Author/s:
Cameron, G;Godfrey, DI

Title:
Differential surface phenotype and context-dependent reactivity of functionally diverse NKT cells

Date:
2018-08-01

Citation:
Cameron, G. & Godfrey, D. I. (2018). Differential surface phenotype and context-dependent reactivity of functionally diverse NKT cells. Immunology and Cell Biology, 96 (7), pp.759-771. https://doi.org/10.1111/imcb.12034.

Article type : Original Article

Differential surface phenotype and context-dependent reactivity of functionally diverse NKT cells

Garth Cameron[1,2] and Dale I. Godfrey[1,2,*]

AFFILIATIONS:

[1]Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, University of Melbourne, Parkville, Victoria 3010, Australia.

[2]Australian Research Council Centre of Excellence in Advanced Molecular Imaging, University of Melbourne, Parkville, Victoria 3010, Australia.

*Corresponding author: Dale I. Godfrey, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, 792 Elizabeth Street, Melbourne, VIC 3000, Australia. E-mail: godfrey@unimelb.edu.au.

RUNNING TITLE: Functionally diverse NKT cell subsets

FUNDING: This work was supported by the National Health and Medical Research Council of Australia (NHMRC; 1013667, 1063587, 1113293) and the Australian Research Council (ARC; CE140100011). DIG is supported by an NHMRC Senior Principal Research Fellowship (1020770 and 1117766).

KEY WORDS: NKT, NKT1, NKT2, NKT10, NKT17, NKT cell subsets, T cell plasticity, context-dependent T cell function

Abstract

Natural Killer T (NKT) cells are a functionally diverse population that recognize lipid-based antigens in association with the antigen-presenting molecule CD1d. Here we define a technique to separate the functionally distinct thymic NKT1, NKT2 and NKT17 cell subsets by their surface expression of CD279 (ICOS) and the activation-associated glycoform of CD43, enabling the investigation of subset-specific effector-functions. We report that all three subsets express the transcription factor GATA-3 and the potential to produce IL-4 and IL-10 following activation. This questions the notion that NKT2 cells are the predominant source of IL-4 within the NKT cell pool, and suggests that IL-10-production may be more indicative of NKT cell plasticity than the existence of a distinct regulatory lineage or subset. We also show that many NKT17 cells are CD4+ and are biased towards Vβ8.3 TCR gene usage. Lastly, we demonstrate that the toll-like receptor (TLR) ligand lipopolysaccharide (LPS) can induce a NKT17 cell-biased response, even in the absence of exogenous antigen, and that combining LPS with α-GalCer resulted in enhanced IL-17A-production, and reduced levels of the immunosuppressive cytokine IL-10. This study provides a novel means to examine the context-dependent reactivity of the functionally heterogeneous NKT cell population and provides important new insight into the functional biology of these subsets.

INTRODUCTION

Natural Killer T (NKT) cells are a CD1d-restricted population present in mice and humans with broad functional activity, including the production of Th1, Th2 and Th17-type cytokines [reviewed in (1)]. This is reflected by their contribution to a diverse array of diseases including infection, autoimmunity, allergy and cancer [reviewed in (2)]. Despite this broad functional scope, NKT cells exhibit limited TCR sequence diversity due to the expression of a largely invariant TCR α-chain, paired with a constrained selection of TCR Vβ-genes [reviewed in (3)]. These cells are highly activated by certain α-linked glycolipid antigens, the

best-known example being α-GalactosylCeramide (α-GalCer). Moreover, it is well established that structurally distinct synthetic analogues of α-GalCer can impact on the balance of Th1/Th2-type cytokines produced following in vivo glycolipid administration.[4-7] Although individual NKT cell TCR usage has the potential to influence the recognition of CD1d in an antigen-dependent manner -[8-10] there is no evidence of selective activation of functionally discrete NKT cells when challenged with specific glycolipid-antigens. Therefore, while a desirable goal for more tailored NKT cell based therapies, mechanisms that could potentially induce subset-specific activation within this functionally heterogeneous population remain unknown.

Whilst the effector-function of distinct NKT cells has been investigated by exploiting their differential transcription factor expression,[11] the cell-destructive nature of this intracellular labelling process limits the potential to carry out any additional functional analysis. Recently, the variable expression of CD122 and CD4,[12] or NK1.1, CD4, and CD27[13] amongst NKT cells have been used as surrogates for transcription factor staining. However, these strategies are unable to stringently isolate functionally discrete sub-populations, thereby compromising the attribution of ensuing responses.

In this study, we show that segregating thymic NKT cells based on their differential expression of CD279 (ICOS) and the activation-associated glycoform of CD43 mediates strict separation of NKT1 (T-bet+, RORγT-), NKT2 (T-bet-, RORγT-) and NKT17 (T-bet-, RORγT+) cells. Using this approach, our findings have revealed clear differences in activation, and IFN-γ and IL-17 production following antigenic stimulation in direct correlation with their associated TCR expression levels; yet conserved IL-4, IL-10 and IL-13 cytokine production across otherwise functionally distinct NKT cells. Furthermore, we show that the presence of the TLR4 agonist LPS skews the NKT cell cytokine response by preferentially stimulating NKT17 cells, with or without α-GalCer-mediated stimulation, whilst limiting production of the immunoregulatory cytokine IL-10, suggesting that the context of activation may be a key factor in shaping the NKT cell response.

RESULTS

Functionally distinct NKT cell subsets can be distinguished by cell-surface phenotype

NKT cells produce a broad range of cytokines including IFN-γ, IL-4, IL-10 and IL-17 following activation [reviewed in (1)], and this diversity is in part thought to reflect the existence of distinct subsets that differ in their transcription factor profiles.[11, 14] We found that the relative levels of the 130-KDa highly glycosylated isoform of CD43 (CD43HG) and the inducible co-stimulatory receptor ICOS (CD278) segregated thymic NKT cells from BALB/c mice into 3 clear sub-populations (Figure 1a), which exhibited markedly different transcription factor expression profiles that align with the previously described NKT1, NKT2 and NKT17 cell subsets as defined in Lee et al 2013. Thus, CD43HG- ICOS^{lo} NKT cells were T-bet+ RORγT- NKT1 cells; CD43HG^{int} ICOS+ cells were T-bet- RORγT-, corresponding to NKT2 cells; and CD43HG+ ICOS+ NKT cells were T-bet- RORγT+ aligning with NKT17 cells (Figure 1a-b).

NKT1 cells have been reported as being low to negative for expression of the Th2-associated transcription factor GATA-3 when assessed in relation to DP thymocytes,[11] however DP thymocytes are known to express variable levels of this transcription factor.[15] We found that GATA-3 was clearly expressed by all thymic NKT cell subsets in comparison to GATA-3– B cells within this organ (Figure 1c).

Another key transcription factor that is thought to distinguish NKT cell subsets is PLZF.[11] Differences in PLZF expression levels between subsets were more distinct than GATA-3, with NKT1 cells being PLZF^{lo}, NKT2 cells PLZF^{hi} and NKT17 cells PLZF^{int} (Figure 1c), in accordance with previous reports.[11-13]

Prior attempts to identify functional NKT cell subsets via their surface phenotype have relied on the premise that NKT17 cells lack CD4 expression.[12, 13, 16] Despite this, we consistently found CD4+ NKT17 cells within the thymus and lymph nodes of BALB/c and C57BL/6 mice (Figure 1d-e), suggesting that CD4 expression is not stringently linked to NKT cell function.

