Published in final edited form as:

Immunol Lett. 2011 September 30; 139(1-2): 33–41. doi:10.1016/j.imlet.2011.04.012.

Critical role for Invariant chain in CD1d-mediated selection and maturation of Vα14-invariant NKT cells

Fenna C. M. Sillé^{a,b}, Constance Martin^{c,d}, Pushpa Jayaraman^c, Alissa Rothchild^{c,e}, Gurdyal S. Besra^f, Samuel M. Behar^{c,d,1}, and Marianne Boes^{a,b,1,*}

^a Department of Dermatology, Brigham and Women's Hospital, Boston, MA 02115, USA ^b Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, 3584 EA, Utrecht, The Netherlands ^c Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA ^d Department of Immunology and Infectious Disease, Harvard School of Public Health, Boston, MA 02115, USA ^e Department of Immunology, Harvard Medical School, Boston, MA 02115, USA ^f School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

Abstract

The development and maturation of Vα14 invariant (i)NKT cells in mice requires CD1d-mediated lipid antigen presentation in the thymus and the periphery. Cortical thymocytes mediate positive selection, while professional APCs are involved in thymic negative selection and in terminal maturation of iNKT cells in the periphery. CD1d requires entry in the endosomal pathway to allow antigen acquisition for assembly as lipid/CD1d complexes for display to iNKT cells. This process involves tyrosine-based sorting motifs in the CD1d cytoplasmic tail and invariant chain (Ii) that CD1d associates with in the endoplasmic reticulum. The function of Ii in iNKT cell thymic development and peripheral maturation had not been fully understood. Using mice deficient in Ii and the Ii-processing enzyme cathepsin S (catS), we addressed this question. Ii^{-/-} mice but not catS^{-/-} mice developed significantly fewer iNKT cells in thymus, that were less mature as measured by CD44 and NK1.1 expression. Ii^{-/-} mice but not catS^{-/-} mice developed fewer Vβ7⁺ cells in their iNKT TCR repertoire than WT counterparts, indicative of a change in endogenous glycolipid antigen/CD1d-mediated iNKT cell selection. Finally, using a *Mycobacterium tuberculosis* infection model in macrophages, we show that iNKT developed in Ii^{-/-} but not catS^{-/-} mice have defective effector function. Our data support a role for professional APCs expressing Ii, but no role for catS in the thymic development and peripheral terminal maturation of iNKT cells.

Keywords

CD1d; development; invariant chain; mouse; *Mycobacterium tuberculosis*; Vα invariant Natural Killer T (iNKT) cells

© 2011 Elsevier B.V. All rights reserved.

*Corresponding author. Mailing address: Department of Pediatric Immunology, University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands. Phone: +31-88-75-53328. Fax +31-88-75-55350. mboes@umcutrecht.nl.

¹These authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The development of iNKT cells is a strictly thymus-dependent process that involves the presentation of antigenic glycolipid/CD1d complexes to developing T cells. The antigen presenting cells (APCs) that mediate the positive selection of iNKT cells are cortical thymocytes, while professional APCs (i.e., dendritic cells, DCs) are involved in negative selection and terminal maturation [1,2]. CD1d-mediated lipid antigen presentation by APCs is probably an important contributor to host defense against pathogens [3], and may have equally important roles in triggering iNKT cells under conditions of non-infectious cellular stress, such as in type I diabetes [4-6].

CD1d/lipid complexes are assembled in the endoplasmic reticulum (ER), composed of CD1d heavy chain, beta2-microglobulin ($\beta 2m$) and endogenous lipids. After assembly, stable CD1d/lipid complexes are allowed to transit to the cell surface, followed by the rapid, within 30 minutes, introduction into the endocytic pathway, mediated by a tyrosine-based motif present in the CD1d cytosolic tail or through interaction with invariant chain (Ii)/Class II MHC complexes [7,8]. Most mouse CD1d protein is found in endosomal compartments under steady-state conditions with low levels of CD1d present at the cell surface. Lysosomal enzymes are involved in the processing and degradation of glycolipids for exchange and binding to CD1d [8-12], after which CD1d/lipid complexes traffic back to the plasma membrane for presentation. Cathepsin S (catS) is a lysosomal enzyme that cleaves the N-terminal segment of Ii. In absence of catS, the Class II-associated fragment Ii-p10 that contains the Class II MHC endosomal sorting signals is retained, resulting in the accumulation of Class II MHC complexes in endosomal compartments [13-15]. As a consequence, build up of Ii/Class II MHC complexes causes the enlargement of endosomal compartments, possibly affecting the loading of CD1d with antigenic lipid cargo.

The involvement of the Class II MHC chaperone Ii in CD1d sorting had been suggested to allow for iNKT cell development, selection and maturation, but is not entirely clear. Mice that lack catS appeared to have decreased numbers of thymic iNKT cells [16], a finding that was not supported by others [9,17] and was not investigated further. In professional APCs, CD1d transport to the endosomal pathway requires association with Ii [4,7,8,18], supporting the possibility that Ii mediates CD1d endosomal transport if professional APCs play a role in thymic development of iNKT cells. Similarly, there is data to suggest a role for Ii in peripheral maturation of iNKT cells [4,7,8,18], but the effector function of iNKT cells matured in the absence of Ii has not been evaluated.

We set out to resolve the role of Ii and catS in CD1d-mediated selection and development of iNKT cells. By using $Ii^{-/-}$ and $catS^{-/-}$ mice, we show a role for Ii but not catS in the thymic selection and peripheral terminal differentiation of CD1d-restricted iNKT cells in spleen. $Ii^{-/-}$ but not $catS^{-/-}$ mice exhibit a V β -bias in the TCR repertoire of thymic iNKT cells. As readout for iNKT cell effector function, we exploited a model of *Mycobacterium tuberculosis* (*Mtb*) infected macrophages (M ϕ), for which we earlier showed that iNKT cells are both necessary and sufficient to suppress *Mtb* replication [19]. Addition of $catS^{-/-}$ or WT splenocytes to *Mtb*-infected M ϕ resulted in a reduction in bacterial CFU. Splenocytes from $Ii^{-/-}$ mice, however, did not suppress intracellular *Mtb* replication in infected M ϕ . Only WT splenocytes were able to produce interferon- γ (IFN- γ) and tumor necrosis factor (TNF) upon stimulation by *Mtb*-infected WT M ϕ . Furthermore, we tested iNKT cell effector function by stimulating iNKT cells with α Galactosylceramide (α GalCer), a potent stimulant of CD1d-restricted iNKT cells [20,21]. Both $catS^{-/-}$ and $Ii^{-/-}$ iNKT cells were able to produce IFN- γ but produced less TNF. It is well established that cortical thymocytes mediate iNKT cell positive selection [2,22-24], while professional APCs mediate thymic negative selection [1] and peripheral terminal maturation of iNKT cells [2]. We show here

that Ii, but not catS, expression in APCs facilitates the generation of functional CD1d-restricted iNKT cells.

