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Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria

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Natural killer T cells (NKT cells) recognize glycolipid antigens presented by CD1d. These cells express an evolutionarily conserved, invariant T cell antigen receptor (TCR), but the forces that drive TCR conservation have remained uncertain. Here we show that NKT cells recognized diacylglycerol-containing glycolipids from *Streptococcus pneumoniae*, the leading cause of community-acquired pneumonia, and group B *Streptococcus*, which causes neonatal sepsis and meningitis. Furthermore, CD1d-dependent responses by NKT cells were required for activation and host protection. The glycolipid response was dependent on vaccenic acid, which is present in low concentrations in mammalian cells. Our results show how microbial lipids position the sugar for recognition by the invariant TCR and, most notably, extend the range of microbes recognized by this conserved TCR to several clinically important bacteria.

Natural killer T cells (NKT cells) fascinate immunologists because they have several unique features[1–5]. For example, NKT cells are responsive to glycolipids presented by CD1d, a nonpolymorphic major histocompatibility complex class I–like antigen-presenting molecule, in contrast to conventional T cells, which recognize peptide antigens. Furthermore, instead of the diverse antigen receptors expressed by most T cell populations, most NKT cells express an invariant T cell antigen receptor (TCR) α-chain formed by α-chain variable region 14–α-chain joining region 18 (V_α14-J_α18) rearrangement in mice and V_α24-J_α18 rearrangement in humans. Therefore, mouse NKT cells that express an invariant V_α14 TCR are called 'V_α14*i* NKT cells' and their human counterparts are called 'V_α24*i* NKT cells' and this population is collectively called '*i*NKT cells'. Additionally, rodent and primate *i*NKT cells recognize the same antigens, and there is interspecies cross-reactivity[6]. This unusual degree of conservation of antigen recognition suggests that this specificity has a particularly important function.

Many reports have shown that *i*NKT cells participate in the response to microbial pathogens[1,2,5,7]. In some such cases, *i*NKT cells were probably responding not to a microbial glycolipid but instead were probably activated by inflammatory cytokines acting alone[8] and/or with self antigens presented by CD1d[2,7,9]. In contrast, two types of bacteria, *Sphingomonas* species[10,11] and *Borrelia burgdorferi*[12], have been shown to have glycolipid antigens for the *i*NKT cell TCR. *Helicobacter pylori* is also reported to have such antigens[13], although we have not confirmed reactivity with synthetic or purified material (J.L.V., A.K. and M.K., data not shown). Despite that discrepancy, none of these microbes cause lethal diseases and therefore it is unlikely that they are major drivers for the conservation of the *i*NKT cell TCR specificity throughout much of mammalian evolution.

Because of the conservation noted above, we reasoned that the *i*NKT cell TCR would recognize antigens of certain highly pathogenic bacteria. Worldwide, the most lethal bacterial pathogen is *Streptococcus pneumoniae*, an agent of pneumonia, bloodstream infection and meningitis in children and the elderly, now estimated to cause 11% of all deaths in children from 1 month to 5 years of age[14]. Notably, V_α14*i* NKT cell–deficient J_α18-deficient mice challenged with *S. pneumoniae* have considerable impairment in bacterial clearance from the lung and much lower survival[15]. The mechanism is related in part to interferon-γ (IFN-γ) derived from V_α14*i* NKT cells, which facilitates bacterial clearance by stimulating tumor necrosis

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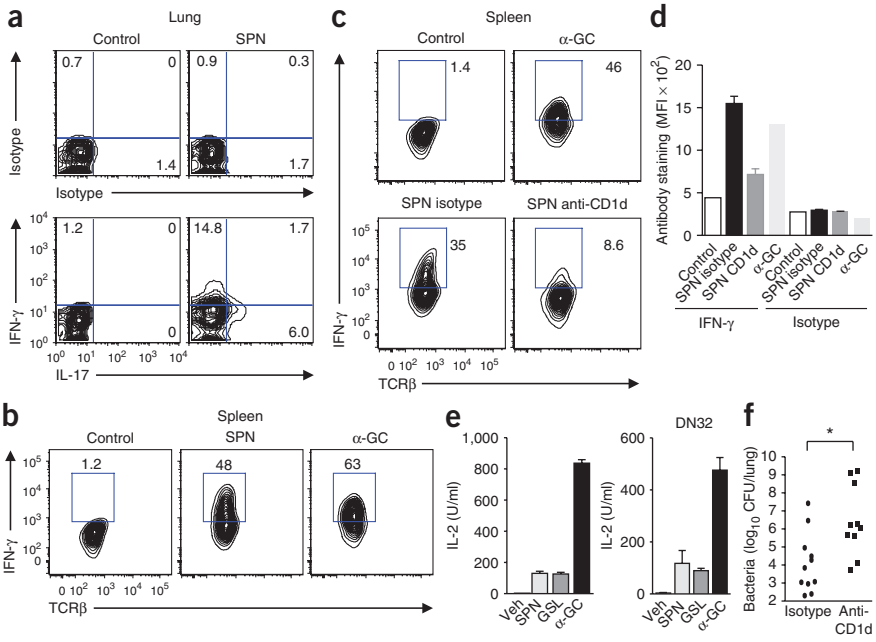