Whilst NKT1 cells were relatively rare in the thymus of BALB/c mice (~5%), they were the major population (>90%) in C57BL/6 thymus, consistent with previous reports.[11] Furthermore, essentially all NKT cells in the liver of both strains were NKT1 cells (Supplementary Figure 1a-b). In contrast, peripheral lymph node NKT cells in BALB/c mice were biased towards the NKT2 subset while those in C57BL/6 mice were biased towards NKT17 cells (Supplementary Figure 1a-b). These experiments highlighted the strain and organ-specific representation of these functionally distinct NKT cell subsets.

The correlation between CD43HG and ICOS expression and the transcription factor profiles of NKT cells was clearest in BALB/c thymus; however, a similar, albeit less stringent, pattern was observed in peripheral tissues such as of this strain (Supplementary Figure 1c-e). For C57BL/6 NKT cells, while the pattern of ICOS expression was the same as in BALB/c mice, CD43HG was more promiscuous showing bimodal expression on NKT1 and NKT2 cells, whereas NKT17 cells were CD43HG^{hi} ICOS+, similar to BALB/c mice (Supplementary Figure 1c-e). These findings expand upon previous reports,[11-13] and reveal complex tissue and strain-specific factors that regulate the distribution of functionally discrete NKT cells.

CD43HG and ICOS expression aligns with differential NKT cell effector-function

We next sought to establish whether NKT cell subsets defined by their levels of CD43HG and ICOS showed differential cytokine production in accordance with their unique transcription factor expression. Thymocytes were enriched for NKT cells and stimulated in vitro for 4 hours with PMA and ionomycin in the presence of GolgiStop, and the production of IL-4, IL-10, IL-13, IL-17A and IFN-γ amongst α-GalCer-loaded CD1d-tetramer+ TCRβ+ cells was assessed via intracellular cytokine staining (ICS). In line with their distinct transcription factor profiles (Figure 1a), CD43HG- ICOS^{lo} (NKT1) cells produced high levels of IFN-γ (>60%). CD43HG+ ICOS+ (NKT17) cells produced high levels of IL-17A (>80%), whereas CD43HG^{int}, ICOS+ (NKT2) cells produced very little of either IFN-γ or IL-17A (Figure 2a-b). While CD43HG^{int} ICOS+ cells did produce IL-4, as expected for the NKT2 subset, CD43HG+ ICOS+ (NKT17) cells produced similar amounts of IL-4, whereas the CD43HG- ICOS^{lo} (NKT1) subset produced the lowest levels of IL-4. Importantly, by double labelling for IL-4 with IFN-γ or IL-17A, we showed that bona-fide IFN-γ-producing NKT1 and IL-17-producing NKT17 cells (rather than contaminating NKT2 cells) were producers of IL-4. The Th2-associated cytokine IL-13 was also produced by each NKT cell subset, albeit most abundantly by CD43^{int} ICOS+ (NKT2) cells (Figure 2a-b), whereas IL-10-production was not detected from any subset in these short-term stimulation assays.

We also tested cytokine production by NKT subsets following antigenic-challenge with α-GalCer. Thymic NKT cells were purified by flow cytometric sorting based on their differential CD43HG and ICOS expression (Figure 3a), labelled with CellTrace Violet and co-cultured with Jα18-/- splenocytes (that lack NKT cells) as a source of antigen-presenting cells, pulsed overnight with α-GalCer (100 ng/ml). After 48 hours - the final 4 hours in the presence of GolgiStop, the production of IL-4, IL-10, IL-13, IL-17A and IFN-γ cytokines

were assessed via ICS (Figure 3b-c). Differences in IFN-γ and IL-17A-production were consistent with the PMA and ionomycin-induced cytokine production (Figure 2) and the ex vivo transcription factor expression (Figure 1a) of each NKT cell subset. Thus, CD43HG-ICOS^{lo} (NKT1) cells were the prominent IFN-γ-producers (~15%), CD43HG+ ICOS+ (NKT17) cells produced the most IL-17A (~13%), whilst the CD43HG^{int} ICOS+ (NKT2) subset produced very little (<1%) of either cytokine (Figure 3b-c). Notably, all NKT cell subsets produced IL-4 and IL-13 in these cultures, supporting the fact that RORγT or T-bet expression does not preclude the production of Th2-type cytokines by NKT cells. The co-production of IL-4 with IL-17A and IFN-γ was also detected from α-GalCer-challenged thymic NKT cells upon re-stimulation with PMA and ionomycin (Supplementary Figure 2), and from peripheral NKT cells either, ex vivo or following in vitro activation with α-GalCer (Supplementary Figure 3). Despite IL-10 not being detected from ex vivo thymic NKT cells (Figure 2), proliferating cells from each subset were clearly producing this cytokine by 48hrs (Figure 3b-c), suggesting that this phenotype is rapidly acquired following α-GalCer-mediated activation, regardless of the ability of these cells to produce other cytokines.

Functionally distinct NKT cell subsets show distinct TCR expression

The segregation of BALB/c thymic NKT cells based on their transcription factor expression also revealed marked differences in TCR expression levels. Whereas, NKT1 cells were TCR^{lo}, NKT2 cells were TCR^{hi} and NKT17 cells were TCR^{int} (Figure 4a-b), which correlated with differences in CD3 expression among BALB/c thymic NKT cell subsets reported recently.[12] This trend was also reflected in C57BL/6 thymus, although less prominently compared to BALB/c. Interestingly, TCR and PLZF expression levels were closely correlated among NKT cells (Figure 4c), suggesting that they may be co-regulated during the thymic differentiation process. The T-bet- RORγT- (NKT2) subset also expressed the highest levels of TCR within peripheral lymph nodes of BALB/c and C57BL/6 mice (Figure 4d). However, in contrast to thymic subsets, lymph node NKT1 and NKT17 cells showed comparable TCR expression levels in both strains (Figure 4d), suggesting that the TCR expression levels may be modulated following thymic egress. Interestingly, differences in TCR expression levels in the C57BL/6 strain were more noticeable in the periphery (Figure 4d), compared to the thymus (Figure 4b).

Differences in TCRVβ gene usage were observed among (BALB/c) functional thymic NKT cell subsets in accordance with a previous report.[11] For example, NKT1 cells were biased

toward Vβ7 expression, whereas NKT2 cells were biased towards Vβ2 (Figure 4e). However, while Vβ8 expression was previously reported as unbiased amongst NKT cell subsets,[11] we found that NKT17 cells were significantly biased towards the expression of Vβ8.3. A potential explanation for these conflicting data is that the presence of anti-Vβ8.3 can severely inhibit α-GalCer-loaded CD1d-tetramer binding; therefore, the ability to detect Vβ8.3+ NKT cells can vary considerably based on the staining technique employed (Supplementary Figure 4). These data support an association between TCR expression levels and TCR-Vβ usage in the divergent development of functionally distinct NKT cells.

TCR expression levels correlate with antigen-reactivity

To determine whether the differential TCR composition and expression levels amongst NKT cell subsets impacted on their proliferative response to antigen, these cells were separated, labelled with CellTrace Violet, and stimulated with Jα18-/- splenocytes as antigen-presenting cells pulsed with α-GalCer (100 ng/ml) in vitro and the extent of proliferation assessed at 24, 36 and 48hr time-points (Figure 5a). While very little proliferation was seen for any subset at 24hrs, by 36hrs the majority of CD43HG^{int} ICOS+ (NKT2) cells had divided, residing in the first or second proliferative-generations. In contrast, most CD43HG+ ICOS+ (NKT17) cells had undergone only one division, whereas CD43HG- ICOS^{lo} (NKT1) cells remained mostly undivided. The majority of NKT cells from each subset were proliferating by 48 hours, however, the division index, which gauges the extent of proliferation within each sample exposed a hierarchy of CD43HG^{int} ICOS+ (NKT2) > CD43HG+ ICOS+ (NKT17) > CD43HG- ICOS^{lo} (NKT1). The variability in proliferative responses observed correlated closely with the relative differences in TCR expression levels between NKT cell subsets assessed following sort-purification (Figure 5b). These data suggest that the kinetics of the NKT cell response to CD1d-restricted antigen may be influenced by subset-specific differences in TCR expression.