1. Materials and Methods

1.1. Mice

We used six- to eight- week old mice, all on C57Bl/6 background. WT mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). Cathepsin S knockout (catS^{-/-}) mice [25], CD1d-knockout (CD1d^{-/-}) mice [26] and Ii-knockout (Ii^{-/-}) mice [27] were described previously. Mice were bred and maintained in a barrier facility and studies were performed according to institutional guidelines for animal use and care.

1.2. Antibodies and reagents

The National Institute of Allergy and Infectious Disease MHC Tetramer Core facility (Atlanta) kindly provided the CD1d:PBS-57 (α galactosylceramide, α GalCer analog) tetramers. Dr G. Besra generated α GalCer for culture experiments. The following antibodies were used in this study: rat anti-mouse CD1d (1B1, Biolegend), rat anti-mouse CD4 (RM4-5, BD Pharmingen), rat anti-mouse CD8 (53-6.7, BD Pharmingen), Armenian Hamster anti-mouse CD11c (HL3, BD Pharmingen), rat anti-mouse CD44 (IM7, eBioscience), Armenian hamster anti-mouse CD69 (H1.2F3, Biolegend), rat anti-mouse B220 (RA3-6B2, Biolegend), rat anti-mouse F4/80, NK1.1 (PK136, Biolegend), rat anti-mouse V β 7 (TR310, BD Pharmingen), rat anti-mouse V β 8 (KJ16, Serotec) and Armenian Hamster anti-mouse TCR β (H57-597, Biolegend).

1.3. V α 14 iNKT cell enrichment from the liver

Mouse livers were perfused with PBS and homogenized through a stainless steel mesh. The liver cells were spun for 5 min at 2000 rpm and taken up in 9 ml of PBS, mixed with 5ml of 80% Percoll. This cell/PBS/Percoll suspension was layered over 3 ml 80% Percoll and the gradient was spun for 20 min at 2000 rpm. Liver lymphocytes accumulating at the interface were collected and washed with PBS after which red blood cells were lysed using ammonium chloride.

1.4. Flow cytometry

Single-cell suspensions from thymus, spleen and liver were resuspended in FACS buffer (2% FCS, 2 mM sodium azide and anti-Fc γ RII/III Ab (2.4G2) in PBS) and incubated for 20 min with the relevant conjugated mAb on ice. *Mtb*-infected cells were fixed with 1% paraformaldehyde overnight before analysis. At least 100,000 cells per sample were analyzed on a FACScanto (Becton Dickinson) using FlowJo software.

1.5. In vitro culture of peritoneal M ϕ

Mice received intraperitoneal injections with sterile 3% thioglycolate medium. After 4 days elicited peritoneal cells were harvested by intraperitoneal lavage as described earlier [19]. M ϕ , purified by positive selection with CD11b-microbeads (Miltenyi Biotec) were >95% pure (F4/80⁺ CD11b⁺), as determined by flow cytometry (data not shown). 5 $\times$ 10⁵ purified M ϕ were cultured in 24-well flat-bottom plates (or 1 $\times$ 10⁵ in 96-well plates) in complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% fetal calf serum (HyClone), penicillin/streptomycin, L-glutamine, sodium-pyruvate, 2-ME, nonessential amino acids, essential amino acids, and HEPES buffer (all from Gibco).

1.6. In vitro bacteria infections

Virulent *Mtb* (H37Rv) were grown to mid-log phase in Middlebrook 7H9 medium containing 10% albumin/dextrose/catalase enrichment (BD Biosciences) under shaking conditions, 37°C / 5% CO₂. After opsonization for 5 min using RPMI 1640 medium with 2% human serum (Gemini Bio-Products), 10% FBS, and 0.05% Tween 80, mycobacteria were washed twice with complete medium without antibiotics. After passage through a 5- μ m syringe filter (Millipore), mycobacteria were counted in a Petroff-Hausser chamber and added to purified M ϕ at a multiplicity of infection (MOI) of 10:1. After 2hrs, unbound *Mtb* were removed by extensive washing with complete RPMI medium without antibiotics. Infected M ϕ were cultured overnight before the addition of splenocytes (see below) and the next day all wells were washed twice with RPMI medium without antibiotics.

1.7. Splenocytes

Spleens were aseptically removed and mechanically homogenized with a 3-ml syringe plunger. Erythrocytes were lysed with RBC lysis buffer (1 mM KHCO₃, 0.15 M NaCl and 0.1 mM sodium-EDTA, pH 7.3). After washing, cell viability was determined using trypan blue. Splenocytes, as a source of iNKT cells, were resuspended in complete RPMI 1640 medium without antibiotics and 2.5 $\times$ 10⁶ splenocytes/well were added to cultures of *Mtb*-infected M ϕ in 24-well plates (5:1, or 5 $\times$ 10⁵ in 96-well plates).

1.8. Determination of colony forming units

Mycobacterial growth was quantified 24 hrs after M ϕ infection (day 1) and 72 hr after co-culture with or without naïve splenocytes (day 4). Cells were lysed by removing the culture supernatant and adding distilled water containing 1% Triton X-100 for 5 min. 10-fold dilutions, made in PBS containing 0.02% Tween-80, were plated on Middlebrook 7H11 agar plates. Three weeks after incubation at 37°C in a humidified CO₂ atmosphere, the numbers of colonies on plates were counted.

1.9. iNKT stimulation assays

CD11b⁺ MACS-purified thioglycolate-induced M ϕ (1 $\times$ 10⁵ in 96-well plates) were stimulated for 4 h with 3-30 ng/ml α GalCer. After two washes, 1 $\times$ 10⁵ freshly isolated and enriched (B220⁻, CD11b⁻ and CD11c⁻) spleen iNKT cells were added to the culture for 48 h. Enrichment was performed by negative depletion of B220⁺, CD11b⁺ and CD11c⁺ cells with magnetic beads using autoMACs. After 48 h supernatants were harvested for cytokine detection.