Figure 1 CD1d-dependent cytokine production by $V_\alpha14i$ NKT cells. (**a**) Expression of intracellular IFN-γ and IL-17 by $CD19^-$ lung mononuclear cells positive for α-GalCer-loaded CD1d tetramer, measured in uninfected mice (Control) or 13 h after intratracheal infection with *S. pneumoniae* (SPN; cells combined from at least five mice per condition). Isotype, isotype-matched control antibody. Numbers in quadrants indicate percent cells in each throughout. (**b**) Expression of intracellular IFN-γ by tetramer-positive $CD19^-$ spleen cells from an uninfected mouse (Control), a mouse 6 h after intravenous infection with *S. pneumoniae* (SPN; $n = 3$) or a mouse injected with α-GalCer 1.5 h before tissue collection (α-GC). (**c**,**d**) Expression of intracellular IFN-γ by tetramer-positive $CD19^-$ spleen cells from an uninfected mouse or a mouse injected with α-GalCer as in **b**, and from mice treated with anti-CD1d (SPN anti-CD1d) or isotype-matched control antibody (SPN isotype) and infected intravenously with *S. pneumoniae*, assessed 6 h later ($n = 3$ per group). MFI, mean fluorescence intensity. Numbers adjacent to outlined areas (**b**,**c**) indicate percent $IFN\text{-}\gamma^+TCR\beta^+$ cells. (**e**) Enzyme-linked immunosorbent assay of IL-2 produced by $V_\alpha14i$ NKT cell hybridoma clones 1.2 and DN32 cultured with $CD11c^+$ cells from spleens of mice injected with buffer containing Tween 20 vehicle (Veh), infected 16 h earlier with *S. pneumoniae* (SPN), injected 16 h earlier with synthetic glycosphingolipid antigen from *Sphingomonas* bacteria (GSL) or injected with α-GalCer (α-GC). (**f**) Bacterial burden in lungs of mice treated with anti-CD1d or isotype-matched control antibody (immunoglobulin G), assessed 3 d after infection with *S. pneumoniae*. Each symbol represents an individual mouse. CFU, colony-forming units. $^*P < 0.05$ (Mann-Whitney test). Data are representative of at least two experiments with similar results (**a**–**d**; mean and s.d. in **d**) or two independent experiments (**e**; mean and s.d. of triplicate wells) or are from two independent experiments (**f**).

factor and production of the chemokine CXCL2 (MIP-2), leading to enhanced recruitment of neutrophils to the lung[16].

Here we demonstrate CD1d-dependent activation of $V_\alpha14i$ NKT cells *in vivo* after infection with *S. pneumoniae*, which indicates antigen-dependent activation of these cells. Furthermore, we identify the unique structures of glycolipids from *S. pneumoniae* and another Gram-positive pathogen, group B *Streptococcus*, which were recognized by the *i*NKT cell TCR. Our data demonstrate a requirement in these glycolipid antigens for a fatty acid that is infrequent in mammalian cells. Additionally, we determine the unique binding mode of these antigens to mouse CD1d by solving the crystal structure of the antigen-CD1d complex. We propose that the *i*NKT cell TCR is a particularly useful and conserved specificity in part because it recognizes glycolipids from important pathogens that cause invasive, rapid and potentially lethal infections.

RESULTS

$V_\alpha14i$ NKT cells produce IFN-γ after infection

The protective effect of $V_\alpha14i$ NKT cells after infection with *S. pneumoniae* is dependent on the ability of mononuclear cells to produce IFN-γ[16], but several cell types can produce IFN-γ, and its production by activated $V_\alpha14i$ NKT cells has not been demonstrated directly. We therefore determined if $V_\alpha14i$ NKT cells synthesize cytokines after intratracheal infection of mice with *S. pneumoniae*. At 13 h after infection with *S. pneumoniae*, we stained lung mononuclear cells with CD1d tetramers loaded with α-galactosylceramide (α-GalCer), which specifically detect *i*NKT cells, then analyzed the stained cells for intracellular cytokines immediately after isolation, without restimulation or treatment with brefeldin A. A substantial frequency of $V_\alpha14i$ NKT cells in the lungs had intracellular IFN-γ or interleukin 17 (IL-17) at 13 h after infection (**Fig. 1a** and **Supplementary Fig. 1a**). We also detected intracellular IFN-γ in $V_\alpha14i$ NKT cells in the spleen at 6 h after intravenous infection with *S. pneumoniae* (**Fig. 1b** and **Supplementary Fig. 1b**), although under these circumstances intracellular IL-17 was not detectable (data not shown). Similarly, we also detected intracellular IFN-γ in $V_\alpha14i$ NKT cells in the liver after systemic infection (data not shown).

