid agonists. Understanding the mechanism/s of self-tolerance in this lineage is of immunological importance because NKT cells are self-reactive, and mediate microbial and sterile inflammation. An unbridled inflammatory response could then lead to a chronic state that initiates and perpetuates inflammatory disease. Hence, it is imperative that the immune system ensures that iNKT cells do not go

rogue and cause autoimmune or overt autoinflammatory responses. This study focused on Nur77 as a potential candidate transcription factor that controls tolerance induction in iNKT cells because, (a) Nur77 is expressed at high levels within developing iNKT cells that wanes through differentiation; (b) the role of Nur77 in iNKT cells has not yet been defined; and (c) Nur77 has been implicated in negative selection of thymocytes and tolerance induction in peripheral but not thymic conventional T cells. In contrast to this view, Nur77 was found central to self-tolerance induction in developing, thymic iNKT cells making these cells hyporesponsive to agonistic stimulation in the periphery. Most importantly, Nur77 also controlled effector differentiation and functions of iNKT cells, features that distinguish them from conventional T cells emerging from the thymus.

iNKT cell development was intact in mice lacking Nur77 alone or all three Nur77 family members, but a detailed analysis of iNKT cell differentiation was not possible because the TKO mice succumbed to disseminated autoimmune diseases [19, 42]. Hence, we investigated iNKT cell development, differentiation and function in mice expressing a Nur77 transgene within thymocytes under the control of the proximal *Lck* promoter. We found that Nur77 mediated caspase 3-dependent cell death in this T cell lineage but did not alter the proliferative capacity of developing iNKT cells. The noted cell death is reminiscent of Nur77-

mediated negative selection of conventional T cells [17-20]. This new result suggests that Nur77 mediates the pruning of pathogenic, self-reactive cells from the functional repertoire. Consistent with this view, iNKT cells were depleted by the addition of exogenous α GC to 16.5-day fetal thymic organ cultures established from wild type mice or by in vivo α GC injections into neonatal mice [39, 41][41]. Hence, both positive and negative selection likely sculpt the functional iNKT cell repertoire.

Results from mixed BM chimera experiments suggest both cell extrinsic and intrinsic roles for Nur77 in iNKT cell development. Cell extrinsic factors that affect NKT cell development could involve defects in CD1d-restricted selecting ligand presentation, SLAM co-receptor help, and DP thymocyte and thymic medullary cellularity/architecture. The integrity of the thymic medullary compartment relies on RANK-L (receptor activator of nuclear factor- κ B ligand or TNFSF11) produced by NKT2 and NKT17 cells [12]. As the B6 partner in the mixed Nur77^{tg};V α 14^{tg}/B6 chimeras developed functional iNKT cell subsets. RANK-L so produced could have “helped” iNKT cell development from the Nur77^{tg};V α 14^{tg} partner as well but did not. Hence, the thymic medullary environment had little contribution, if any, to Nur77-mediated impaired iNKT cell development and differentiation. The above findings also suggest that the cell extrinsic role of Nur77 is DP thymocyte

mediated and independent of the nature of the endogenous agonist and its presentation by DP thymocytes originating from Nur77-expressing mice.

Homotypic SLAM-SLAM interactions between NKT cell precursors and DP thymocytes are critical for the selection and differentiation of this lineage [5, 29-36, 67]. Hence, this interaction is fine-tuned at the level of SLAM family member expression on the two cell types. For example, three-fold higher than normal SLAMF1 expression yielded higher than normal NKT cell output, potentially by enhancing survival [68]. In contrast, a seven-fold higher than normal SLAM expression resulted in lower than normal NKT cell yield [68]. In this context, it is noteworthy that Nur77-expressing mouse strains and derived chimeras showed up to 1.5–2.25-fold higher SLAM (SLAMF1), Ly108 (SLAMF6) and CD48 (SLAMF2) expression. This level of SLAM expression was much below the threshold for NKT cell attrition by cell death signaled via SLAM-SAP-Fyn signaling, but, if anything, closer to the threshold of expansion. Therefore, it is unlikely that defective homotypic SLAM interactions impaired NKT cell development and differentiation. This notion is further supported by the finding that Nur77 overexpression did not alter the proliferative capacity of developing NKT cells.

A recent study demonstrated that SLAM receptors temper TCR signal strength for proper NKT cell development. Mice lacking all the SLAM family receptors developed NKT cells with enhanced Nur77 expression. Further, enhanced Nur77 expression correlated with inhibitory immune checkpoint receptors such as PD-1, TIM-3 and CD160 expression [5]. Thus, it is likely that elevated expression of SLAMF1 and SLAMF6 on NKT cells that overexpress Nur77 were induced as a negative feedback loop to counter Nur77 mediated apoptosis. But this was not the case as increased SLAM receptor expression did not prevent apoptosis or NKT cell hypo-responsiveness in Nur77^{tg} mice. This notion is consistent with increased SLAM receptor expression by IL-10-producing NKT10 cells [13]. It is therefore, probable that enhanced SLAM receptor expression and SLAM signaling might play a role in the induction of NKT cell tolerance.