1.10. Cytokine detection

Culture supernatants were assayed for IFN- γ and TNF by standard sandwich ELISA. ELISAs were done in accordance with the manufacturer's instructions (eBiosciences). Samples were read at 405 nm on SoftMax Pro ELISA analysis software (Molecular Devices).

1.11. Statistical analysis

Data are shown as mean $\pm$ standard error of the mean (SEM). Unpaired two-tailed t-test was used to compare two groups. To compare multiple different groups to their control, we used one-way ANOVA. A p-value of at least 0.05 was considered statistically significant. Analysis was performed using Prism 4.0 for Mac software (GraphPad Software, Inc).

2. Results

2.1. Reduction in thymic development of iNKT cells in $Ii^{-/-}$ mice

Mice deficient in Ii and $catS$ exhibit modifications in the endosomal pathway in professional APCs: $Ii^{-/-}$ cells exhibit defects in endosomal fusion and resulting in small Class II MHC-positive endosomal compartments, while $catS^{-/-}$ cells are defective in endosomal degradation of Ii remnants resulting in enlarged endosomal compartments that lack multivesicular structures [14,15]. As CD1d localization to specialized endosomal compartments is required for the loading of antigenic lipids onto CD1d, the absence of Ii or $catS$ may affect CD1d-driven iNKT cell development. Multiple studies had attempted to address a role for Ii or $catS$ in iNKT cell biology, mostly by staining for iNKT cell numbers or use of iNKT cell hybridomas in culture experiments [7,9,16,17]. Our own approach was to analyze the phenotype and function of primary iNKT cells developed in the complete absence of Ii or $catS$. We first confirmed the number of iNKT cells in WT, $Ii^{-/-}$ and $catS^{-/-}$ mice using α GalCer-loaded CD1d-tetramers. CD1d $^{-/-}$ mice served as controls that do not support the development of CD1d-restricted iNKT cells. We found that lack of Ii or $catS$ does not significantly alter the percentage or absolute number of iNKT cells in spleen and liver (Fig. 1). The percentage of thymic iNKT cells in $Ii^{-/-}$ mice was reduced compared to WT (* $p < 0.05$). However, this trend was not recapitulated in absolute iNKT cell numbers, perhaps as a consequence of the biased thymocyte repertoire in $Ii^{-/-}$ mice, lacking most Class II MHC-restricted CD4 T cells [27,29].

2.2. Reduction in thymic and peripheral maturation of iNKT cells in $Ii^{-/-}$ mice

While Ii -deficient mice develop slightly fewer iNKT cells, there is still a sizeable population present in thymus. In $Ii^{-/-}$ spleen and liver, normal iNKT cell percentages and numbers are present, probably because of homeostatic CD1d-independent expansion [28] and supported by the lymphopenic CD4 T cell-deficient environment in $Ii^{-/-}$ mice [27,29]. We asked whether CD1d-restricted iNKT cells that develop in the absence of Ii or $catS$ have a distinction in maturation phenotype. To this end, we stained cells from thymus, spleen and liver with α GalCer-loaded CD1d-tetramers and antibodies against TCR β and the maturation markers CD44, CD69 and NK1.1. Thymic iNKT cell development can be divided into three successive stages: stage 1: CD44 $^{-}$ NK1.1 $^{-}$, stage 2: CD44 $^{+}$ NK1.1 $^{-}$, and mature stage 3: CD44 $^{+}$ NK1.1 $^{+}$ CD69 $^{+}$, as described in previous studies [4,30-34]. The transition in development from immature NK1.1 $^{-}$ to mature NK1.1 $^{+}$ V α 14 iNKT cells is an important step during the terminal differentiation of iNKT cells, which continues outside the thymus and requires the presence of CD1d [2,35,36]. There are slightly lower percentages of iNKT cells expressing CD69 in spleen from $catS^{-/-}$ mice (Fig. 2A). However, the maturation of thymic and liver iNKT cells from $catS^{-/-}$ mice, based on expression of CD69 is similar to WT (Fig. 2A). The maturation of iNKT cells from $catS^{-/-}$ mice, based on expression of NK1.1 is similar to WT (Fig. 2B). The iNKT cells from $Ii^{-/-}$ mice are delayed in maturation with significantly lower percentages expressing CD69 and NK1.1 during stage 3 of maturation in the thymus (** $p < 0.01$). Correspondingly, we found elevated percentages of iNKT cells in stage 2 (CD44 $^{+}$ NK1.1 $^{-}$) in $Ii^{-/-}$ thymus compared to WT (** $p < 0.01$). However, peripheral maturation, measured in the spleen and liver, is only marginally, if at all decreased (* $p < 0.05$ in spleen). We observed that in $Ii^{-/-}$ liver, more iNKT cells than in WT liver express a CD44 $^{+}$ NK1.1 $^{+}$ phenotype. This possibly reflects local activation of liver-resident iNKT cells, rather than increased migration of mature iNKT cells, since absolute iNKT cell numbers are similar in $Ii^{-/-}$ and WT livers (Fig. 1). Thus, although Ii may influence iNKT cell differentiation from stage 2 (CD44 $^{+}$ NK1.1 $^{-}$) to the mature stage 3 (CD44 $^{+}$ NK1.1 $^{+}$ CD69 $^{+}$) in the thymus, this delay in iNKT cell maturation is partially overcome in the periphery. We have previously reported similar kinetics for iNKT cell

development in mice in which thymic CD1d endosomal trafficking was delayed due to presence of a cytosolic tag [4].

2.3. Bias in TCR V β chain usage of iNKT cells in *Ii*^{-/-} mice

We considered that a change in intracellular sorting could expose CD1d to endogenous glycolipid antigens that were not normally encountered. This raised the possibility that such a change in binding of endogenous glycolipid antigens by CD1d would alter iNKT cell selection. The selection of V β TCR domains assembled with the invariant V α 14 chain shapes the avidity of TCRs on iNKT cells for endogenous ligand/CD1d complexes, to which developing iNKT cells are exposed during thymic selection [28]. A change in V β usage would suggest a role for *Ii* and *catS*-expressing professional APCs in thymic selection of iNKT cells. We found that *Ii*^{-/-} but not *catS*^{-/-} mice display significantly less V β 7 positive iNKT cells in the thymus compared to *catS*^{-/-} and WT mice ($p < 0.01$), but normal contribution of V β 7 iNKT cells in spleen and liver (Fig. 2C). Thus, thymic selection of iNKT cells by lipid/CD1d complexes appears influenced by *Ii*, but not *catS*-expressing professional APCs.