$V_\alpha14i$ NKT cells can be activated by cytokines, particularly IL-12, even in the absence of antigen presentation by CD1d and engagement of the TCR[8,9,17]. Therefore, we sought evidence that a TCR-dependent response was contributing to the activation of the $V_\alpha14i$ NKT cells after infection. To achieve this, we injected a blocking antibody to CD1d (anti-CD1d) into infected mice. The frequency of $IFN\text{-}\gamma^+$ tetramer-positive cells was much lower after blockade of CD1d (**Fig. 1c**,**d** and **Supplementary Fig. 1c**), which indicated involvement of TCR recognition in the activation of the $V_\alpha14i$ NKT cells *in vivo* in the early phases of *S. pneumoniae* infection. To confirm the idea that an antigen that engages the $V_\alpha14i$ NKT cell TCR is formed after infection, we purified $CD11c^+$ cells from spleen of *S. pneumoniae*–infected mice. We then analyzed these antigen-presenting cells (APCs) for their ability to activate $V_\alpha14i$ NKT cell hybridomas for IL-2 release, a response that is dependent only on TCR engagement. Although $V_\alpha14i$ NKT cell hybridomas were not responsive to IL-12 or lipopolysaccharide (data not shown), we found that APCs stimulated the release of IL-2 from $V_\alpha14i$ NKT cell hybridomas and that they were as effective as APCs preloaded with synthetic glycosphingolipid (GSL) antigen from *Sphingomonas* bacteria (**Fig. 1e**). To determine if CD1d-dependent activation of $V_\alpha14i$ NKT cells also has a role in clearance of bacteria, we treated mice with monoclonal antibody (mAb) to CD1d or isotype-matched control antibody and infected the mice intratracheally with *S. pneumoniae*, then counted bacteria in the lungs at 3 d after infection. Injection of mAb to CD1d resulted in significantly more bacterial colonies (**Fig. 1f**).

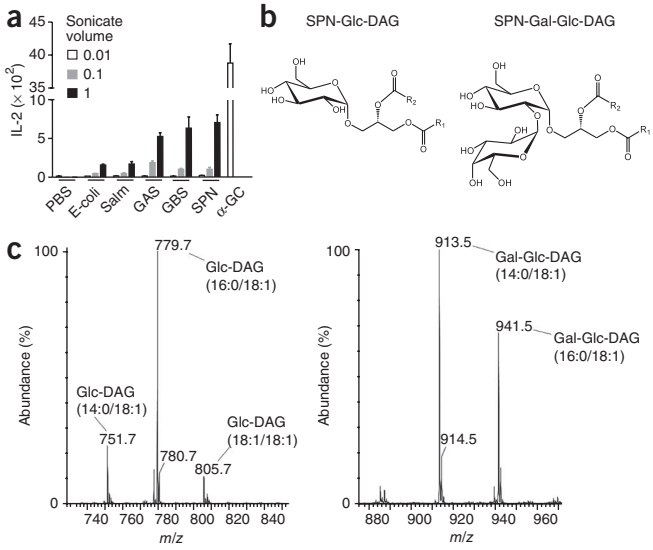

Figure 2 Structure of *S. pneumoniae* glycolipids. (**a**) Enzyme-linked immunosorbent assay of IL-2 in supernatants of $V_\alpha14i$ NKT cell hybridoma 1.2 assessed in an APC-free assay of PBS alone, *E. coli* (E-coli), *S. typhimurium* (Salm), group A *Streptococcus* (GAS), group B *Streptococcus* (GBS), *S. pneumoniae* (SPN) or α-GalCer (α-GC; 5 ng/well). The sonicate volumes 0.01, 0.1 and 1 (key) are equivalent to 1×10^6, 1×10^7 and 1×10^8 bacteria per well, respectively. (**b**) Structure of *S. pneumoniae* glycolipids SPN-Glc-DAG (left) and SPN-Gal-Glc-DAG (right). (**c**) Electrospray-ionization mass spectrometry analysis of SPN-Glc-DAG (left) and SPN-Gal-Glc-DAG (right); fatty acid composition is in parentheses. *m/z*, mass/charge. Data are representative of two experiments (**a**; mean and s.e.m. of triplicate wells) or three experiments (**c**).

In sum, these data suggest that infection of DCs with *S. pneumoniae* *in vivo* leads to the generation of an antigen that can stimulate the TCR of $V_\alpha14i$ NKT cells and that antigen recognition is important in the clearance of *S. pneumoniae*.

$V_\alpha14i$ NKT cell hybridomas respond to bacterial sonicates

Because *i*NKT cells can be activated by either self or foreign antigens[2], we determined if *S. pneumoniae* contain compounds that can stimulate the *i*NKT cell TCR. We prepared sonicates from a *S. pneumoniae* clinical isolate whose clearance is impaired in $V_\alpha14i$ NKT cell–deficient mice[15]. We incubated those bacterial sonicates in microwell plates coated with soluble mouse CD1d molecules. We observed dose-dependent IL-2 responses to the *S. pneumoniae* sonicates with two $V_\alpha14i$ NKT cell hybridomas with different $V_\beta8.2$ rearrangements (**Fig. 2a** and **Supplementary Fig. 2a**) and found that the response was inhibited by mAb to CD1d (**Supplementary Fig. 2b**). A CD1d-reactive hybridoma that does not bear the $V_\alpha14i$ TCR and does not recognize α-GalCer[18] did not respond to either bacterial sonicate (**Supplementary Fig. 2c**).