The context of activation impacts the NKT cell response

To examine if varying the context of activation could impact subset-specific responses, NKT cell-enriched thymocytes were labelled with CFSE and co-cultured with Jα18-/- splenocytes, pulsed with LPS (100 ng/ml) and/or α-GalCer (100 ng/ml) for 48 hours, by which point TCR expression levels were recovered (Supplementary Figure 5). For the final 4 hours, cells were cultured with GolgiStop alone, or re-stimulated with PMA and ionomycin for 4 hours in the presence of GolgiStop. Splenocytes pulsed with LPS alone induced NKT cell expansion and

the production of IL-17A (Figure 6a-c). In contrast, cells stimulated with α-GalCer displayed a heterogeneous cytokine response, including the production of IL-4, IL-10, IL-17A and IFN-γ (Figure 6c-d). Although the combination of LPS and α-GalCer did not typically enhance NKT cell proliferation compared to α-GalCer alone (Figure 6a-b), LPS did enhance the production of IL-17A, whilst reducing the levels of IL-10 (Figure 6c-d). Importantly, the IL-17-bias induced by LPS exposure was more notable following PMA and ionomycin re-stimulation (Figure 6e-f), supporting the possibility that the presence of LPS was having a subset-specific impact.

To investigate if functional responses changed in a dose-dependent manner, NKT cell-enriched thymocytes were stimulated with titrating doses of α-GalCer (0, 1, 10 and 100 ng/ml) with or without LPS (100 ng/ml) and then re-stimulated with PMA and ionomycin. Although the extent of proliferation of NKT cells was similar in response to LPS alone and the lowest dose (1ng/ml) of α-GalCer (Figure 7a-b), the functionality of responding cells was clearly distinct. Whereas, the majority of proliferating NKT cells in response to splenocytes pulsed with LPS alone were IL-17A-producers, those responding to α-GalCer (1 ng/ml) were predominantly IL-10-positive (Figure 7c). Interestingly, in the absence of LPS, the percentages of IL-10-producing NKT cells was inversely correlated with the dose of α-GalCer (Figure 7d). Furthermore, IL-17A production induced by LPS was most evident at lower doses of α-GalCer. Importantly, IL-10-production was blunted by the presence of LPS, regardless of the concentration of α-GalCer (Figure 7d), suggesting that the induction of IL-10 is dependent on the activation-context

To identify whether a shift towards IL-10-production could also be detected from peripheral NKT cells, lymph node (C57BL/6) cells were cultured for 72hrs with titrating doses of α-GalCer. The cytokine profile of NKT cells was then examined following PMA and ionomycin re-stimulation. Although the concentration of α-GalCer clearly influenced the extent of NKT cell expansion, IL-10-producing cells were similarly detected at all doses, whilst being barely detectable from unstimulated cells (Figure 7e). Notably, IL-10-producing lymph node NKT cells displayed broad functionality, which included the co-production of IL-4, IL-17A and IFN-γ (Figure 7f). These data illustrate the potential for functionally dissimilar thymic and peripheral NKT cell to co-produce IL-10 following TCR-mediated activation.

The presence of LPS induces NKT17 cell proliferation

To examine the correlation between the presence of LPS and the biased production of IL-17A by NKT cells in a more refined way, thymocytes were enriched for NKT cells (via the complement-mediated depletion of immature thymocytes), and purified by flow cytometric sorting based on CD43HG and ICOS expression alone to segregate thymocyte populations containing either NKT1, NKT2 and NKT17 cells. This is a major advantage of having cell surface markers for these subsets, because in vitro culture experiments would not be possible if the cells had been permeabilised for transcription factor staining. Thymocytes were isolated in these experiments without α-GalCer-loaded CD1d-tetramer staining to avoid TCR-mediated stimulation. These populations were then co-cultured with Jα18-/- splenocytes, pulsed with LPS (100 ng/ml) with or without a low concentration of α-GalCer (1 ng/ml). The extent of CellTrace Violet dilution amongst α-GalCer-loaded CD1d-tetramer+ TCRβ+ cells was then assessed after 72 hours. CD43HG+ ICOS+ (NKT17) cells showed robust proliferation in response to LPS-pulsed splenocytes, whereas CD43HG- ICOS^{lo} (NKT1) and CD43HG^{int} ICOS+ (NKT2) cells did not respond (Figure 8a-b). Further, the addition of LPS enhanced the α-GalCer-mediated proliferation of NKT17 cells, but not the NKT1 or NKT2 cell subsets.

These data demonstrate how the presence of certain pro-inflammatory stimuli can potentially shape NKT cell responses. This approach also highlights the utility of segregating thymic NKT cell subsets by their expression of CD43HG and ICOS for functional analysis.

DISCUSSION

To fully explore the contribution of functionally distinct NKT subsets to immunity requires methods to viably isolate these cells, rather than relying on the destructive process of intracellular transcription factor staining. Previously, examining the function of NKT cell subsets using PLZF and RORγT staining required IL-4-reporter mice to investigate the subset-specific production of this cytokine,[11] which led to the conclusion that NKT cells were segregated into IFN-γ-producing NKT1 cells, IL-4-producing NKT2 cells and IL-17-producing NKT17 cells.[11] However, more recent techniques designed to isolate functional subsets by their surface phenotypes have indicated that IL-4-production is not restricted to NKT2 cells.[12, 13] Further, we have shown by separating thymic NKT cells via their differential CD43HG and ICOS expression, that similar levels of IL-4, IL-10 and IL-13-production are

detectable from NKT1, NKT2 and NKT17 cells - the true extent of which only being evident when their cytokine output was assessed downstream of TCR-mediated activation.

However, the value of any such methodology is reliant on the stringency with which it purifies target populations. For example, the segregation of NKT cells based on their CD122 and CD4 expression isolates 'NKT2' cells that clearly produce IL-17A,[12] likely due to the detection of CD4+ NKT17 cells demonstrated herein. However, we found that NKT2 cells purified based on their CD43HG and ICOS expression produced negligible levels of IL-17A. In addition, the use of NK1.1, CD4, and CD27 expression to purify 'NKT2' cells isolated a population that contained an undefined proportion of T-bet+ cells,[13] that by definition are not NKT2 cells.[11] These data suggest that identifying NKT cell subsets by their relative levels of CD43HG and ICOS expression represents a more precise means to isolate, and explore the individual functionality of NKT1, NKT2 and NKT17 cells. The data also demonstrate that the term 'NKT2' is potentially confusing, because these cells are no better than NKT1 or NKT17 cells at producing IL-4 or IL-13 following activation.

Co-expression of the T cell 'lineage defining' transcription factors GATA-3 and T-bet by NKT1 cells, and GATA-3 and RORγT by NKT17 cells provides an explanation for the co-production of IL-4 by these populations, although it is unclear why only some NKT cells produce IL-4 when all of them express GATA-3. While direct and indirect molecular interactions that influence the activity of GATA-3 and T-bet within co-expressing cells have been described,[17] the means that control the cytokine output of NKT1 cells remain unclear. Similarly, mechanisms that may regulate the differential expression of GATA-3 and RORγT-associated genes within NKT17 cells have not been identified. Thus, establishing how environmental cues may impact the activity of these transcription factors in both NKT1 and NKT17 cells[18] is required in order to fully appreciate the inherent diversity, and potential functional plasticity that exists within these broadly-defined subsets.