2.4. No suppression of *M. tuberculosis* replication by iNKT cells from *Ii*^{-/-} mice

We earlier showed that CD1d-restricted iNKT cells are able to suppress intracellular *Mtb* replication [19]. We asked whether iNKT cells that developed in the complete absence of *Ii* or *catS* could suppress the replication of *Mtb* in M ϕ . Confirming previous findings [19], we found that splenocytes from CD1d^{-/-} mice, which lack iNKT cells, are unable to restrict intracellular *Mtb* replication compared to WT splenocytes (Fig. 3A). Although to a lesser extent the addition of *catS*^{-/-} splenocytes to *Mtb*-infected WT M ϕ still led to a significant reduction in bacterial CFU after 4 days (** $p < 0.001$), compared to the addition of WT splenocytes (** $p < 0.001$) (Fig. 3A). However, *Ii*^{-/-} splenocytes did not suppress intracellular *Mtb* replication in infected WT M ϕ , and the growth of *Mtb* was not statistically different compared to M ϕ that were cultured in the absence of splenocytes (Fig. 3A).

2.5. Impaired cytokine production by iNKT cells from *catS*^{-/-} and *Ii*^{-/-} mice

M ϕ activation is an important part of the host defense against *Mtb* mediated by IFN- γ [37] and TNF [38], which are both readily produced by activated iNKT cells. We determined cytokine production by spleen iNKT cells upon stimulation by *Mtb*-infected M ϕ *in vitro*. The addition of WT splenocytes, but not *catS*^{-/-} or *Ii*^{-/-} splenocytes, resulted in significant production of TNF and IFN- γ (Fig. 3B). We did not detect any significant levels of IL-4 in the *in vitro* *Mtb* infection assays (data not shown). We additionally tested iNKT cell activation by stimulating purified spleen iNKT cells with α GalCer presented by WT M ϕ . We did not find any significant difference in IFN γ production by *Ii*^{-/-} or *catS*^{-/-} iNKT cells compared to WT iNKT cells. However, we observed significantly lower levels of TNF produced by iNKT cells from mice deficient for *Ii* or *catS* (Fig. 3C). Given the bias in immature iNKT cells (CD44⁺NK1.1⁻) in *Ii*^{-/-} thymus and spleen and the demonstrated bias of immature iNKT cells towards T-helper 2 (T_H2) phenotype [32], an increase in IL4 production upon activation was expected. However, we did not observe any significant IL4 production in these assays (data not shown).

2.6. Inability of naïve purified iNKT cells from *Ii*^{-/-} mice to suppress *M. tuberculosis* replication in WT macrophages

Finally, we addressed directly whether purified iNKT cells from WT and *Ii*^{-/-} spleens can suppress *Mtb* replication in infected WT M ϕ . *Mtb*-infected WT M ϕ were cultured alone or in the presence of purified iNKT cells from uninfected WT or *Ii*^{-/-} mice. We added purified iNKT cells to the *Mtb*-infected WT M ϕ at 1 day post-infection and determined growth of

Mtb in infected M ϕ by determining CFU on day 1 and day 4 post-infection. Addition of WT iNKT cells (***) but not *Ii*^{-/-} splenocytes (NS) led to a significant reduction in bacterial CFU after 4 days. Thus, iNKT cells developed in the complete absence of *Ii* are not only deficient in cytokine production, but are defective at inhibition of *Mtb* replication.

Discussion

Invariant (i)NKT cells are a unique subset of T lymphocytes that use a conserved TCR that recognizes both self and foreign glycolipids when presented in complex with the antigen presenting molecule CD1d. It is now well established that endosomal localization of CD1 molecules including mouse CD1d, is important for the presentation of glycolipid antigen/CD1 complexes at the cell surface [4,39-41]. From the endoplasmic reticulum, where CD1d heavy chain is assembled into stable complexes with beta-2-microglobulin and endogenous lipid, CD1d complexes are transported via the trans-Golgi network to the cell surface. CD1d complexes are rapidly thereafter introduced into endosomal compartments through association with the Class II MHC-associated invariant chain (*Ii*), and by use of a tyrosine-based endosomal sorting motif present in the cytoplasmic tail of CD1d [4,7,8]. Exchange of self-lipids for antigenic lipid in CD1d complexes occurs in specialized compartments to which endocytosed CD1d has access, and that contain necessary co-factors including lysosomal lipid-transfer proteins such as saposins and microsomal triglyceride transfer protein [12,42,43]. CD1d, through its ability to bind selective glycolipid antigens from endosomal compartments, controls the development, thymic selection and maturation as well as the peripheral maturation and function of iNKT cells.

The development of iNKT cell precursors separates from mainstream thymocytes at the CD4⁺CD8⁺ thymocyte stage, and involves the random rearrangement and selection of the canonical V α 14 TCR. Expression of this V α 14-invariant TCR allows for the positive selection of these CD4⁺CD8⁺ thymocytes on neighboring CD1d-expressing thymocyte counterparts [2,22-24], cells that do not express *Ii*. The periphery is seeded primarily with immature iNKT cells that undergo further maturation in the periphery [30,34]. The terminal differentiation of iNKT cells involves antigen presentation by professional APCs, but the necessity for *Ii* in these cells to develop fully functional iNKT cells, able to fight pathogen infection was not yet known.

iNKT cells themselves do not express *Ii* or the *Ii* proteolytic enzyme catS, but rely on CD1d-mediated lipid antigen presentation, which may be controlled by function of *Ii* or catS. Multiple lines of research suggest a role for *Ii* in CD1d endosomal transport. First, *Ii* controls endosomal architecture and transport of endosomal content [14,44,45]. Second, *Ii* can promote the adherence between *Ii* molecules embedded in distinct vesicular membranes and thereby promoting endosomal docking and fusion [46,47]. Third, *Ii* alone or *Ii*/Class II MHC complexes can mediate entry of CD1d in late endosomal compartments: mouse CD1d can be co-immunoprecipitated with *Ii* [7] and human CD1d associates with *Ii*/Class II MHC complexes in the endoplasmic reticulum and late endosomal compartments [18]. Finally, concerning CD1d specifically, *Ii* can introduce CD1d into endosomal compartments [4,7]. CatS has also been implicated in CD1d endosomal processes. CatS proteolysis is critical for endosomal antigen loading of Class II MHC complexes by cleavage of Class II-associated *Ii*-p10 to CLIP, and was implicated in CD1d-mediated antigen presentation [16]. However, a study from the same year did not confirm this data [17]. Endosomal architecture in catS^{-/-} APCs is changed dramatically, causing enlarged compartments devoid of multivesicular structures [14]. Either morphological changes or a direct proteolytic defect in catS^{-/-} APCs may conceivably cause aberrant glycolipid loading of CD1d. A study carefully delineating CD1d-mediated iNKT cell thymic and peripheral development and function in *Ii*^{-/-} or catS^{-/-} mice has not been reported.