We compared those *S. pneumoniae* sonicates with ones prepared from several other bacteria, including important Gram-positive pathogens such as group A *Streptococcus*, which is estimated to cause over 500 million cases of pharyngitis and 600,000 invasive infections annually worldwide[19], and group B *Streptococcus*, the leading cause of life-threatening bacterial infections such as sepsis and meningitis in newborn humans[20]. When tested in the CD1d-coated-plate assay, sonicates of group A *Streptococcus* and group B *Streptococcus* also reproducibly induced the release of IL-2 from $V_\alpha14i$ NKT cell hybridomas. The Gram-negative bacteria *Escherichia coli* and *Salmonella typhimurium* are widely believed not to have glycolipid antigens for *i*NKT cells[11,17], and they produced much weaker responses, although in some assays *E. coli* sonicates produced completely negative results (**Fig. 2a** and data not shown). On the basis of these results, we cannot exclude the possibility that there is a weak antigen in *S. typhimurium* and perhaps in *E. coli* as well, but these sonicates did have less stimulatory activity.

Structure of microbial glycolipids

We prepared crude lipid extracts from a panel of Gram-positive strains of clinical origin, including *S. pneumoniae* strains of serotypes 3, 12 and 17, a group B *Streptococcus* serotype IA strain, and a strain of the Gram-positive zoonotic pathogen *Streptococcus suis*. We fractioned lipids from these bacteria as described[21] and analyzed them by electrospray mass spectrometry, one- and two-dimensional nuclear magnetic resonance and gas chromatography–mass spectrometry (additional analysis details, **Supplementary Figs. 3–7**). We detected two main fractions of *S. pneumoniae* glycolipids. One had a single glucose sugar α-linked to diacylglycerol (DAG; 1,2-di-*O*-acyl-(α-glucopyranosyl)-(1→3)-glycerol) (called 'SPN-Glc-DAG' here; **Fig. 2b**). The second fraction was identical except it had a disaccharide moiety attached to the DAG, with galactose (Gal) α1→2 linked to the glucose sugar, resulting in (α-galactopyranosyl)-(1→2)-(α-glucopyranosyl)-(1→3)-glycerol) (called 'SPN-Gal-Glc-DAG' here; **Fig. 2b**). Analysis of SPN-Glc-DAG by electrospray mass spectrometry identified two main fatty acids: hexadecanoic acid ($C_{16:0}$) and octadecenoic acid ($C_{18:1}$; **Fig. 2c**). We confirmed that composition by gas chromatography–mass spectrometry analysis of fatty acid methyl esters (data not shown). We found the same fatty acids in disaccharide-containing SPN-Gal-Glc-DAG, but this glycolipid also had a substantial amount of tetradecanoic acid ($C_{14:0}$). Notably, we identified the structure of the octadecenoic acid as *cis*-vaccenic (octadecen-11-oic acid or $C_{18:1}$(n-7)), which has an unsaturated bond between the carbons at positions 11 and 12 (**Supplementary Fig. 5a**). Oleic acid, the more common $C_{18:1}$ fatty acid in mammalian cells[22], also present in the *Borrelia* DAG antigen, has a *cis* unsaturated bond between the carbons at positions 9 and 10 ($C_{18:1}$(n-9)). The group B *Streptococcus* DAG glycolipids did not differ from those obtained from *S. pneumoniae*, including the presence of vaccenic acid. One of the main glycolipids purified had an α-linked glucose monosaccharide (called 'GBS-Glc-DAG' here). As in *S. pneumoniae*, the other main chemical species had a disaccharide, but with two glucose sugars (Glc α1→2 Glc); we call this compound (α-glucopyranosyl)-(1→2)-(α-glucopyranosyl)-(1→3)-glycerol) (called 'GBS-Glc-Glc-DAG' here). Finally, the DAG glycolipid from *S. suis* had a monosaccharide with α-linked mannose. It is noteworthy that glucosylated DAG glycolipids are not found only in pathogens. We also analyzed the glycolipid content of a Gram-positive commensal organism, *Lactobacillus casei*, which had similar DAG glycolipids with α-linked glucose (data not shown).

Microbial glycolipids stimulate $V_\alpha14i$ NKT cell hybridomas

To determine if the purified glycolipids were able to stimulate *i*NKT cells, we cultured $V_\alpha14i$ NKT cell hybridomas with A20 mouse B lymphoma cells transfected to express CD1d. $CD1d^+$ cells incubated with SPN-Glc-DAG or SPN-Gal-Glc-DAG induced CD1d-dependent release of IL-2 from the hybridomas (**Fig. 3a** and **Supplementary Fig. 8a**). To confirm that the purified *S. pneumoniae* glycolipids stimulated the invariant TCR of $V_\alpha14i$ NKT cells, we also tested them in the CD1d-coated-plate assay. *S. pneumoniae* glycolipids stimulated the release of IL-2 from all four $V_\alpha14i$ NKT cell hybridomas tested when added to CD1d-coated plates (**Fig. 3b**, **Supplementary Fig. 8b** and data