Whilst the markers CD43HG and ICOS were used in this study as a means to segregate functionally distinct sub-populations of NKT cells, ICOS is a costimulatory molecule that interacts with B7-H2 (CD275), an interaction important for the expansion and functional maturation of thymic NKT cells.[19] CD43 exists in at least two distinct glycoforms; while the 115kDa form is expressed by all T cells, expression of the 130kDa highly glycosylated

isoform (CD43HG) is reportedly restricted to activated T cells and DP thymocytes.[20, 21] Following the positive selection of MHC-restricted cells, distinct TCR usage can correlate with the differential down-regulation of CD43HG, suggesting that contrasting selection events may impact on CD43HG expression levels.[21] Both glycoforms of CD43 are ligands for Galectin-1, an apoptosis-inducing molecule expressed by a range of cell types including thymic epithelial cells.[22] Furthermore, CD43HG is also a ligand for E-selectin, an interaction that has been reported to promote Th1 cell-mediated inflammatory responses.[23] Thus, an important area for future study is whether the differential expression of ICOS and CD43HG by NKT cell sublineages relates to the distinct developmental requirements and/or functional potential of these populations.

Exploring how the functionally diverse NKT cell pool responds under different conditions is key to understand their natural role during disease, and to provide insight to more effectively manipulate this population in the clinic. Here we provide evidence that the NKT cell population can respond precisely and appropriately to different forms of antigenic stimuli. For example, whilst production of IL-10 was undetectable from naive NKT cells, we found that challenge with CD1d-restricted antigen elicited production of this immunoregulatory cytokine from otherwise functionally dissimilar NKT cells. These data raise the possibility that IL-10-production by NKT cells may, at least in some circumstances, represent activated NKT1, 2 or 17 cells, rather than the existence of a distinct regulatory subset of NKT10 cells.[24] Importantly, acquisition of IL-10 production was blunted when NKT cells were challenged with α-GalCer in combination with the TLR ligand LPS, whilst conversely, LPS enhanced the production of IL-17A by NKT cells, regardless of the presence of α-GalCer. Indeed, by isolating thymic NKT1, NKT2 and NKT17 cells via their CD43HG and ICOS expression, we show that exposing antigen-presenting cells to LPS alone was sufficient to elicit specific and robust proliferation of NKT17 cells, demonstrating how the environmental context can shape the response of the functionally heterogeneous populations of NKT cells.

In conclusion, the discovery that functionally diverse thymic NKT cells can be specifically identified, and purified as viable cells based on their CD43HG and ICOS expression represents a novel means to investigate how combinations of different biological inputs impact NKT cell development and function, and should enhance our understanding of their contribution to the immune response.

METHODS

Mice

C57BL/6 mice were bred in-house at the Department of Microbiology and Immunology Animal House. BALB/c mice were bred in-house or obtained from the Animal Resources Centre, Melbourne, Australia. BALB/c Jα18-deficient mice were bred in-house (originally provided by M. Taniguchi; backcrossed 10 times). All procedures were approved by the University of Melbourne Animal Ethics Committee.

Glycolipids

α-GalCer (C26) was purchased from Enzo Life Sciences. α-GalCer (C24:1)/PBS-44 was kindly provided by Prof. Paul Savage, Brigham Young University, UT, USA. For in vitro cultures, glycolipid stocks were prepared using Tween 20-based [0.5% (v/v) Tween 20, sucrose (56 mg/ml), and L-histidine (7.5 mg/ml) in PBS] vehicle reagent. For CD1d-tetramer manufacture, glycolipid stocks were prepared in Tyloxapol-based vehicle (0.5% Tyloxapol in TRIS-buffered saline) reagent.

Flow cytometry and antibodies

Single cell suspensions were prepared from: thymocytes enriched for NKT cell via complement-mediated depletion of anti-HSA and anti-CD24 labelled cells, followed by Histopaque-1083 (Sigma-Aldrich) gradient centrifugation; hepatic mononuclear cells subject to red blood cell lysis (Sigma-Aldrich) and 33% isotonic Percoll (GE Healthcare) gradient centrifugation; or pooled lymph nodes (cervical, brachial, axillary and inguinal). Antibody cocktails included α-GalCer (C24:1)/PBS-44-loaded CD1d-tetramer, 7-aminoactinomycin D (Sigma) and anti-CD16-CD32 (clone 2.4G2; grown in-house). mAbs against CD4 (clone RM4-5), CD19 (clone 1D3), B220/CD45R (clone RA3-6B2), ICOS/CD278 (clone 7E.17G9), βTCR (clone H57-597), NK1.1 (clone PK136), IL-4 (clone 11B11), IL-10 (clone JES5-16E3), IL-17A (clone TC11), IFN-γ (clone XMG1.2), Vβ2 (B20.6), Vβ7 (TR310), Vβ8.1/8.2 (MR5-2) and Vβ8.3 (IB3.3) were purchased from BD Biosciences. mAbs against IL-13 (clone eBio13A), T-bet (clone eBio4B10), RORγT (clone B2D), GATA-3 (clone TWAJ) and PLZF (clone Mags.21F7) were purchased from eBiosciences. mAb against the 130kDa glycoform of CD43 (clone 1B11) was purchased from BioLegend. Intracellular cytokine staining was performed using BD Biosciences Cytofix/Cytoperm kit. Intranuclear staining

was performed using the eBioscience transcription factor staining buffer set. Cell-sorting experiments were performed using a BD FACS Aria III. Flow cytometry experiments were performed using a BD LSR Fortessa, and analysed using FlowJo software (TreeStar).

In vitro proliferation assays

Splenocytes from Jα18-/- (BALB/c) mice were pulsed overnight with α-GalCer (1, 10 or 100 ng/ml) and/or LPS (100 ng/ml) in 48-well flat-bottom plates (3 x 10^6 cells per well), harvested and washed with fresh media. Wild-type (BALB/c) thymocytes were enriched for NKT cells by complement-mediated depletion of anti-CD24 and anti-CD8-labelled cells, with dead cells being removed via Histopaque-1083 gradient centrifugation. Thymic NKT cells were sort-purified based on TCRβ and α-GalCer (PBS-44)-loaded CD1d-tetramer staining, and CD43HG and ICOS expression (sort purities ≥ 99%), labelled with 1 μM CellTrace Violet (Invitrogen) and incubated for 10 min at 37°C. Jα18-/- (BALB/c) splenocytes (3 x 10^5 per well) and sort-purified cells (1 x 10^4 per well) were then co-cultured in 96-well round-bottom plates. In some experiments, NKT cell-enriched thymocytes were sorted based on CD43HG and ICOS expression alone (sort purities ≥ 98%), then labelled with 1 μM CellTrace Violet. Jα18-/- (BALB/c) splenocytes (3 x 10^5 per well) and FACS-purified thymocytes [1 x 10^4 ($CD43HG^{int}$ ICOS+ and CD43HG+ ICOS+) or 2 x 10^5 (CD43HG- ICOSlo) per well] were co-cultured in 96-well round-bottom plates. Unsorted NKT cell-enriched thymocytes were labelled with 1 μM CFSE (Molecular Probes). Jα18-/- (BALB/c) splenocytes (3 x 10^5 per well) and NKT cell-enriched thymocytes (5 x 10^4 per well) were then co-cultured in 96-well round-bottom plates. Lymph node cells were cultured in 96-well round-bottom plates (1 x 10^6 per well) in 120 μL media. The next day, 50 μl of media was removed from each well and replaced with 100 μl of fresh media to limit the presence of unprocessed antigen. Cells were then cultured for an additional 2 days prior to analysis.