Even though the majority of NKT cells were arrested at the thymic precursor stage 0 in Nur77^{tg};V α 14^{tg} mice, a few thymic NKT1, NKT2, and NKT17 cells with a mature phenotype had escaped this maturation arrest. Further, the periphery was lacking in NKT1 and NKT2 subsets; yet NKT17 development was unaffected. Based on the CD5, CD6 and Ly6C expression pattern, a recent study reported that the differentiation of the three NKT subsets required distinct TCR signal strength: NKT2>NKT17>NKT1[27]. Consistent with this hierarchy, Nur77 expression correlated with TCR signal strength integrating cell activation to downstream differentiation signals [16, 27]. Thus, it was intriguing that sustained Nur77

overexpression in the mixed BM chimeras abrogated NKT2 cell development whereas NKT17 subset development was intact. It is conceivable that strong TCR signals induced other factor/s and signaling module/s apart from inducing Nur77. Or, alternatively, Nur77 may play distinct roles in different NKT cell subsets, or both. This function of Nur77 in effector differentiation of NKT cells strikingly contrasts no known role for Nur77 in effector differentiation within conventional T cells.

Nur77 controls the induction of self-tolerance at multiple levels: it controlled negative selection of developing NKT cells as discussed above. Further, Nur77 induced an exhausted phenotype in NKT cells as the developing cells expressed PD1, which is associated with NKT cell anergy induction [60-62]. Two recent studies demonstrated a role for Nr4a family transcription factors (TF) in the induction of T cell hypo-responsiveness [24]. The Nr4a-TFs binding motif, ~23 kilobases upstream of the *Pdcd1* transcription start site, is highly enriched in several models of T cell exhaustion but is absent in *Nr4a* TKO mice[24]. Similarly, Nur77 expression is strongly correlated with H3K4me3 (accessible chromatin) marks near tolerance related genes [24]. Thus, Nur77 induction with thymic NKT cells appears to program a tolerant or exhausted gene expression profile. Enhanced expression of FR4, NRP1, and CD244—markers of T/NKT cell tolerance—on NKT cells over-expressing Nur77 further supports this notion. NKT cell tolerance so induced is distinct in that this process occurs in the periphery of

conventional T cells and not the thymus as occurs in the early stages of developing NKT cells.

iNKT cells in mouse strains and derived chimeras that overexpressed Nur77 were hyporesponsive to agonistic stimulation in vivo with α GC. CD4⁺ T cell precursors from *Nr4a* TKO mice produce substantially high IL-4 compared to wild type mice [19, 69]. Further, *Nr4a* TKO CD8⁺ CAR T cells have enhanced IFN- γ production compared to their wild type counterparts [24]. A Nur77 binding motif in the *Ifng* locus is enriched in *Nr4a*-TKO CD8⁺ CAR T cells [24], which suggests Nur77 directly binds to regulatory the regions and repress expression. In addition, other cytokine gene loci, such as *Il21* and *Tnfa*, also showed enrichment for Nur77 without a direct Nur77 binding motif, suggesting that Nur77 can induce the expression of negative regulators of cytokine genes and, thereby, control T cell responsiveness [24, 69].

Lastly, Nur77 overexpression induced Foxp3 expression on a subset of NKT cells and the emergence of cells that resemble conventional Tregs. We predict that Nur77-induced cell death in the thymus induces transforming growth factor- β , which is known to induce Foxp3 expression on iNKT cells [70]. Further, IL-10-producing Foxp3⁺ regulatory iNKT cells are produced in a paracrine indoleamine 2, 3-dioxygenase-dependent manner in an acute liver injury model [71]. Hence,

multiple mechanisms ensure the induction of self-tolerance in this self-reactive iNKT cell lineage.

In summary, this study unveiled a critical, yet hitherto unknown, role for Nur77 in iNKT cell development, effector subset differentiation, and function. Nur77 controlled negative selection by inducing caspase-3-mediated cell death and iNKT cell hypo-responsiveness as a means of self-tolerance induction. Nur77 did so by cell intrinsic and extrinsic mechanisms that were distinct from its role in conventional T cell development. These new findings assure that iNKT cells do not go rogue to cause autoimmune or autoinflammatory diseases and pinpoint Nur77 as a target for clinical intervention should that occur.

Materials and Methods

Mice. Age-matched, 8—10-week old mice were used for experiments described herein. B6-Nur77^{tg} (Nur77^{tg}) [17], and B6-J α 18^{-/-} (J α 18^{-/-}) [72] mice were generous gifts from A. Winoto, and M. Taniguchi, respectively. B6-V α 14^{tg} (V α 14^{tg}) [35] and B6-CD1d1^{-/-} (CD1d^{-/-}) [73] mice have been reported. B6 (C57BL/6J) mice were purchased from the Jackson Laboratory. Nur77^{tg} mice were crossed with B6 or V α 14^{tg} mice to obtain Nur77^{tg} or Nur77^{tg};V α 14^{tg} mice, respectively. A single copy of each transgene was maintained for the studies.