In this study, we combined detailed phenotype analysis of iNKT cells with study of effector function with standard iNKT cell activation assays involving presentation of α GalCer as well as in an established *Mycobacterium tuberculosis* macrophage infection model [19]. iNKT cells are frequently activated early during infection and have an immunomodulatory role that can either exacerbate or ameliorate the outcome of infection (reviewed in [48]; [49]). While the absence of CD1d-restricted T cells does not worsen the outcome to tuberculosis infection in the mouse model, specific activation of iNKT cells with α GalCer, significantly prolongs the survival of infected mice [21,50]. Other data suggests that iNKT cells make a contribution to anti-mycobacterial immunity early during infection [19,51]. One possible explanation for why CD1d^{-/-} mice, which lack the iNKT cells, are not more susceptible to *Mtb* than WT mice is that the contribution of iNKT cells to anti-mycobacterial immunity is redundant. However, iNKT cells obtained from uninfected mice are unique in their ability to recognize *Mtb*-infected macrophages in vitro and suppress mycobacterial replication [19]. The capacity of iNKT cells to inhibit *Mtb* growth requires CD1d expression by the infected macrophage, although whether a self/endogenous or microbial antigen is recognized, is unknown. We have used this well characterized model to determine whether the alteration of iNKT cell numbers in *Ii*-deficient mice results in a functional alteration [9,16].

Our work complements earlier studies using *Ii*^{-/-} or *catS*^{-/-} APCs co-cultures involving iNKT cell hybridomas and use of model antigens [7,9,16]. Our data shows that *Ii* but not *catS* may be involved in thymic development of fully functional iNKT cells. iNKT cells developed in absence of *Ii* were slightly decreased in numbers and exhibited a bias in TCR V β repertoire coupled to the TCR V α 14, suggesting a change in endogenous glycolipid/CD1d-mediated selection. *Ii*^{-/-} mice moreover displayed increased percentages of iNKT cells in the immature stage 2 (CD44⁺NK1.1⁻) at the expense of mature 3 (CD44⁺NK1.1⁺) iNKT cells. It has been demonstrated that the Tec family tyrosine kinase *Itk* is necessary for optimal NK1.1 up-regulation and hence final maturation of NKT cells [32]. Future experiments may verify any convergence of Tec family kinase *Itk* and *Ii*⁺ APCs in directing iNKT cell maturation. iNKT cells from *Ii*^{-/-} mice were unable to restrict *Mycobacterium tuberculosis* replication in infected macrophages. In addition, they failed to produce IFN- γ or TNF, cytokines which have been shown to play a role in the host response against *Mtb* [37,38]. Furthermore, despite increased percentages of immature (CD44⁺NK1.1⁻) iNKT cells in the spleen, we did not observe significant IL4 production by *Ii*^{-/-} iNKT cells. *CatS*^{-/-} mice, however, developed iNKT cells in normal ratios and were still able to suppress *Mtb* replication despite impaired production of IFN- γ and TNF, suggesting a non-critical role for *catS* in iNKT cell development and effector function. We conclude that *Ii*-expressing professional APCs support thymic development of iNKT cells. It is well established that iNKT cells positive selection occurs by CD4⁺CD8⁺ double positive cortical thymocytes [2,22,23]. Targeted expression of CD1d limited to professional APCs did not support the development of iNKT cells [24]. However, CD1d-mediated presentation by professional APCs is important for iNKT cell development. Experiments in which human CD1d was expressed on thymic CD11c⁺ APCs suggested a role for professional APCs in the negative selection of thymic iNKT cells [1]. In the periphery, professional APCs mediate the terminal maturation of newly formed iNKT cells, for acquisition of full effector function [2]. Our experiments described here establish a role for *Ii* during thymic development of iNKT cells, which is independent of *catS*. Moreover, we show an essential requirement for *Ii* in the periphery to iNKT cell acquisition of full effector function, in the ability to fight *Mtb* infection.

Acknowledgments

This work was supported by grants from NIH RO1-AR052810 (to M.B.) and 5 R01 HL080330, R01 HL080312 (to S.B.). We thankfully acknowledge the members of the Boes and Behar laboratories for helpful discussions. We thank Dr. M. Exley for providing CD1d^{-/-} mice and The NIH Tetramer Facility for supplying the CD1d-tetramers.

Vitae

Fenna C. M. Sillé received her combined B.A. and M.Sc. in biology in 2004 from the University of Groningen, The Netherlands. She performed her PhD thesis research in the Brigham and Women's Hospital in Boston under supervision of Marianne Boes. In 2010 she received her Ph.D. in immunology from the Utrecht University, The Netherlands.

Constance Martin received her B.S. in Biology from Case Western Reserve University in Cleveland, OH in 2006. She is currently a Ph.D. student in the Department of Immunology & Infectious Diseases at the Harvard School of Public Health, Boston MA.

Pushpa Jayaraman received her Ph. D. in Pathobiology in 2006 from the University of Washington. She is currently a post-doctoral fellow working with Sam Behar, Brigham and Women's Hospital, Boston on the role of Tim3 mediated immunoregulation of innate and adaptive immunity towards Mycobacterium tuberculosis. She is a recipient of post-doctoral research training fellowship from American Lung Association (RT-123085-N).

Alissa Rothchild received her B.S. in biology in 2005 from Brown University and is currently a PhD graduate student in the immunology program in the Division of Medical Science at Harvard University.

Samuel M. Behar is an Associate Professor in Rheumatology, Immunology, and Allergy at Brigham and Women's Hospital, Harvard Medical School, and in the Department of Immunology and Infectious Diseases at the Harvard School of Public Health.

Marianne Boes was Assistant Professor in Dermatology at Brigham and Women's Hospital during part of these studies, and is currently Associate Professor in Immunology at the University Medical Centre in Utrecht, the Netherlands.