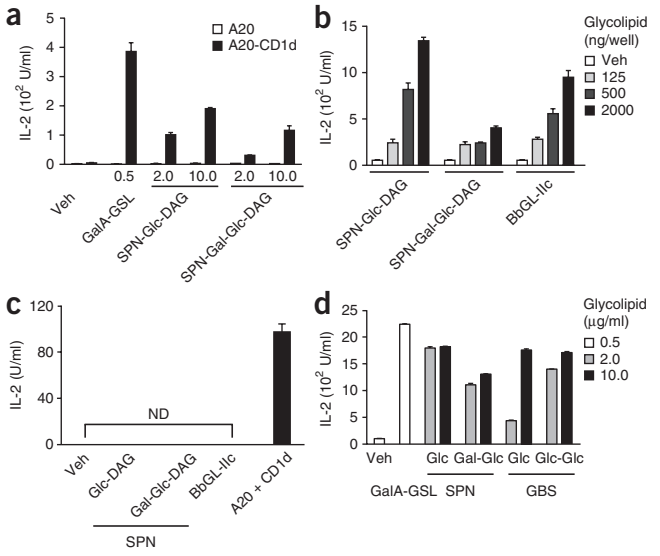

Figure 3 Microbial glycolipids stimulate $V_\alpha14i$ NKT cells *in vitro*. (**a**) Release of IL-2 from cells of the $V_\alpha14i$ NKT cell hybridoma 1.2 cultured with A20 cells (A20) or A20 cells transfected to express mouse CD1d (A20-CD1d), pulsed for 20 h with buffer containing Tween 20 (vehicle), *Sphingomonas* GalA-GSL, SPN-Glc-DAG or SPN-Gal-Glc-DAG at various concentrations (horizontal axis; in μg/ml). (**b**) Release of IL-2 from cells of $V_\alpha14i$ NKT cell hybridoma 1.2 cultured for 20 h in mouse CD1d–coated wells with SPN-Glc-DAG, SPN-Gal-Glc-DAG or BbGL-IIc at various concentrations (key). (**c**) Release of IL-2 from cells of the non-$V_\alpha14$-expressing but CD1d-reactive hybridoma 19 in wells coated with CD1d, cultured with Tween 20 (vehicle) alone, SPN-Glc-DAG, SPN-Gal-Glc-DAG or BbGL-IIc (2,000 ng/well) or self antigen presented by A20 cells transfected to express mouse CD1d. ND, not detected. (**d**) Release of IL-2 from cells of the $V_\alpha14i$ NKT hybridoma 1.2 cultured with A20 cells, transfected to express mouse CD1d and pulsed with buffer containing Tween 20 (vehicle), *Sphingomonas* GalA-GSL, SPN-Glc-DAG (Glc), SPN-Gal-Glc-DAG (Gal-Glc), GBS-Glc-DAG (Glc) or GBS-Glc-Glc-DAG (Glc-Glc) at various concentrations (key). Data are representative of at least three (**a**,**b**,**d**) or two (**c**) experiments (mean and s.e.m. of triplicate wells).

not shown). In the coated-plate assay, the disaccharide-containing SPN-Gal-Glc-DAG stimulated weaker responses (**Fig. 3b**). On the basis of published work[23], we predicted these compounds might require lysosomal processing to generate a stimulatory monosaccharide antigen. The diminished responses to SPN-Gal-Glc-DAG were consistent with our prediction, and the residual response could have been due to contaminating monosaccharide. In agreement with the results obtained with the whole bacterial sonicates, none of these purified glycolipids stimulated two CD1d-reactive but non-$V_\alpha14i$ NKT cell hybridomas (**Fig. 3c** and data not shown), which demonstrated specific activation of T cells expressing the invariant TCR. Furthermore, GBS-Glc-DAG, GBS-Glc-Glc-DAG and the commensal-derived Glc-DAG of *L. casei* also stimulated $V_\alpha14i$ NKT cell hybridomas when cultured with $CD1d^+$ A20 cells pulsed with these compounds (**Fig. 3d** and **Supplementary Fig. 9a**,**b**).

It has been shown that GSL antigens containing α-linked glucose and galactose are antigenic, whereas those containing α-linked mannose are not[24]. The DAG lipid from *S. suis* containing α-linked mannose was not antigenic (**Supplementary Fig. 9c**). These data on DAG antigens suggest that the recognition of α-linked sugars in the DAG bacterial antigens is similar to the well-characterized recognition of the α-linked carbohydrates in GSLs. Consistent with that, elucidation of the trimolecular structures of the invariant TCR bound to complexes of mouse CD1d with a *Sphingomonas* GSL or a *Borrelia* DAG antigen indicates a similar binding mode for the TCR[25].

Glycolipids stimulate $V_\alpha14i$ NKT cells *in vivo*

We determined if purified *S. pneumoniae* glycolipids were able to stimulate $V_\alpha14i$ NKT cells *in vivo*. We pulsed bone marrow–derived dendritic cells (DCs) with SPN-Glc-DAG, the disaccharide SPN-Gal-Glc-DAG or a synthetic version of the natural *Sphingomonas* galacturonic acid–containing GSL (GalA-GSL)[10] and transferred the cells into C57BL/6 mice; 14 h later, we analyzed activation of $V_\alpha14i$ NKT cells in liver and spleen. As a positive control, we transferred DCs pulsed with α-GalCer, the highly potent synthetic GSL antigen similar to the *Sphingomonas* antigens[10,11]. We analyzed cells that stained with α-GalCer-loaded CD1d tetramers for their expression of activation markers by flow cytometry. The expression of CD25 and CD69 on CD1d tetramer–positive cells was higher in mice treated with DCs pulsed with SPN-Glc-DAG than in mice treated with vehicle-pulsed DCs (**Fig. 4a** and **Supplementary Fig. 10a**). SPN-Gal-Glc-DAG also induced higher expression of CD25 and CD69, although to a lesser extent (**Fig. 4a** and **Supplementary Fig. 10a**). SPN-Glc-DAG induced intracellular expression of IFN-γ by most $V_\alpha14i$ NKT cells when they were analyzed immediately after isolation, equivalent to that induced by α-GalCer (**Fig. 4b**), which indicated that most $V_\alpha14i$ NKT cells responded to this antigen. We note that to obtain an optimal response, we incubated the DCs with more SPN-Glc-DAG than α-GalCer. We also observed intracellular IL-4 and tumor necrosis factor in activated liver $V_\alpha14i$ NKT cells, albeit on a smaller percentage of cells, but we did not detect IL-17 (**Supplementary Fig. 10b**,**c**). We observed lower cytokine responses in response to the disaccharide compound (**Fig. 4b**), in agreement with the lower induction of expression of activation markers. Purified glycolipid from group B *Streptococcus* induced a similar response *in vivo* (**Fig. 4c** and **Supplementary Fig. 11**), reflective of its essentially identical structure.