Study approval. All procedures for use of mice in the study were approved by the Institutional Animal Care and Use Committee at Vanderbilt University School of Medicine.

Reagents and experimental procedures. Details are provided in Supplemental Information.

Acknowledgments

We thank A. Winoto (UC Berkeley) and M. Taniguchi (RIKEN, Japan) for B6- B6-Nur77^{tg} (Nur77^{tg}) and B6-J α 18^{-/-} (J α 18^{-/-}) mice, respectively. We thank Vanderbilt University Medical Centre Flow Cytometry Shared Resources, supported by Vanderbilt Ingram Cancer Center (CA68485) for assistance with cell sorting, NIH Tetramer Core CD1d tetramers. This work was supported by NIH Grants (AI061721, AI042284 to S.J.; DK081536 to L.W. and L.V.K.; DK104817 and AI139046 to L.V.K.); American Heart Association Grant 19TPA34910078 to L.V.K. and VA Merit BX001444 to S.J.).

References

1. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nature reviews Immunology* **13**, 101-117 (2013).
2. Crosby CM, Kronenberg M. Tissue-specific functions of invariant natural killer T cells. *Nature reviews Immunology* **18**, 559-574 (2018).
3. Kumar A, Suryadevara N, Hill TM, Bezbradica JS, Van Kaer L, Joyce S. Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective. *Frontiers in immunology* **8**, 1858 (2017).
4. Van Kaer L, Wu L, Joyce S. Mechanisms and Consequences of Antigen Presentation by CD1. *Trends in immunology* **37**, 738-754 (2016).

5. Bedard M, *et al.* Sterile activation of invariant natural killer T cells by ER-stressed antigen-presenting cells. *Proceedings of the National Academy of Sciences of the United States of America*, (2019).
6. Bennstein SB. Unraveling Natural Killer T-Cells Development. *Frontiers in immunology* **8**, 1950 (2017).
7. Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Current opinion in immunology* **25**, 161-167 (2013).
8. Matsuda JL, *et al.* Homeostasis of V alpha 14i NKT cells. *Nature immunology* **3**, 966-974 (2002).
9. Gordy LE, *et al.* IL-15 regulates homeostasis and terminal maturation of NKT cells. *Journal of immunology (Baltimore, Md : 1950)* **187**, 6335-6345 (2011).
10. Lee YJ, Holzapfel KL, Zhu J, Jameson SC, Hogquist KA. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature immunology* **14**, 1146-1154 (2013).
11. Lee YJ, Wang H, Starrett GJ, Phuong V, Jameson SC, Hogquist KA. Tissue-Specific Distribution of iNKT Cells Impacts Their Cytokine Response. *Immunity* **43**, 566-578 (2015).
12. Wang H, Hogquist KA. CCR7 defines a precursor for murine iNKT cells in thymus and periphery. *eLife* **7**, (2018).
13. Sag D, Krause P, Hedrick CC, Kronenberg M, Wingender G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *The Journal of clinical investigation* **124**, 3725-3740 (2014).
14. Lynch L, *et al.* Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nature immunology* **16**, 85-95 (2015).
15. Engel I, *et al.* Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nature immunology* **17**, 728-739 (2016).
16. Moran AE, *et al.* T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *The Journal of experimental medicine* **208**, 1279-1289 (2011).
17. Calnan BJ, Szychowski S, Chan FK, Cado D, Winoto A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity* **3**, 273-282 (1995).
18. Winoto A. Genes involved in T-cell receptor-mediated apoptosis of thymocytes and T-cell hybridomas. *Seminars in immunology* **9**, 51-58 (1997).
19. Sekiya T, *et al.* Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis. *Nature immunology* **14**, 230-237 (2013).

20. Thompson J, Winoto A. During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. *The Journal of experimental medicine* **205**, 1029-1036 (2008).
21. Tao R, Hancock WW. Resistance of Foxp3⁺ regulatory T cells to Nur77-induced apoptosis promotes allograft survival. *PloS one* **3**, e2321 (2008).
22. Baldwin TA, Hogquist KA. Transcriptional analysis of clonal deletion in vivo. *Journal of immunology (Baltimore, Md : 1950)* **179**, 837-844 (2007).
23. Zhou T, *et al.* Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *The Journal of experimental medicine* **183**, 1879-1892 (1996).
24. Liu X, *et al.* Genome-wide analysis identifies NR4A1 as a key mediator of T cell dysfunction. *Nature* **567**, 525-529 (2019).
25. Seo H, *et al.* TOX and TOX2 transcription factors cooperate with NR4A transcription factors to impose CD8(+) T cell exhaustion. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 12410-12415 (2019).
26. Stanic AK, Bezbradica JS, Park JJ, Van Kaer L, Boothby MR, Joyce S. Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase C theta and NF-kappa B. *Journal of immunology (Baltimore, Md : 1950)* **172**, 4667-4671 (2004).
27. Tuttle KD, *et al.* TCR signal strength controls thymic differentiation of iNKT cell subsets. *Nature communications* **9**, 2650 (2018).
28. Zhao M, *et al.* Altered thymic differentiation and modulation of arthritis by invariant NKT cells expressing mutant ZAP70. *Nature communications* **9**, 2627 (2018).
29. Bendelac A. Positive selection of mouse NK1⁺ T cells by CD1-expressing cortical thymocytes. *The Journal of experimental medicine* **182**, 2091-2096 (1995).
30. Wei DG, *et al.* Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *The Journal of experimental medicine* **202**, 239-248 (2005).
31. Nichols KE, *et al.* Regulation of NKT cell development by SAP, the protein defective in XLP. *Nature medicine* **11**, 340-345 (2005).
32. Pasquier B, *et al.* Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *The Journal of experimental medicine* **201**, 695-701 (2005).