References

- [1]. Schumann J, Pittoni P, Tonti E, Macdonald HR, Dellabona P, Casorati G. Targeted expression of human CD1d in transgenic mice reveals independent roles for thymocytes and thymic APCs in positive and negative selection of Vα14i NKT cells. *J Immunol.* 2005; 175:7303–7310. [PubMed: 16301636]
- [2]. Wei DG, Lee H, Park SH, Beaudoin L, Teyton L, Lehuen A, Bendelac A. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *The Journal of experimental medicine.* 2005; 202:239–248. [PubMed: 16027237]
- [3]. Kinjo Y, Kronenberg M. V α14 i NKT cells are innate lymphocytes that participate in the immune response to diverse microbes. *J Clin Immunol.* 2005; 25:522–533. [PubMed: 16380816]
- [4]. Sille FC, Boxem M, Sprengers D, Veerapen N, Besra G, Boes M. Distinct requirements for CD1d intracellular transport for development of V(α)14 iNKT cells. *J Immunol.* 2009; 183:1780–1788. [PubMed: 19587020]
- [5]. Falcone M, Yeung B, Tucker L, Rodriguez E, Sarvetnick N. A defect in interleukin 12-induced activation and interferon gamma secretion of peripheral natural killer T cells in nonobese diabetic mice suggests new pathogenic mechanisms for insulin-dependent diabetes mellitus. *The Journal of experimental medicine.* 1999; 190:963–972. [PubMed: 10510086]
- [6]. Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, Leite-De-Moraes M, Gouarin C, Zhu R, Hameg A, Nakayama T,

- Taniguchi M, Lepault F, Lehuen A, Bach JF, Herbelin A. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med*. 2001; 7:1057–1062. [PubMed: 11533711]
- [7]. Jayawardena-Wolf J, Benlagha K, Chiu YH, Mehr R, Bendelac A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity*. 2001; 15:897–908. [PubMed: 11754812]
- [8]. Chiu YH, Park SH, Benlagha K, Forestier C, Jayawardena-Wolf J, Savage PB, Teyton L, Bendelac A. Multiple defects in antigen presentation and T cell development by mice expressing cytoplasmic tail-truncated CD1d. *Nature immunology*. 2002; 3:55–60. [PubMed: 11731798]
- [9]. Elewaut D, Lawton AP, Nagarajan NA, Maverakis E, Khurana A, Honing S, Benedict CA, Sercarz E, Bakke O, Kronenberg M, Prigozy TI. The adaptor protein AP-3 is required for CD1d-mediated antigen presentation of glycosphingolipids and development of Valpha14i NKT cells. *The Journal of experimental medicine*. 2003; 198:1133–1146. [PubMed: 14557411]
- [10]. Zhou D, Cantu C 3rd, Sagiv Y, Schrantz N, Kulkarni AB, Qi X, Mahuran DJ, Morales CR, Grabowski GA, Benlagha K, Savage P, Bendelac A, Teyton L. Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins. *Science (New York, NY)*. 2004; 303:523–527.
- [11]. Kang SJ, Cresswell P. Saposins facilitate CD1d-restricted presentation of an exogenous lipid antigen to T cells. *Nature immunology*. 2004; 5:175–181. [PubMed: 14716312]
- [12]. Zhou D, Mattner J, Cantu C 3rd, Schrantz N, Yin N, Gao Y, Sagiv Y, Hudspeth K, Wu YP, Yamashita T, Teneberg S, Wang D, Proia RL, Lavery SB, Savage PB, Teyton L, Bendelac A. Lysosomal glycosphingolipid recognition by NKT cells. *Science (New York, NY)*. 2004; 306:1786–1789.
- [13]. Bakke O, Nordeng TW. Intracellular traffic to compartments for MHC class II peptide loading: signals for endosomal and polarized sorting. *Immunol Rev*. 1999; 172:171–187. [PubMed: 10631946]
- [14]. Boes M, van der Wel N, Peperzak V, Kim YM, Peters PJ, Ploegh H. In vivo control of endosomal architecture by class II-associated invariant chain and cathepsin S. *European journal of immunology*. 2005; 35:2552–2562. [PubMed: 16094690]
- [15]. Driessen C, Bryant RA, Lennon-Dumenil AM, Villadangos JA, Bryant PW, Shi GP, Chapman HA, Ploegh HL. Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J Cell Biol*. 1999; 147:775–790. [PubMed: 10562280]
- [16]. Riese RJ, Shi GP, Villadangos J, Stetson D, Driessen C, Lennon-Dumenil AM, Chu CL, Naumov Y, Behar SM, Ploegh H, Locksley R, Chapman HA. Regulation of CD1 function and NK1.1(+) T cell selection and maturation by cathepsin S. *Immunity*. 2001; 15:909–919. [PubMed: 11754813]
- [17]. Honey K, Benlagha K, Beers C, Forbush K, Teyton L, Kleijmeer MJ, Rudensky AY, Bendelac A. Thymocyte expression of cathepsin L is essential for NKT cell development. *Nature immunology*. 2002; 3:1069–1074. [PubMed: 12368909]
- [18]. Kang SJ, Cresswell P. Regulation of intracellular trafficking of human CD1d by association with MHC class II molecules. *The EMBO journal*. 2002; 21:1650–1660. [PubMed: 11927549]
- [19]. Sada-Ovalle I, Chiba A, Gonzales A, Brenner MB, Behar SM. Innate invariant NKT cells recognize Mycobacterium tuberculosis-infected macrophages, produce interferon-gamma, and kill intracellular bacteria. *PLoS Pathog*. 2008; 4:e1000239. [PubMed: 19079582]
- [20]. Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, Kronenberg M. CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *The Journal of experimental medicine*. 1998; 188:1521–1528. [PubMed: 9782129]
- [21]. Chackerian A, Alt J, Perera V, Behar SM. Activation of NKT cells protects mice from tuberculosis. *Infect Immun*. 2002; 70:6302–6309. [PubMed: 12379709]
- [22]. Bendelac A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *The Journal of experimental medicine*. 1995; 182:2091–2096. [PubMed: 7500054]
- [23]. Coles MC, Raulet DH. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol*. 2000; 164:2412–2418. [PubMed: 10679077]