Activation of *i*NKT cells can be achieved in the absence of foreign glycolipid antigens by endogenous antigen(s) presented by CD1d and/or by inflammatory cytokines, such as IL-12, which are produced by APCs stimulated with Toll-like receptor (TLR) ligands[8,17]. At a relatively early time (4 h after the transfer of SPN-Glc-DAG–pulsed DCs into mice), many of the cytokine-producing $V_\alpha14i$ NKT cells were positive for both intracellular IFN-γ and IL-4 (**Supplementary Fig. 12a**,**b**) when analyzed immediately after isolation. This is consistent with TCR-mediated activation of these cells because IL-4 production has not been observed in $V_\alpha14i$ NKT cells stimulated only indirectly by inflammatory cytokines such as IL-12 (refs. 8,17). To confirm that TLR-mediated activation of APCs is not required for the activation of $V_\alpha14i$ NKT cells induced by *S. pneumoniae* glycolipids, we pulsed DCs from TLR signaling–defective ($Myd88^{-/-}Trif^{Lps2/Lps2}$) mice with SPN-Glc-DAG or SPN-Gal-Glc-DAG and transferred the DCs into $Myd88^{-/-}$ mice or wild-type mice, then stained $V_\alpha14i$ NKT cells for intracellular cytokines. DCs from $Myd88^{-/-}Trif^{Lps2/Lps2}$ mice were able to stimulate equal production of IFN-γ and IL-4 by $V_\alpha14i$ NKT cells from recipient $Myd88^{-/-}$ mice or wild-type mice (**Fig. 4d** and **Supplementary Fig. 13**). Furthermore, the magnitude of the response to TLR signaling–defective DCs was similar to the response obtained when both donor DCs and recipient mice were wild-type. Consistent with that, IL-12-deficient DCs that had been pulsed with SPN-Glc-DAG also were still able to induce cytokines from $V_\alpha14i$ NKT cells when transferred into IL-12-deficient mice (**Fig. 4e** and **Supplementary Fig. 14a**,**b**). Therefore, the *in vivo* activation of $V_\alpha14i$ NKT cells by the purified glycolipids was due to TCR-dependent activation by microbial glycolipid antigens and did not require activation of the innate immune response and IL-12.