33. Chen S, *et al.* Dissection of SAP-dependent and SAP-independent SLAM family signaling in NKT cell development and humoral immunity. *The Journal of experimental medicine* **214**, 475-489 (2017).
34. Nunez-Cruz S, *et al.* Differential requirement for the SAP-Fyn interaction during NK T cell development and function. *Journal of immunology (Baltimore, Md : 1950)* **181**, 2311-2320 (2008).
35. Griewank K, *et al.* Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* **27**, 751-762 (2007).
36. Kageyama R, *et al.* The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. *Immunity* **36**, 986-1002 (2012).
37. Mallevaey T, *et al.* A molecular basis for NKT cell recognition of CD1d-self-antigen. *Immunity* **34**, 315-326 (2011).
38. Mallevaey T, *et al.* T cell receptor CDR2 beta and CDR3 beta loops collaborate functionally to shape the iNKT cell repertoire. *Immunity* **31**, 60-71 (2009).
39. Chun T, *et al.* CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *The Journal of experimental medicine* **197**, 907-918 (2003).
40. Napolitano A, *et al.* Functional education of invariant NKT cells by dendritic cell tuning of SHP-1. *Journal of immunology (Baltimore, Md : 1950)* **190**, 3299-3308 (2013).
41. Pellicci DG, *et al.* Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *European journal of immunology* **33**, 1816-1823 (2003).
42. Hanna RN, *et al.* The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. *Nature immunology* **12**, 778-785 (2011).
43. Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *The Journal of experimental medicine* **195**, 835-844 (2002).
44. Kumar A, Bezbradica JS, Stanic AK, Joyce S. Characterization and Functional Analysis of Mouse Semi-invariant Natural T Cells. *Current protocols in immunology* **117**, 14.13.11-14.13.55 (2017).
45. Bezbradica JS, Hill T, Stanic AK, Van Kaer L, Joyce S. Commitment toward the natural T (iNKT) cell lineage occurs at the CD4+8+ stage of thymic

- ontogeny. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 5114-5119 (2005).
46. D'Cruz LM, Knell J, Fujimoto JK, Goldrath AW. An essential role for the transcription factor HEB in thymocyte survival, Tcra rearrangement and the development of natural killer T cells. *Nature immunology* **11**, 240-249 (2010).
 47. Egawa T, *et al.* Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. *Immunity* **22**, 705-716 (2005).
 48. Hu T, Simmons A, Yuan J, Bender TP, Alberola-Ila J. The transcription factor c-Myb primes CD4+CD8+ immature thymocytes for selection into the iNKT lineage. *Nature immunology* **11**, 435-441 (2010).
 49. Stanic AK, *et al.* Defective presentation of the CD1d1-restricted natural Va14Ja18 NKT lymphocyte antigen caused by beta-D-glucosylceramide synthase deficiency. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1849-1854 (2003).
 50. Kim S, Lalani S, Parekh VV, Vincent TL, Wu L, Van Kaer L. Impact of bacteria on the phenotype, functions, and therapeutic activities of invariant NKT cells in mice. *The Journal of clinical investigation* **118**, 2301-2315 (2008).
 51. Malhotra N, *et al.* SOX4 controls invariant NKT cell differentiation by tuning TCR signaling. *The Journal of experimental medicine* **215**, 2887-2900 (2018).
 52. Becker AM, *et al.* Invariant NKT cell development requires a full complement of functional CD3 zeta immunoreceptor tyrosine-based activation motifs. *Journal of immunology (Baltimore, Md : 1950)* **184**, 6822-6832 (2010).
 53. Vahl JC, *et al.* NKT cell-TCR expression activates conventional T cells in vivo, but is largely dispensable for mature NKT cell biology. *PLoS biology* **11**, e1001589 (2013).
 54. Dashtsoodol N, Bortoluzzi S, Schmidt-Supprian M. T Cell Receptor Expression Timing and Signal Strength in the Functional Differentiation of Invariant Natural Killer T Cells. *Frontiers in immunology* **10**, 841 (2019).
 55. Myers DR, *et al.* Tonic LAT-HDAC7 Signals Sustain Nur77 and Irf4 Expression to Tune Naive CD4 T Cells. *Cell reports* **19**, 1558-1571 (2017).
 56. Nowyhed HN, Huynh TR, Thomas GD, Blatchley A, Hedrick CC. Cutting Edge: The Orphan Nuclear Receptor Nr4a1 Regulates CD8+ T Cell Expansion and Effector Function through Direct Repression of Irf4. *Journal of immunology (Baltimore, Md : 1950)* **195**, 3515-3519 (2015).