- [24]. Forestier C, Park SH, Wei D, Benlagha K, Teyton L, Bendelac A. T cell development in mice expressing CD1d directed by a classical MHC class II promoter. *J Immunol.* 2003; 171:4096–4104. [PubMed: 14530331]
- [25]. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity.* 1999; 10:197–206. [PubMed: 10072072]
- [26]. Exley MA, Bigley NJ, Cheng O, Tahir SM, Smiley ST, Carter QL, Stills HF, Grusby MJ, Koezuka Y, Taniguchi M, Balk SP. CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. *Journal of leukocyte biology.* 2001; 69:713–718. [PubMed: 11358978]
- [27]. Viville S, Neefjes J, Lotteau V, Dierich A, Lemeur M, Ploegh H, Benoist C, Mathis D. Mice lacking the MHC class II-associated invariant chain. *Cell.* 1993; 72:635–648. [PubMed: 7679955]
- [28]. Matsuda JL, Gapin L, Sidobre S, Kieper WC, Tan JT, Ceredig R, Surh CD, Kronenberg M. Homeostasis of V alpha 14i NKT cells. *Nature immunology.* 2002; 3:966–974. [PubMed: 12244311]
- [29]. Bikoff EK, Huang LY, Episkopou V, van Meerwijk J, Germain RN, Robertson EJ. Defective major histocompatibility complex class II assembly, transport, peptide acquisition, and CD4+ T cell selection in mice lacking invariant chain expression. *The Journal of experimental medicine.* 1993; 177:1699–1712. [PubMed: 8098731]
- [30]. Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science (New York, NY.* 2002; 296:553–555.
- [31]. Benlagha K, Wei DG, Veiga J, Teyton L, Bendelac A. Characterization of the early stages of thymic NKT cell development. *The Journal of experimental medicine.* 2005; 202:485–492. [PubMed: 16087715]
- [32]. Gadue P, Stein PL. NK T cell precursors exhibit differential cytokine regulation and require Itk for efficient maturation. *J Immunol.* 2002; 169:2397–2406. [PubMed: 12193707]
- [33]. Gapin L, Matsuda JL, Surh CD, Kronenberg M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nature immunology.* 2001; 2:971–978. [PubMed: 11550008]
- [34]. 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.* 2002; 195:835–844. [PubMed: 11927628]
- [35]. McNab FW, Berzins SP, Pellicci DG, Kyparissoudis K, Field K, Smyth MJ, Godfrey DI. The influence of CD1d in postselection NKT cell maturation and homeostasis. *J Immunol.* 2005; 175:3762–3768. [PubMed: 16148122]
- [36]. McNab FW, Pellicci DG, Field K, Besra G, Smyth MJ, Godfrey DI, Berzins SP. Peripheral NK1.1 NKT cells are mature and functionally distinct from their thymic counterparts. *J Immunol.* 2007; 179:6630–6637. [PubMed: 17982053]
- [37]. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. *The Journal of experimental medicine.* 1993; 178:2249–2254. [PubMed: 7504064]
- [38]. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity.* 1995; 2:561–572. [PubMed: 7540941]
- [39]. Sugita M, Grant EP, van Donselaar E, Hsu VW, Rogers RA, Peters PJ, Brenner MB. Separate pathways for antigen presentation by CD1 molecules. *Immunity.* 1999; 11:743–752. [PubMed: 10626896]
- [40]. Gumperz JE. The ins and outs of CD1 molecules: bringing lipids under immunological surveillance. *Traffic.* 2006; 7:2–13. [PubMed: 16445682]
- [41]. Boes M, Stoppelenburg AJ, Sille FC. Endosomal processing for antigen presentation mediated by CD1 and Class I major histocompatibility complex: roads to display or destruction. *Immunology.* 2009; 127:163–170. [PubMed: 19476512]

- [42]. Schrantz N, Sagiv Y, Liu Y, Savage PB, Bendelac A, Teyton L. The Niemann-Pick type C2 protein loads isoglobotrihexosylceramide onto CD1d molecules and contributes to the thymic selection of NKT cells. *The Journal of experimental medicine*. 2007; 204:841–852. [PubMed: 17389239]
- [43]. Sagiv Y, Bai L, Wei DG, Agami R, Savage PB, Teyton L, Bendelac A. A distal effect of microsomal triglyceride transfer protein deficiency on the lysosomal recycling of CD1d. *The Journal of experimental medicine*. 2007; 204:921–928. [PubMed: 17403933]
- [44]. Vascotto F, Lankar D, Faure-Andre G, Vargas P, Diaz J, Le Roux D, Yuseff MI, Sibarita JB, Boes M, Raposo G, Mougneau E, Glaichenhaus N, Bonnerot C, Manoury B, Lennon-Dumenil AM. The actin-based motor protein myosin II regulates MHC class II trafficking and BCR-driven antigen presentation. *J Cell Biol*. 2007; 176:1007–1019. [PubMed: 17389233]
- [45]. Zwart W, Peperzak V, de Vries E, Keller AM, van der Horst G, Veraar EA, Geumann U, Janssen H, Janssen L, Naik SH, Neefjes J, Borst J. The invariant chain transports TNF family member CD70 to MHC class II compartments in dendritic cells. *J Cell Sci*. 2010; 123:3817–3827. [PubMed: 20971706]
- [46]. Nordeng TW, Gregers TF, Kongsvik TL, Meresse S, Gorvel JP, Jourdan F, Motta A, Bakke O. The cytoplasmic tail of invariant chain regulates endosome fusion and morphology. *Mol Biol Cell*. 2002; 13:1846–1856. [PubMed: 12058053]
- [47]. Lagaudriere-Gesbert C, Newmyer SL, Gregers TF, Bakke O, Ploegh HL. Uncoating ATPase Hsc70 is recruited by invariant chain and controls the size of endocytic compartments. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:1515–1520. [PubMed: 11818572]
- [48]. Tupin E, Kinjo Y, Kronenberg M. The unique role of natural killer T cells in the response to microorganisms. *Nat Rev Microbiol*. 2007; 5:405–417. [PubMed: 17487145]
- [49]. Behar SM, Porcelli SA. CD1-restricted T cells in host defense to infectious diseases. *Curr Top Microbiol Immunol*. 2007; 314:215–250. [PubMed: 17593663]
- [50]. Sada-Ovalle I, Skold M, Tian T, Besra GS, Behar SM. Alpha-galactosylceramide as a therapeutic agent for pulmonary *Mycobacterium tuberculosis* infection. *Am J Respir Crit Care Med*. 2010; 182:841–847. [PubMed: 20508216]
- [51]. Szalay G, Ladel CH, Blum C, Brossay L, Kronenberg M, Kaufmann SH. Cutting edge: anti-CD1 monoclonal antibody treatment reverses the production patterns of TGF-beta 2 and Th1 cytokines and ameliorates listeriosis in mice. *J Immunol*. 1999; 162:6955–6958. [PubMed: 10358132]

Fig. 1. Defective iNKT cell development in *Ii*^{-/-} mice

Representative flow cytometry dot-plots of thymus, spleen and liver are shown for different mouse genotypes. iNKT cells are detected by using anti-TCR β antibody and α -galactosylceramide (α GalCer)-loaded CD1d-tetramers. Absolute numbers of iNKT cells in thymus, spleen and liver were calculated and displayed in scatter plots. While thymic iNKT cell percentages in *Ii*^{-/-} mice are slightly decreased, *catS*^{-/-} mice exhibit normal iNKT cell percentages in thymus. Average percentages $\pm$ SEM are summarized in each dot-plot (n=6-7). Shown is one representative experiment out of three. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT.