Cytokine analysis

Prior to intracellular cytokine staining (ICS) ex vivo or in vitro cultured cells were exposed for 4 hrs to 10 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). Ex vivo hepatic cells were cultured in vitro for 4 hrs with PMA, ionomycin in the presence of GolgiStop and 100 nM P2X7R inhibitor A-438079 (Santa Cruz Biotechnology) used to maintain the viability of hepatic NKT cells.[25] Lymph node cultures were re-stimulated for the final 4 hrs with PMA and ionomycin in the presence

of GolgiStop. Jα18-/- splenocyte and NKT cell-purified or NKT cell-enriched thymocyte co-cultures were either exposed to GolgiStop alone (4hrs), or re-stimulated for the final 4 hrs with PMA and ionomycin in the presence of GolgiStop.

ACKNOWLEDMENTS: We are grateful to Dr Paul Savage (Brigham Young University, UT, USA) for providing α-GalCer analogue PBS-44. We thank staff from the Flow Cytometry facility at the Department of Microbiology and Immunology at The University of Melbourne.

CONFLICT OF INTEREST: The authors declare no conflicts of interest.

Supplementary information is available at *Immunology and Cell Biology*'s website.

References

1. Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* 2013; **13:** 101-117.

2. Godfrey DI, Uldrich AP, McCluskey J *et al.* The burgeoning family of unconventional T cells. *Nat Immunol* 2015; **16:** 1114-1123.

3. Rossjohn J, Pellicci DG, Patel O *et al.* Recognition of CD1d-restricted antigens by natural killer T cells. *Nat Rev Immunol* 2012; **12:** 845-857.

4. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 2001; **413:** 531-534.

5. Schmieg J, Yang G, Franck RW *et al.* Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand α-Galactosylceramide. *J Exp Med* 2003; **198:** 1631-1641.

6. Yu KO, Im JS, Molano A *et al.* Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of α-galactosylceramides. *Proc Natl Acad Sci U S A* 2005; **102:** 3383-3388.

7. Aspeslagh S, Nemcovic M, Pauwels N *et al.* Enhanced TCR footprint by a novel glycolipid increases NKT-dependent tumor protection. *J Immunol* 2013; **191:** 2916-2925.

8. Florence WC, Xia C, Gordy LE *et al.* Adaptability of the semi-invariant natural killer T-cell receptor towards structurally diverse CD1d-restricted ligands. *EMBO J* 2009; **28:** 3579-3590.

9. Cameron G, Pellicci DG, Uldrich AP *et al.* Antigen Specificity of Type I NKT Cells Is Governed by TCR β-Chain Diversity. *J Immunol* 2015; **195:** 4604-4614.

10. Clancy-Thompson E, Chen GZ, Tyler PM *et al.* Monoclonal Invariant NKT (iNKT) Cell Mice Reveal a Role for Both Tissue of Origin and the TCR in Development of iNKT Functional Subsets. *J Immunol* 2017; **199:** 159-171.

11. Lee YJ, Holzapfel KL, Zhu J *et al.* Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat Immunol* 2013; **14:** 1146-1154.

12. Georgiev H, Ravens I, Benarafa C *et al.* Distinct gene expression patterns correlate with developmental and functional traits of iNKT subsets. *Nat Commun* 2016; **7:** 13116.

13. Engel I, Seumois G, Chavez L *et al.* Innate-like functions of natural killer T cell subsets result from highly divergent gene programs. *Nat Immunol* 2016; **17:** 728-739.

14. Lynch L, Michelet X, Zhang S *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. *Nat Immunol* 2015; **16:** 85-95.

15. Hernandez-Hoyos G, Anderson MK, Wang C *et al.* GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. *Immunity* 2003; **19:** 83-94.

16. Coquet JM, Chakravarti S, Kyparissoudis K *et al.* Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* 2008; **105:** 11287-11292.

17. Li P, Spolski R, Liao W *et al.* Complex interactions of transcription factors in mediating cytokine biology in T cells. *Immunol Rev* 2014; **261:** 141-156.

18. Fang D, Zhu J. Dynamic balance between master transcription factors determines the fates and functions of CD4 T cell and innate lymphoid cell subsets. *J Exp Med* 2017; **214:** 1861-1876.

19. Chung Y, Nurieva R, Esashi E *et al.* A critical role of costimulation during intrathymic development of invariant NK T cells. *J Immunol* 2008; **180:** 2276-2283.

20. Jones AT, Federsppiel B, Ellies LG *et al.* Characterization of the activation-associated isoform of CD43 on murine T lymphocytes. *J Immunol* 1994; **153:** 3426-3439.

21. Ellies LG, Tao W, Fellinger W *et al.* The CD43 130-kD peripheral T-cell activation antigen is downregulated in thymic positive selection. *Blood* 1996; **88:** 1725-1732.

22. Perillo NL, Uittenbogaart CH, Nguyen JT *et al.* Galectin-1, an endogenous lectin produced by thymic epithelial cells, induces apoptosis of human thymocytes. *J Exp Med* 1997; **185:** 1851-1858.

23. Alcaide P, King SL, Dimitroff CJ *et al.* The 130-kDa glycoform of CD43 functions as an E-selectin ligand for activated Th1 cells in vitro and in delayed-type hypersensitivity reactions in vivo. *J Invest Dermatol* 2007; **127:** 1964-1972.

24. Sag D, Krause P, Hedrick CC *et al.* IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* 2014; **124:** 3725-3740.

25. Kawamura H, Aswad F, Minagawa M *et al.* P2X7 receptors regulate NKT cells in autoimmune hepatitis. *J Immunol* 2006; **176:** 2152-2160.

Figure Legends

Figure 1. CD43HG and ICOS are correlated amongst functional NKT cell subsets. (**a**) Representative plots of the CD43HG and ICOS expression amongst (BALB/c) NKT cells from NKT cell-enriched thymocytes (top), and RORγT and T-bet expression amongst the indicated NKT cell subset (bottom). (**b**) Representative plots of the segregation of function NKT cell subsets based on their differential transcription factor expression (left) and the CD43HG (middle) and ICOS (right) expression levels amongst the indicated NKT cell subsets in relation to B cells and TCRβ+, CD4+, α-GalCer (PBS-44)-loaded CD1d-tetramer-cells from NKT cell-enriched thymocytes (BALB/c). (**c**). GATA-3 (left) and PLZF (right) expression of thymic (BALB/c) NKT cell subsets (gauged by their T-bet and RORγT expression), B cells and DP thymocytes. Data representative of at least 4 independent experiments. (**d**) Plot depicts the CD4 expression amongst the indicated (BALB/c) thymic NKT cell subset. (**e**) Graph depicts the percentages of CD4+ NKT17 cells from the thymus and lymph nodes of BALB/c and C57BL/6 mice. Data pooled from 3 to 10 independent experiments (n=7-16).

Figure 2. Differential CD43HG and ICOS expression aligns with NKT cell cytokine production. Thymocytes (BALB/c) were enriched for NKT cells and stimulated in vitro for 4 hours with PMA and ionomycin in the presence of GolgiStop, and the production of IL-4, IL-10, IL-13, IL-17A and IFN-γ was assessed via ICS. (**a**) Representative plots depicting the percentages of the indicated NKT cell subset producing cytokine. (**b**) Graph depicts the percentages (mean ± SEM) of each NKT cell subsets producing the indicated cytokine. Data pooled from 3 independent experiments (n=6).