57. Parekh VV, *et al.* Glycolipid antigen induces long-term natural killer T cell anergy in mice. *The Journal of clinical investigation* **115**, 2572-2583 (2005).
58. Fujii S, Shimizu K, Kronenberg M, Steinman RM. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nature immunology* **3**, 867-874 (2002).
59. Uldrich AP, *et al.* NKT cell stimulation with glycolipid antigen in vivo: costimulation-dependent expansion, Bim-dependent contraction, and hyporesponsiveness to further antigenic challenge. *Journal of immunology (Baltimore, Md : 1950)* **175**, 3092-3101 (2005).
60. Chang WS, *et al.* Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. *Journal of immunology (Baltimore, Md : 1950)* **181**, 6707-6710 (2008).
61. Parekh VV, *et al.* PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *Journal of immunology (Baltimore, Md : 1950)* **182**, 2816-2826 (2009).
62. Wang J, Cheng L, Wondimu Z, Swain M, Santamaria P, Yang Y. Cutting edge: CD28 engagement releases antigen-activated invariant NKT cells from the inhibitory effects of PD-1. *Journal of immunology (Baltimore, Md : 1950)* **182**, 6644-6647 (2009).
63. Huang JR, *et al.* alpha-Galactosylceramide but not phenyl-glycolipids induced NKT cell anergy and IL-33-mediated myeloid-derived suppressor cell accumulation via upregulation of egr2/3. *Journal of immunology (Baltimore, Md : 1950)* **192**, 1972-1981 (2014).
64. Kojo S, Elly C, Harada Y, Langdon WY, Kronenberg M, Liu YC. Mechanisms of NKT cell anergy induction involve Cbl-b-promoted monoubiquitination of CARMA1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17847-17851 (2009).
65. Van Kaer L, Parekh VV, Wu L. The Response of CD1d-Restricted Invariant NKT Cells to Microbial Pathogens and Their Products. *Frontiers in immunology* **6**, 226 (2015).
66. Barber DL, *et al.* Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* **439**, 682-687 (2006).
67. DeVault VL, *et al.* Regulation of invariant NKT cell development and function by a 0.14 Mbp locus on chromosome 1: a possible role for Fcgr3. *Genes and immunity* **20**, 261-272 (2019).

68. Jordan MA, *et al.* Role of SLAM in NKT cell development revealed by transgenic complementation in NOD mice. *Journal of immunology (Baltimore, Md : 1950)* **186**, 3953-3965 (2011).
69. Bandukwala HS, Rao A. 'Nurr'ishing Treg cells: Nr4a transcription factors control Foxp3 expression. *Nature immunology* **14**, 201-203 (2013).
70. Monteiro M, *et al.* Identification of regulatory Foxp3⁺ invariant NKT cells induced by TGF-beta. *Journal of immunology (Baltimore, Md : 1950)* **185**, 2157-2163 (2010).
71. Milosavljevic N, *et al.* Mesenchymal stem cells attenuate acute liver injury by altering ratio between interleukin 17 producing and regulatory natural killer T cells. *Liver transplantation : official publication of the American Association for the Study of Liver Diseases and the International Liver Transplantation Society* **23**, 1040-1050 (2017).
72. Cui J, *et al.* Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. *Science (New York, NY)* **278**, 1623-1626 (1997).
73. Mendiratta SK, Martin WD, Hong S, Boesteanu A, Joyce S, Van Kaer L. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* **6**, 469-477 (1997).