Fig. 2. Defective iNKT cell maturation in $Ii^{-/-}$ mice

Cells from thymus, spleen and liver from different mouse genotypes were stained with α GalCer-loaded CD1d-tetramers and antibodies against TCR β , CD44, CD69 (A) and NK1.1 (B) and analyzed by flow cytometry. Dots in the scatter-plots represent individual mice. Representative flow cytometry dot-plots displaying iNKT cell maturation based on CD44 and NK1.1 expression are shown for the different mouse genotypes. iNKT cells from $Ii^{-/-}$ mice are delayed in maturation with significantly lower percentages expressing CD69 and NK1.1 in the thymus and spleen (** $p < 0.01$ and * $p < 0.05$, respectively), but not in liver. iNKT cells from $catS^{-/-}$ mice display lower percentages expressing CD69 in the spleen (* $p < 0.05$), but not in thymus or liver, nor for NK1.1. (C) Skewing of TCR V β repertoire in $Ii^{-/-}$ mice. Cells from thymus, spleen and liver, from different mice were stained with α GalCer-loaded CD1d-tetramers in combination with antibodies against TCR β , V β 7 and V β 8, and analyzed by flow cytometry. Bars represent mean percentages $\pm$ standard deviation of V β 7 (black, left axis) and V β 8 (white, right axis) positive iNKT cells ($5 \leq n \leq 7$). $Ii^{-/-}$ (** $p < 0.01$) mice display significantly less V β 7 positive iNKT cells in the thymus, but not in the periphery compared to $catS^{-/-}$ and WT mice. Shown are results from one representative experiment out of three. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT.

Fig. 3. Naïve splenocytes from $Ii^{-/-}$ mice are functionally impaired and are unable to suppress *M. tuberculosis* replication in WT macrophages

Splenocytes (spl) from $CD1d^{-/-}$ or invariant chain knockout ($Ii^{-/-}$) mice are unable to limit *M. tuberculosis* (*Mtb*) replication (A) and fail to produce cytokines upon stimulation (B).

Mtb-infected WT Mφ were cultured alone or in the presence of splenocytes from uninfected WT, $CD1d^{-/-}$, $Ii^{-/-}$ or $catS^{-/-}$ mice. Splenocytes were added to the *Mtb*-infected WT Mφ 1 day post-infection. (A) Growth of *Mtb* in the infected Mφ was assessed by determining CFU on day 1 and day 4 post-infection. Addition of WT or $catS^{-/-}$ splenocytes (***) but not $CD1d^{-/-}$ or $Ii^{-/-}$ splenocytes (NS) led to a significant reduction in bacterial CFU after 4 days. Statistical testing was done using a one-way ANOVA with Dunnett's post-test comparing each experimental group to WT *Mtb*-infected Mφ cultured alone for 4 days (d4). Shown is one representative experiment out of three. Dots in the scatter-plots represent individual CFU counts of replicate cultures ($n = 3-6$, *** $p < 0.001$). (B) Day 4 IFN- γ and TNF production by spleen iNKT cells stimulated by *Mtb*-infected WT Mφ was assessed by ELISA. $Ii^{-/-}$ and $catS^{-/-}$ iNKT cells fail to produce IFN- γ and TNF upon stimulation with *Mtb*-infected WT Mφ. Statistical testing was done using one-way ANOVA with Dunnett's post-test comparing each experimental group to WT per condition. WT Mφ + mutant splenocytes and mutant splenocytes alone function as negative controls. (C) iNKT cells from $Ii^{-/-}$ and $catS^{-/-}$ mice display altered cytokine production upon stimulation with α GalCer by Mφ. WT peritoneal Mφ were cocultured with purified spleen iNKT cells from WT, $CD1d^{-/-}$, $Ii^{-/-}$ or $catS^{-/-}$ mice, in the presence of different amounts of α GalCer. WT Mφ + mutant iNKT and mutant iNKT alone were included as negative controls. Results displayed in bar graphs are representative of two experiments. Shown are the mean of triplicate values with SEM. $Ii^{-/-}$ and $catS^{-/-}$ iNKT cells were capable of producing normal levels of IFN- γ , but produced significantly lower amounts of TNF upon activation with α GalCer (***) $p < 0.001$ and ** $p < 0.01$). Statistical testing was done using one-way ANOVA with Dunnett's post-test comparing each experimental group to WT per condition.

Fig. 4. Naïve purified iNKT cells from $li^{-/-}$ mice are unable to suppress *M. tuberculosis* replication in WT macrophages

Purified iNKT cells from invariant chain knockout ($li^{-/-}$) mice are unable to limit *M. tuberculosis* (*Mtb*) replication. *Mtb*-infected WT M ϕ were cultured alone or in the presence of purified iNKT cells from uninfected WT or $li^{-/-}$ mice. Enrichment of iNKT cells was performed by negative depletion of B220⁺, CD11b⁺ and CD11c⁺ cells with magnetic beads using autoMACs. Purified iNKT cells were added to the *Mtb*-infected WT M ϕ 1 day post-infection. Growth of *Mtb* in the infected M ϕ was assessed by determining CFU on day 1 and day 4 post-infection. Addition of WT iNKT cells (***) but not $li^{-/-}$ splenocytes (NS) led to a significant reduction in bacterial CFU after 4 days. Statistical testing was done using unpaired two-tailed t-test comparing the $li^{-/-}$ group to WT *Mtb*-infected M ϕ cultured alone for 4 days (d4). Dots in the scatter-plots represent individual CFU counts of replicate cultures (n = 3, *** p < 0.001).