Figure 3. Cytokine production by NKT cell subsets following CD1d-mediated activation. (**a**) NKT cells (left) were sort-purified from NKT cell-enriched thymocytes (BALB/c) based on their CD43HG and ICOS expression (right), labelled with CellTrace Violet and (**b**) co-cultured in vitro for 48hrs (the final 4hrs in the presence of GolgiStop) with Jα18-/- (BALB/c)

splenocytes pulsed overnight with α-GalCer (100 ng/ml) and the production of IL-4, IL-10, IL-13, IL-17A and IFN-γ amongst NKT cells was assessed via ICS. Quadrants dissect proliferating NKT cells based on CellTrace Violet dilution, and those cells producing the indicated cytokine. (**c**) Graph depicts the percentages (Mean ± SEM) of proliferating NKT cells from each subset producing the indicated cytokine. Data pooled from 3 independent experiments (n=6).

Figure 4. Differential TCR expression levels and usages amongst functional NKT cell subsets. (**a**) Representative plots depicting the TCRβ and α-GalCer (PBS-44)-loaded CD1d-tetramer staining intensity of NKT cell-enriched thymocytes (BALB/c) based on their expression of T-bet and/or RORγT (black) relative to all 7-AAD-, B220- thymocytes (grey). (**b**) Graph depicts the levels of TCRβ expression (median) amongst the indicated NKT cell subset (white circles) from NKT cell-enriched thymocytes (BALB/c) based on their geometric mean fluorescence intensity (GMFI) relative to TCRβ+, CD4+, α-GalCer (PBS-44)-loaded CD1d-tetramer- cells (diagonal stripes) from individual mice. Data pooled from 7 independent experiments (n=14). (**c**) Representative plot of the relationship between PLZF and TCRβ expression amongst NKT cells from NKT cell-enriched thymocytes (BALB/c). (**d**) Graphs depict TCRβ expression levels (median) of the indicated lymph node NKT cell subset relative to TCRβ+, CD4+, α-GalCer-loaded CD1d-tetramer- cells (diagonal stripes) from individual mice the in BALB/c (left) or C57BL/6 (right) strains. Data pooled from 10 independent experiments (n=16). (**e**) Graphs depict the TCR-Vβ usage (median) amongst the indicated NKT cell subset from NKT cell-enriched thymocytes (BALB/c). Data pooled from 4 independent experiments (n=12). Statistical significance assessed via Friedman test followed by Dunn post-test comparisons (* $p<0.05$, ** $p<0.001$, *** $p< 0.0001$).

Figure 5. NKT cell subsets display differential sensitivity to CD1d-restricted antigen. (**a**) NKT cell subsets were sort-purified from NKT cell-enriched thymocytes (BALB/c) based on their CD43HG and ICOS expression, labelled with CellTrace Violet and stimulated for 24, 48 and 36hrs with Jα18-/- (BALB/c) splenocytes pulsed overnight with α-GalCer (100 ng/ml). Numbers on plots indicate the division index [the average number of cell divisions including cells that failed to divide using FlowJo (Tree Star) software]; dotted lines indicate the CellTrace Violet intensity of undivided cells. Data representative of 2-7 independent experiments. (**b**) Graph depicts the division index of the indicated NKT cell subset following

48hrs in vitro culture versus the relative TCRβ expression levels amongst sort-purified thymic NKT cell subsets assessed prior to activation. Data pooled from 7 independent experiments.

Figure 6. The presence of LPS biases the NKT cell cytokine response. NKT cell-enriched thymocytes [BALB/c (10-12 weeks of age)] were labelled with CFSE and co-cultured with Jα18-/- splenocytes pulsed overnight with LPS (100 ng/ml) and/or α-GalCer. (**a**) Representative plots of the CFSE dilution amongst gated NKT cells following 48hr co-culture. Numbers on plots indicate the division index. (**b**) Graph depicts the division index (Mean ± SEM) of NKT cells stimulated for 48hrs with Jα18-/- splenocytes pulsed with α-GalCer alone (white), or α-GalCer plus LPS (black). Data pooled from 5 independent experiments. (**c**) Plots represent the cytokine production amongst proliferating NKT cells (based on CFSE dilution) assessed following 48hrs in vitro culture (the final 4hrs in the presence of GolgiStop). (**d**) Graph depicts the percentage (Mean ± SEM) of proliferating NKT cells producing the indicated cytokine. Data pooled from 3 independent experiments. (**e**) Plots represent the cytokine production amongst proliferating NKT cells (based on CFSE dilution) assessed following 48hrs (re-stimulated with PMA and ionomycin for the final 4hrs in the presence of GolgiStop). (**f**) Graph depicts the percentage (Mean ± SEM) of proliferating NKT cells producing the indicated cytokine. Data pooled from 3 independent experiments.

Figure 7. TCR-mediated activation induces IL-10-producing NKT cells. (**a**) Representative plots depict the CFSE dilution among gated NKT cells following 72hr co-culture with Jα18-/- (BALB/c) splenocytes pulsed overnight with LPS (100 ng/ml) and/or α-GalCer (1 ng/ml). Numbers on plots indicate the division index. (**b**) Graph depicts the division index of thymic NKT cells challenged with the indicated dose of α-GalCer with (black), or without LPS (white). Data representative of 2 independent experiments. (**c**) Plots represent the cytokine production amongst proliferating thymic NKT cells assessed at 72hrs following re-stimulation with PMA and ionomycin for the final 4hrs in the presence of GolgiStop. (**d**) Graphs depict the percentages (Mean ± SEM) of proliferating NKT cells co-cultured with splenocytes pulsed with α-GalCer alone (white), or α-GalCer plus LPS (black) producing the indicated cytokine following re-stimulation. Data representative of 2 independent experiments. (**e**) Representative plots of the cytokine production from peripheral lymph node (cervical, axillary, inguinal and brachial lymph node (C57BL/6) NKT cells cultured for 72hrs in the

presence of the indicated concentration of α-GalCer, following re-stimulation for the final 4hrs with PMA and ionomycin in the presence of GolgiStop. (**f**) Plots represent the cytokine production of IL-10-producing lymph node NKT cells re-stimulated with PMA and ionomycin following culture with α-GalCer (10 ng/ml). Data representative of 3 similar experiments.

Figure 8. LPS specifically induces the proliferation of NKT17 cells. (**a**) NKT cell-enriched thymocytes were sort-purified based on CD43HG and ICOS expression, without the use of CD1d-tetramer and then co-cultured with Jα18-/- splenocytes pulsed overnight with LPS (100 ng/ml) and/or α-GalCer (1 ng/ml) for 72hrs. Numbers on plots indicate the division index based on CellTrace Violet dilution of gated NKT cells. (**b**) Graph depicts the division index (Mean ± SEM) of the indicated NKT cell subset. Data pooled from 3 independent experiments.