Figures and Tables

Figure 1. Introgression of a rearranged V α 14i TCR α -chain gene partially rescues NKT cell development in Nur77^{tg} mice. (A) Thymic and splenic NKT cells from B6 ($n=6$), Nur77^{tg} ($n=4$), Nur77^{tg};V α 14^{tg} ($n=7$) and V α 14^{tg} ($n=4$) mice were identified as CD3 ϵ ⁺tetramer⁺ (fluorophore tagged, mouse CD1d- α GC) cells within electronically gated CD8 α ^{lo} thymocytes or B220^{lo} splenocytes. (B) Absolute cell numbers were calculated from % NKT cells and total cell counts. n , as in A. (C) NKT cell developmental stages were identified as CD44^{NEG}NK1.1^{NEG} stage 0+1, CD44⁺NK1.1^{NEG} stage 2, or CD44⁺NK1.1⁺ stage 3 in the thymus. CD44^{NEG}NK1.1^{NEG} stage 0+1 were further gated to identify CD24^{HI} stage 0 or CD24^{LO-NEG} stage 1 NKT cells. Numbers are % cells among total NKT cells. n , as in A. (D) Absolute numbers were calculated from the absolute NKT cell numbers and % stages 0, 1, 2, and 3 cells in C. n , as in A. Data are mean \pm sem from 3 independent experiments. (E-F) Expression of Nur77 on thymic stages 0-3 and splenic stages 1-3 NKT cells from V α 14^{tg} ($n=3$) and Nur77^{tg};V α 14^{tg} ($n=3$) mice was determined by flow cytometry after surface staining to identify NKT cells (as in A) and NKT cell developmental stages (as in C) after nuclear staining with specific mAb for Nur77. (E) Overlay histograms are representative of 2 independent experiments. (F) Geometric mean fluorescence intensity (gMFI) of Nur77 expression in stages 0-3 NKT cells is plotted as mean gMFI \pm sem; n and replicates as in E. (G-J) Expression of PLZF on thymic stage 0+1 NKT cells from V α 14^{tg} ($n=3$) and Nur77^{tg};V α 14^{tg} ($n=4$) mice as determined by flow cytometry after surface staining to identify NKT cells as in A; and NKT cell developmental stages after nuclear staining with specific mAb for PLZF as in C. (E) Overlay histograms are representative of 2 independent experiments. (H) Geometric mean fluorescence intensity (gMFI) of PLZF expression in thymic stage 0+1 NKT cells plotted as mean gMFI \pm sem; n and replicates as in G. (J) Percentages of thymic PLZF^{HI} stage 0+1 NKT cells is plotted as mean percentages \pm sem; n as in G. ns, not significant ($P > 0.05$), * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$.

Figure 2. Nur77 overexpression alters peripheral NKT cells in Nur77^{tg};Vα14^{tg} mice. (A) NKT cells (upper panel) in indicated organs from Nur77^{tg};Vα14^{tg} (*n*=3) and Vα14^{tg} (*n*=3) mice were identified as in **Fig 1A**. NKT cell stages (lower panel) were identified as in **Fig 1C**. Contour plots are representative of 2 independent experiments. **(B)** Absolute NKT cell numbers were calculated using counting beads (see Materials and Methods [44]). Data are mean±sem from 2 independent experiments. *n*, as in **A**. **(C)** Following surface staining, NKT cells were stained with specific mAb for PLZF, T-bet and RORγt. NKT cell subsets were identified as PLZF^{int}Tbet^{hi} NKT1, PLZF^{hi} NKT2, PLZF^{hi}RORγt⁺ NKT17 cells. Absolute numbers were calculated from absolute NKT cell numbers and NKT1, NKT2, or NKT17 cell frequencies (Representative flow plots are shown in **Supplementary Fig 2c**). Data are mean±sem from two independent experiments. *n*, as in **A**. ns, not significant (*P* > 0.05), **P* ≤ 0.05, ***P* ≤ 0.001, ****P* ≤ 0.0001.

Figure 3. Nur77^{tg};Vα14^{tg} mouse NKT cells recognize αGC but do not respond. (A,B) Thymic NKT cells and NKT cell developmental stages in Vα14^{tg};Nur77^{GFP} (*n*=6) and Nur77^{tg};Vα14^{tg};Nur77^{GFP} (*n*=6) mice were identified as in **Fig 1**, and constitutive Nur77 expression was monitored by evaluating GFP levels. Vα14^{tg};Nur77^{GFP(NEG)} mice were used as negative control for background GFP expression. (A) Overlay histograms are representative of 3 independent experiments. (B) gMFI of Nur77^{GFP} expression by NKT cells and its developmental stages. Data are mean±sem (*n* as in A). (C,D) Constitutive (vehicle) and induced (αGC) Nur77^{GFP} expression in splenic NKT cells and NKT cell developmental stages after i.p. injection with vehicle or αGC. NKT cells were identified as in **Fig 1**. (C) Overlay histograms are representative of 3 independent experiments. Vα14^{tg};Nur77^{GFP} (vehicle *n*=3; αGC *n*=3), Nur77^{tg};Vα14^{tg};Nur77^{GFP} (vehicle *n*=3; αGC *n*=3). (D) gMFI of Nur77^{GFP} expression in NKT cells and NKT cell developmental stages. Data are mean±sem (*n* as in C). (E—F) Splenic NKT cells, identified as in **Fig 1**, from Vα14^{tg} and Nur77^{tg};Vα14^{tg} mice injected i.p. with vehicle or αGC were evaluated for cell surface CD69 expression 6 hours post injection. Overlay histograms are representative of 3—4 independent experiments. Vα14^{tg};Nur77^{GFP} (vehicle *n*=4; αGC *n*=4), Nur77^{tg};Vα14^{tg};Nur77^{GFP} (vehicle *n*=4; αGC *n*=4). (F) gMFI of CD69 expression by NKT cells and NKT cell developmental stages. Data are mean±sem; *n* as in E. (G) αGC or vehicle control was injected i.p. into B6, Nur77^{tg}, Nur77^{tg};Vα14^{tg} and Vα14^{tg} mice as above; at 6 hours post injection, serum was harvested and cytokines measured using meso scale detection kit. Data are cumulative mean±sem from two independent experiments; B6 (vehicle *n*=2; αGC *n*=2), Nur77^{tg} (vehicle *n*=2; αGC *n*=2), Nur77^{tg};Vα14^{tg} (vehicle *n*=2; αGC *n*=4) and Vα14^{tg} (vehicle *n*=2; αGC *n*=3). (H,I) Vα14^{tg} (*n*=3) and Nur77^{tg};Vα14^{tg} (*n*=3) mouse NKT cells within the indicated organs were identified as in **Fig 2** and constitutive PD-1 expression was monitored. Overlay histograms are representative of 3—4 independent experiments (H). gMFI of PD-1 expression by NKT cells (I). Data are mean±sem; *n* as in H. ns, not significant (*P*>0.05), **P* ≤ 0.05, ***P* ≤ 0.001, ****P* ≤ 0.0001.