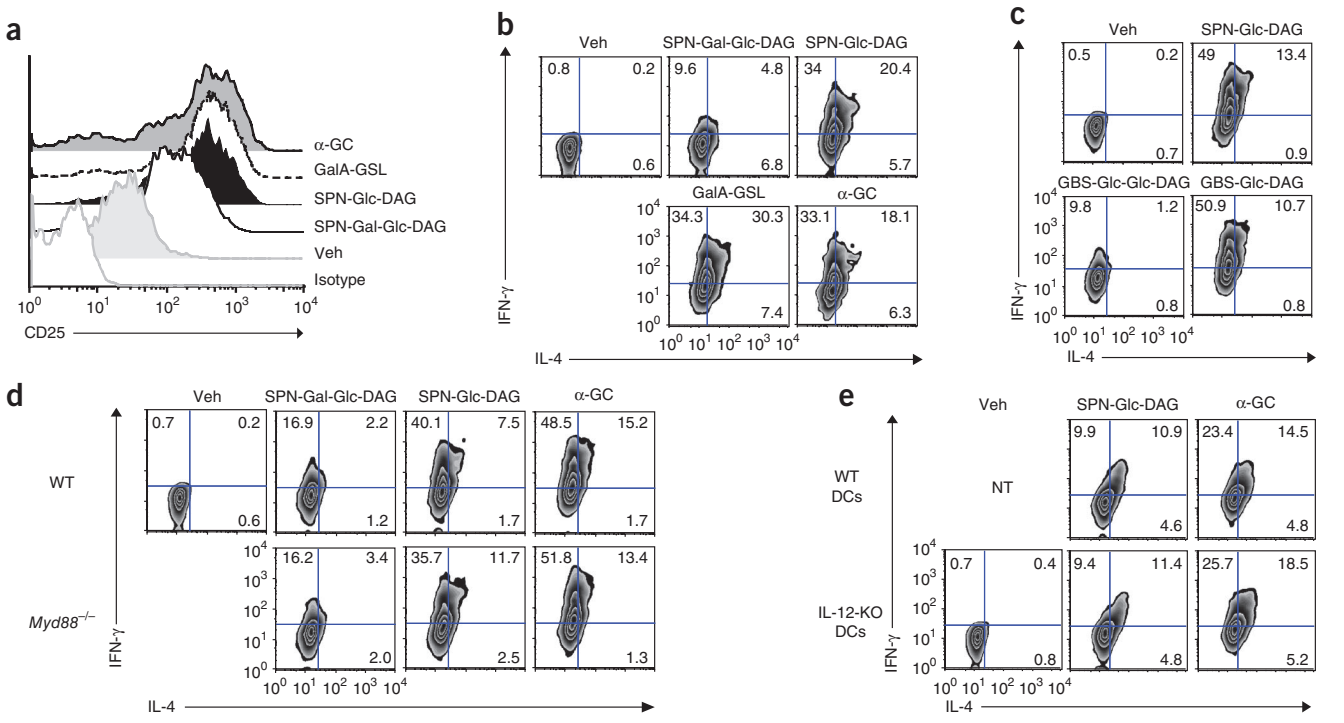

Figure 4 *In vivo* stimulation of $V_\alpha14i$ NKT cells by purified glycolipids. (**a**,**b**) Expression of CD25 (**a**) and intracellular IFN-γ and IL-4 (**b**) by tetramer-positive $V_\alpha14i$ NKT cells (liver mononuclear cells) obtained from mice 14 h after transfer of DCs pulsed with α-GalCer (0.1 μg/ml), *Sphingomonas* GalA-GSL (10 μg/ml), SPN-Glc-DAG (20 μg/ml), SPN-Gal-Glc-DAG (20 μg/ml) or vehicle alone. (**c**) Expression of intracellular IFN-γ and IL-4 by tetramer-positive liver mononuclear cells obtained from mice 14 h after transfer of DCs pulsed with vehicle, SPN-Glc-DAG, GBS-Glc-DAG or GBS-Glc-Glc-DAG (20 μg/ml). (**d**) Intracellular expression of IFN-γ and IL-4 by liver mononuclear cells (positive for CD1d tetramer loaded with α-GalCer) obtained from wild-type or *Myd88*$^{-/-}$ mice 14 h after transfer of *Myd88*$^{-/-}$*Trif*$^{Lps2/Lps2}$ DCs pulsed with vehicle, SPN-Gal-Glc-DAG (20 μg/ml), SPN-Glc-DAG (20 μg/ml) or α-GalCer (0.1 μg/ml). (**e**) Expression of intracellular IFN-γ and IL-4 by tetramer-positive liver mononuclear cells obtained from IL-12p35-deficient mice 4 h after transfer of wild-type or IL-12p35-deficient (IL-12-KO) DCs pulsed with vehicle, SPN-Glc-DAG (20 μg/ml) or α-GalCer (0.1 μg/ml). NT, not tested. Data are representative of at least two independent experiments with similar results ($n = 3$ mice per group, except $n = 2$ mice for α-GalCer in **d**).

Although activation of $V_\alpha14i$ NKT cells by glycolipid-pulsed APCs did not depend on IL-12, activation after *S. pneumoniae* infection did. In agreement with published work[9], the induction of CD69 expression on $V_\alpha14i$ NKT cells was the same in wild-type and IL-12-deficient mice after infection with *S. pneumoniae*; however, IFN-γ production by $V_\alpha14i$ NKT cells was much lower in mice deficient in the p35 subunit of IL-12 (**Supplementary Fig. 15**).

Stimulation of *i*NKT cells by synthetic Glc-DAG antigens

We tested synthetic compounds to verify the identity of the purified material that activated *i*NKT cells. We synthesized versions of SPN-Glc-DAG containing vaccenic acid in the *sn*-1 position (Glc-DAG-s1) or the *sn*-2 position (Glc-DAG-s2), which reflects the structure of the natural antigen, or in both positions (Glc-DAG-s3), with hexadecanoic acid in the remaining position for Glc-DAG-s1 and Glc-DAG-s2 (**Table 1**). To further assess the importance of vaccenic acid, we also tested two compounds with a $C_{18:1}$ oleic acid. In the hybridoma-stimulation assay with APCs transfected to express CD1d, the synthetic version of the naturally occurring antigen, Glc-DAG-s2, was the only compound that induced considerable release of IL-2 (**Supplementary Fig. 16a**). Compounds with vaccenic acid in the *sn*-1 position or linked to both the *sn*-1 and *sn*-2 glycerol positions were ineffective, as were compounds with oleic acid. To test the response of $V_\alpha14i$ NKT cells *in vivo*, we loaded bone marrow DCs with each of the synthetic compounds and injected them into mice, then analyzed cells from the recipient mice immediately after isolation, as with the purified material. In this *in vivo* stimulation assay, we observed a degree of selectivity similar to of the hybridoma assay noted above (**Supplementary Fig. 16a**). Only Glc-DAG-s2 induced surface upregulation of the activation markers CD25 (**Fig. 5a**) and CD69 (**Supplementary Fig. 16b**) and intracellular cytokines (**Fig. 5b** and **Supplementary Fig. 16c**).