Supplementary Figure Legends

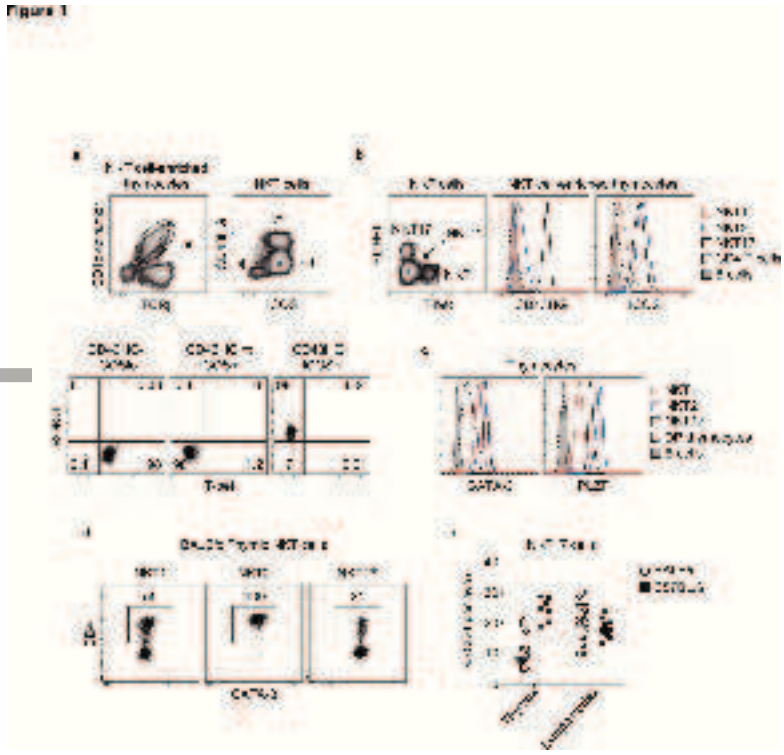
Supplementary Figure 1. The tissue-specific distribution and CD43HG and ICOS expression of functionally diverse NKT cell subsets. (**a**) Representative plots of TCRβ and α-GalCer (PBS-44)-loaded CD1d-tetramer and staining intensity amongst 7-AAD-, B220- NKT cell-enriched thymocytes, hepatic lymphocytes and pooled lymph nodes (cervical, axillary, inguinal and brachial) cells from individual BALB/c (left) or C57BL/6 mice (right), and the T-bet and RORγT expression amongst gated NKT cells (bottom). (**b**) Graph depicts the percentages (median) of NKT cell subsets within NKT cell-enriched thymus (left), liver (middle) and pooled peripheral lymph nodes (cervical, axillary, inguinal and brachial) cells (right) from individual BALB/c (white circles) or C57BL/6 (black squares) mice. Data pooled from 3-13 independent experiments (n=7-21). Statistical significance assessed via Friedman test followed by Dunn post-test comparisons (*** $p< 0.0001$). (**c**) Representative plots depict CD43HG and ICOS expression amongst the indicated NKT cell subset (based on T-bet and RORγT expression) from C57BL/6 thymus. (**d**) Representative plots depict CD43HG and ICOS expression of liver-derived NKT cells from BALB/c (left) and C57BL/6 (right) mice and (**e**) peripheral lymph node (cervical, axillary, inguinal and brachial) NKT cells from BALB/c (left) and C57BL/6 mice (right). Data representative of at least 4 independent experiments.

Supplementary Figure 2. Cytokine co-production by thymic NKT cell subsets. NKT cell-enriched thymocytes (BALB/c) were sort purified based on their CD43HG and ICOS expression then co-cultured for 72 hours (re-stimulated for the final 4hrs with PMA and ionomycin, in the presence of GolgiStop) with Jα18-/- (BALB/c) splenocytes pulsed overnight with α-GalCer (100 ng/ml). Plots depict the production of (**a**) IL-17A, IFN-γ and IL-4 or (**b**) IL-17A, IFN-γ and IL-10. Data representative of at least 3 independent experiments.

Supplementary Figure 3. Cytokine production of peripheral NKT cells. (**a**) Pooled peripheral lymph node (cervical, axillary, inguinal and brachial) cells were stimulated in vitro for 4 hours with PMA and ionomycin in the presence of GolgiStop and P2X7R inhibitor and the production of IL-4, IL-17A and IFN-γ amongst NKT cells was assessed via ICS at Day 0 (left) and following 72hrs in vitro culture with (100 ng/ml) α-GalCer (right) the final 4 hours with PMA, ionomycin, GolgiStop and P2X7R inhibitor. Data representative of 4 similar experiments. (**b**) Hepatic lymphocytes from BALB/c (left) and C57BL/6 mice (right) were stimulated in vitro for 4 hours with PMA and ionomycin in the presence of GolgiStop and P2X7R inhibitor and the production of IL-4, IL-17A and IFN-γ amongst NKT cells was assessed via ICS. Data representative of 3-5 independent experiments.

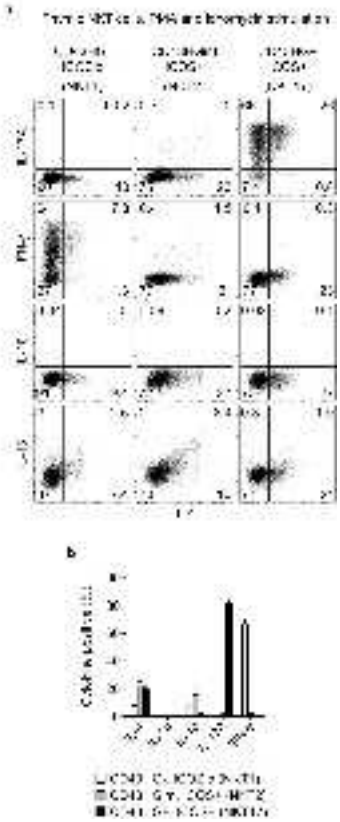
Supplementary Figure 4. The presence of anti-Vβ8.3 can inhibit CD1d-tetramer staining. NKT cell-enriched thymocytes (BALB/c) were stained with anti-Vβ8.3 first, then α-GalCer-loaded CD1d-tetramer (left), or anti-Vβ8.3 and α-GalCer-loaded CD1d-tetramer simultaneously (middle), or α-GalCer-loaded CD1d-tetramer first, then anti-Vβ8.3. Data representative of 2 similar experiments.

Supplementary Figure 5. Thymic NKT cells recover TCR expression 48hrs after activation. (**a**) Representative plots of gated, CFSE-labelled NKT cell-enriched (BALB/c) thymocytes following 48hrs co-culture with Jα18-/- (BALB/c) splenocytes pulsed with LPS, α-GalCer, LPS & α-GalCer or vehicle control. (**b**) Representative plots of sorted, CellTrace Violet-labelled thymic NKT cell subsets following 48hrs co-culture with Jα18-/- splenocytes pulsed α-GalCer (100 ng/ml).