Figure 4. Impaired NKT cell development in Nur77^{tg};Vα14^{tg} mice is due to cell-intrinsic and cell extrinsic defects. (A—D) Analysis of chimeras generated with BM cells from donor B6, Nur77^{tg}, Jα18^{-/-}, Nur77^{tg};Vα14^{tg} or Vα14^{tg} mice transferred into irradiated Jα18^{-/-} recipients alone (single BM chimeras) or mixed with BM from Jα18^{-/-} at 1:1 ratio (mixed BM chimeras). **(A)** Thymic and splenic NKT cells from B6 (*n*=6), Nur77^{tg} (*n*=6), Jα18^{-/-} (*n*=4), Nur77^{tg};Vα14^{tg} (*n*=6), Vα14^{tg} (*n*=6) single BM chimeras and B6 (*n*=5), Nur77^{tg} (*n*=5), Nur77^{tg};Vα14^{tg} (*n*=25), Vα14^{tg} (*n*=20) mixed BM chimeras were identified as in **Fig 1** (rows 1 and 4, respectively). NKT cell developmental stages were identified as stage 0+1, stage 2, or stage 3 in thymus and spleen (rows 2 and 5, respectively). In the thymus, stage 0+1 cells were further gated to distinguish stage 0 from stage 1 NKT cells (row 3). Numbers are % cells among total NKT cells. **(B)** Absolute cell numbers were calculated from % NKT cells and total cell counts. Data are mean±sem from 3—5 independent experiments; *n*, as in **A**. **(C)** Absolute numbers of thymic stages 0, 1, 2, and 3 NKT cells; they were calculated from the absolute NKT cell numbers and % stages 0, 1, 2, and 3 cells, respectively, in **A**. Absolute numbers of splenic NK1.1^{NEG}tetramer⁺ and NK1.1⁺tetramer⁺ NKT cells were calculated from the absolute NKT cell numbers of NK1.1^{NEG}tetramer⁺ and NK1.1⁺tetramer⁺ cells, respectively, in **A**. Data are mean±sem from 3—5 independent experiments; *n*, as in **A**. **(D)** CD122 expression on thymic NKT cells in Vα14^{tg} (*n*=3) and Nur77^{tg};Vα14^{tg} (*n*=3) mixed BM chimeras were identified as above. Overlay histograms are representative of 2 independent experiments (left). Bar graphs showing % CD122⁺ NKT cells (right). Data are mean±sem from 2 independent experiments. ns, not significant (*P* > 0.05), **P* ≤ 0.05, ***P* ≤ 0.001, ****P* ≤ 0.0001.

Figure 5. Enhanced NKT cell apoptosis in Nur77^{tg};Vα14^{tg} mixed BM chimeras. (A) Thymocytes and splenocytes from Vα14^{tg}/Jα18^{-/-} (*n*=5) and Nur77^{tg};Vα14^{tg}/Jα18^{-/-} (*n*=5) mixed BM chimeras were treated with fluorescently-tagged, active caspase 3 or control substrates. After four hours, NKT cells were identified, staged and caspase-3 incorporation monitored. Bar graphs depict mean±sem of the proportion of caspase-3⁺ NKT cells at different stages of NKT maturation in the thymus (left) and spleen (right). (B) Vα14^{tg}/Jα18^{-/-} (*n*=3) and Nur77^{tg};Vα14^{tg}/Jα18^{-/-} (*n*=3) mixed BM chimeras were injected i.p. with 1 mg EdU or PBS (control) and stained for EdU incorporation in NKT cells at various developmental stages. Bar graphs depict mean±sem of the proportion of EdU⁺ NKT cells at different stages of NKT maturation in the thymus (left) and spleen (right). ns, not significant (*P* > 0.05), **P* ≤ 0.05, ***P* ≤ 0.001, ****P* ≤ 0.0001.

Figure 6. Exhausted and functionally impaired NKT cells emerge in mixed BM chimeras. (A—C) $V\alpha 14^{\text{tg}}/Ja18^{-/-}$ and $Nur77^{\text{tg}};V\alpha 14^{\text{tg}}/Ja18^{-/-}$ mixed BM chimeras were injected i.p. with 1 μg αGC or vehicle. Spleens were harvested at the indicated times after injection. Splenocytes were surface stained to identify NKT cells and stained with mAb to detect intracellular IFN- γ and IL-4. (A) Dot plots showing IFN- γ^+ and IL-4 $^+$ NKT cells. Data are representative of 3—5 independent experiments. *n* for 3 hours: $V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (Veh=3, αGC =3), $Nur77^{\text{tg}};V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (Veh=5, αGC =7); *n* for 5 hours: $V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (Veh=5, αGC =6), $Nur77^{\text{tg}};V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (Veh=7, αGC =9). (B) Bar graphs depict mean \pm sem of the proportion of IFN- γ^+ , IL-4 $^+$, and IFN- γ^+ IL4 $^+$ NKT cells. *n* as in A. (C) Bar graphs depict mean \pm sem of gMFI of IFN- γ (left) and IL-4 (right). Data are representative of 3—5 independent experiments. *n* as in A. (D,E) Thymocytes and splenocytes from $V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (*n*=5) and $Nur77^{\text{tg}};V\alpha 14^{\text{tg}}/Ja18^{-/-}$ (*n*=5) mixed BM chimeras were surface stained to identify NKT cells and expression levels of CCR7 and PD-1. Then they were stained with specific mAb for PLZF and ROR γ t. (D) Contour plots showing gating strategy to identify PLZF $^{\text{HI}}$, PLZF $^{\text{INT}}$ and PLZF $^{\text{LO}}$ (all ROR γ t $^{\text{NEG}}$) NKT cells in the thymus and spleen. PLZF $^{\text{HI}}$ population were further gated to identify CCR7 $^{\text{HI}}$ PD1 $^{\text{LO}}$ and CCR7 $^{\text{LO}}$ PD1 $^{\text{HI}}$ NKT2 cells. PD-1 expression was monitored on the indicated NKT cell populations. (E) Overlay histograms are representative of 2 independent experiments. (F) gMFIs of PD-1 expression. Data are mean \pm sem from 2 independent experiments; *n* as above. ns, not significant ($P > 0.05$), * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$.

Figure 7. Overexpression of Nur77 in NKT cells induces a Foxp3⁺ NKT cell subset that responds to α GC stimulation. (A,B) Thymic NKT cells from C57BL/6 ($n=5$), V α 14^{tg} ($n=5$) and Nur77^{tg};V α 14^{tg} ($n=7$) mice were identified as in Fig 1 and surface CD25 and nuclear Foxp3 expression monitored. (A) Contour plots are representative of 3 independent experiments. (B) Bar graphs show cumulative mean \pm sem of % of CD25^{NEG} Foxp3⁺, CD25⁺Foxp3⁺ and CD25⁺Foxp3^{NEG} within NKT cells. n as in A. (C,D) Constitutive Foxp3 and CD25 expression by NKT cells from mice as in A. (C) Overlay histograms are representative of 3—4 independent experiments. (D) gMFIs of Foxp3 (left) and CD25 (right) expression on thymic NKT cells from mice as above. Data are cumulative mean \pm sem from 3 independent experiments. n as in A. (E,F) Splenocytes from C57BL/6, Nur77^{tg}, V α 14^{tg}, and Nur77^{tg};V α 14^{tg} mice injected with vehicle or α GC were stained to identify NKT cells and CD4⁺ T cells as before. CD25 and Foxp3 expression on NKT cells and CD4⁺ T cells were monitored. (E) Representative contour plots from 3 independent experiments showing CD25 and Foxp3 expression on NKT cells (upper two rows) and CD4⁺ T cells (lower two rows). n = C57BL/6 (Veh=5, α GC=5), Nur77^{tg} (Veh=3, α GC=4), V α 14^{tg} (Veh=4, α GC=4) and Nur77^{tg};V α 14^{tg} (Veh=4, α GC=5). (F) Bar graphs showing cumulative mean \pm sem of % of Foxp3⁺ NKT cells (upper panel) and CD4⁺CD25⁺Foxp3⁺ T cells (lower panel). n as in E. (G,H) Inducible Nur77^{GFP} (left) and CD25 (right) expression on Foxp3^{NEG} (G) and Foxp3⁺ (H) NKT cells from Nur77^{tg};V α 14^{tg};Nur77^{GFP+} mice injected i.p with Veh ($n=2$) or α GC ($n=3$). Bar graphs are cumulative mean \pm sem. (I) gMFI of CD25 expression by CD4⁺CD25⁺Foxp3⁺ cells in vehicle or α GC injected mice as in E. Bar graphs are cumulative mean \pm sem, n as in E. ns, not significant ($P > 0.05$), * $P \leq 0.05$, ** $P \leq 0.001$, *** $P \leq 0.0001$.