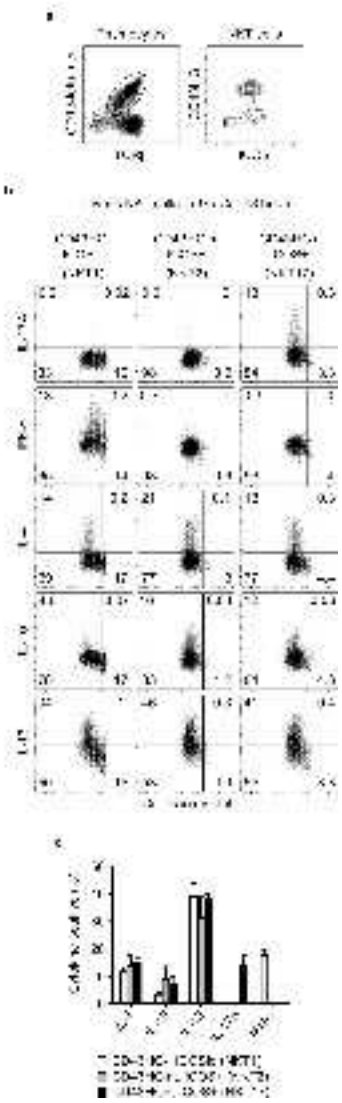


imcb_12034_f1.tif

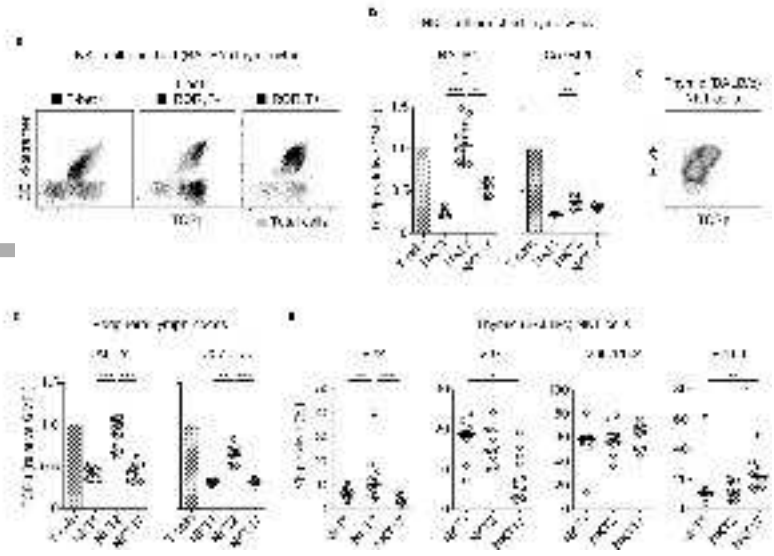
Figure 2



imcb_12034_f2.tif

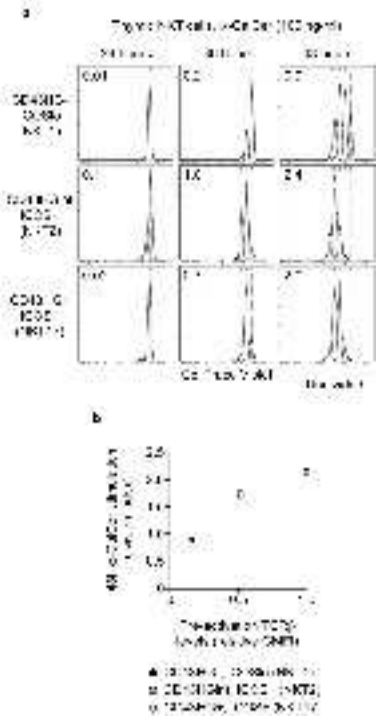


imcb_12034_f3.tif

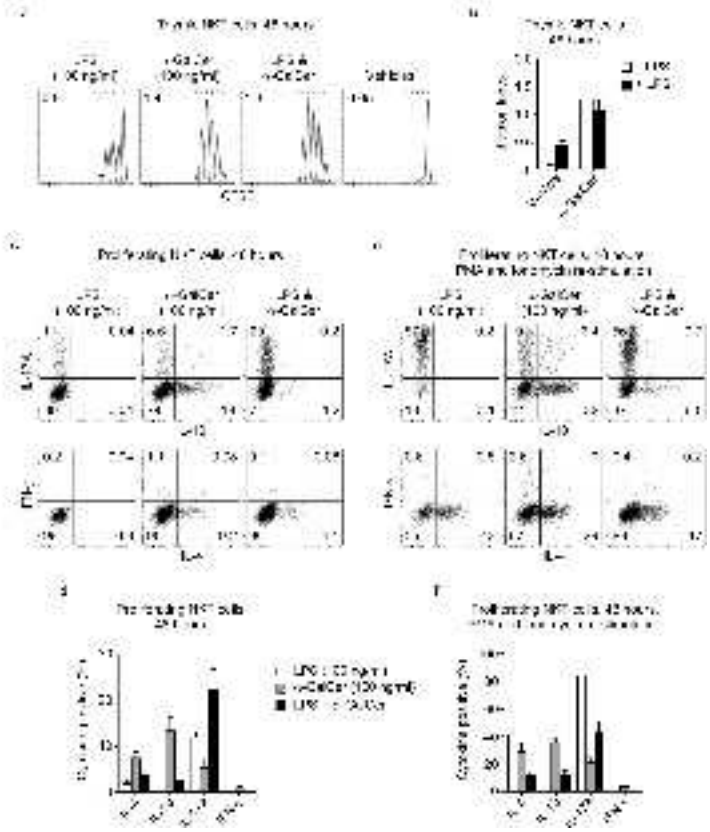


imcb_12034_f4.tif

Figure 5

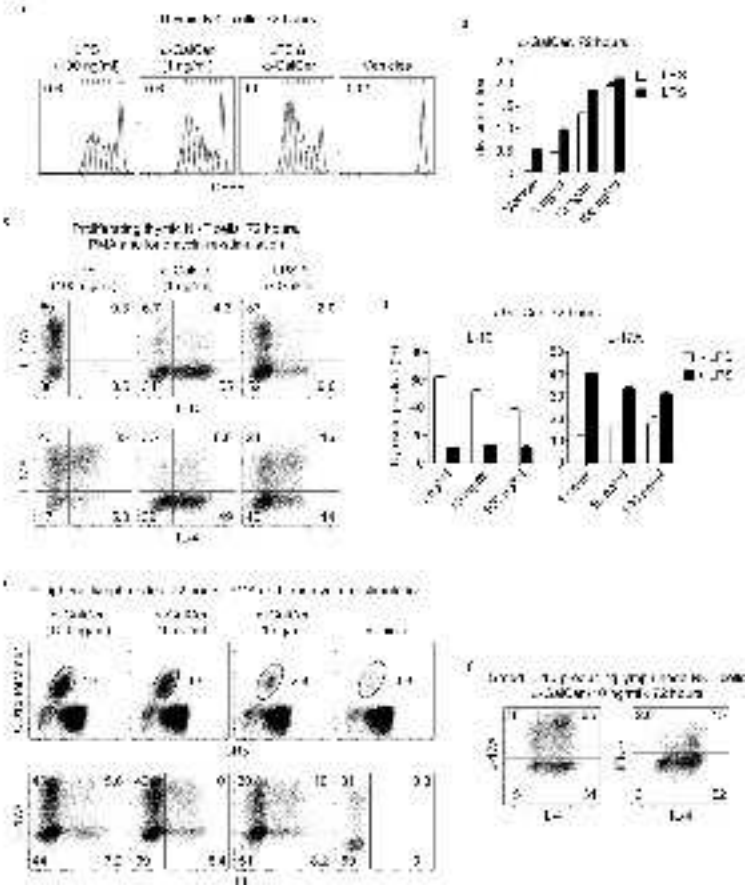


imcb_12034_f5.tif

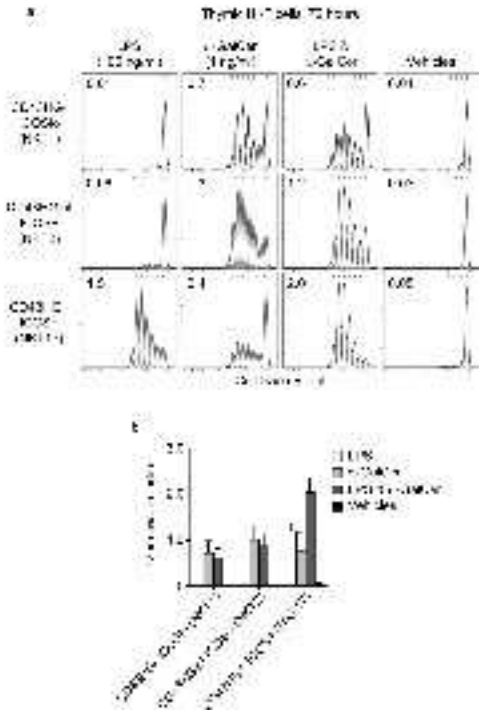


imcb_12034_f6.tif

Figure 7



imcb_12034_f7.tif



imcb_12034_f8.tif