In agreement with the results indicating that the purified glycolipid did not activate the innate immune system, synthetic Glc-DAG-s2 did not stimulate bone marrow–derived dendritic cells to increase their surface expression of CD1d and costimulatory molecules, including CD40 and CD80, whereas lipopolysaccharide induced the upregulation of these molecules (**Supplementary Fig. 17a**). Also, APCs pulsed with Glc-DAG-s2 did not increase the autoreactivity of a CD1d-reactive hybridoma that does not express the invariant TCR (**Supplementary Fig. 17b**). Furthermore, as with the purified compounds, cytokine release was not dependent on secretion of IL-12, which confirmed the requirement for TCR-dependent activation (data not shown). Lipopolysaccharide did cause slightly more reactivity of the hybridoma lacking the invariant TCR (**Supplementary Fig. 17b**), although it did not do so for a $V_\alpha14i$ NKT cell hybridoma. We attribute this result to augmented CD1d expression, although more synthesis of the self antigen for this cell is also possible.

Mouse and human *i*NKT cells tend to recognize the same glycolipids presented by CD1d[1,2,6,7], although differences in the requirement for particular fatty acids in the *B. burgdorferi* DAG antigens have been reported[12,26]. We therefore tested several human $V_\alpha24i$ NKT cell lines, which had been expanded with α-GalCer and IL-2, for reactivity

Table 1 Fatty acids in synthetic variants of Glc-DAG

Glycoplipid	R_1 (*sn*-1)	R_2 (*sn*-2)
Glc-DAG-s1	$C_{18:1}$(n-7)	$C_{16:0}$
Glc-DAG-s2	$C_{16:0}$	$C_{18:1}$(n-7)
Glc-DAG-s3	$C_{18:1}$(n-7)	$C_{18:1}$(n-7)
Glc-DAG-s4	$C_{18:1}$(n-9)	$C_{16:0}$
Glc-DAG-s5	$C_{16:0}$	$C_{18:1}$(n-9)

to the purified and synthetic Glc-DAG glycolipids. $V_\alpha 24i$ NKT cells secreted IFN-γ when cultured with the purified material and, like mouse $V_\alpha 14i$ NKT cells, they responded selectively to Glc-DAG-s2 (with vaccenic acid in the *sn*-2 position; **Fig. 5c**). We obtained similar results with four other $V_\alpha 24i$ NKT cell lines (data not shown) and for the release of IL-4 (**Supplementary Fig. 18**).

The CD1d-binding mode of *S. pneumoniae* glycolipid

Although the α-glucosyl ceramide isomer of α-GalCer differs from α-GalCer only by the orientation of the 4′ OH group of the hexose sugar, it is a weaker antigen[24], with approximately 10% of the affinity for the TCR[27,28]. Despite that, glucose-containing antigens such as Glc-DAG-s2 were approximately as potent as the galactose-containing *B. burgdorferi* DAG antigen BbGL-IIc, working slightly better on CD1d-coated plates (**Fig. 3** and **Supplementary Fig. 8b**) but slightly more weakly with APCs (**Supplementary Figs. 9b** and **16a**). A compound containing an α-linked glucose sugar was not antigenic, however, when linked to the same fatty acids as those in the galactose-containing *B. burgdorferi* antigen, with $C_{18:1}$ oleic acid in the *sn*-1 position and $C_{16:0}$ palmitic acid in *sn*-2 (Glc-DAG-s4; **Fig. 5** and **Supplementary Figs. 16** and **18**). Furthermore, a galactose containing DAG antigens with the same *S. pneumoniae* fatty acids was a less potent stimulator of *i*NKT cells (data not shown). Our data therefore indicate an intricate interaction between the lipid and sugar, with stringent requirements for both in determining antigenic potency, at least for the DAG-containing glycolipid antigens.

Given the requirements for a glucose sugar and an unusual fatty acid, to understand the basis for the activity of Glc-DAG-s2, we determined the crystal structure of the complex of Glc-DAG-s2 with mouse CD1d at a resolution of 1.7Å (**Fig. 6** and **Supplementary Table 1**). The data showed that the uncommon vaccenic acid at the *sn*-2 position of Glc-DAG-s2 was bound in the A′ pocket of mouse CD1d, encircling the A′ pole in a clockwise orientation. This was in contrast to most other CD1d-glycolipid structures, which have a counterclockwise orientation of a hydrophobic chain in the A′ pocket[29,30]. The *sn*-1 linked palmitic acid was bound in the F′ pocket while leaving the *sn*-3 linked α-anomeric glucose exposed for recognition by T cells (**Fig. 6a**,**b**). The opposite binding orientation, with the *sn*-1 and *sn*-2 chains in the A′ and F′ pockets, respectively, was not supported by the electron density in the region of the glycerol moiety and the polar head group (**Fig. 6a**). However, we observed poor electron density at the end of the *sn-1* chain, most probably the result of the various conformations the acyl chain can adopt in this portion of the F′ pocket, as described for several other mouse CD1d ligands[30]. The binding mode of Glc-DAG-s2, probably influenced by the position of the *cis*-unsaturation of the vaccenic acid, differed from the binding of the other known bacterial DAG antigen, BbGL-IIc of *B. burgdorferi*, which has the *sn*-2 fatty acid in the F′ pocket and the *sn*-1 $C_{18:1}$ oleic acid wound in a counterclockwise direction in the A′ pocket[26].

Notably, the position of the glucose head group in Glc-DAG-s2 mouse CD1d complexes was different from the position of the galactose in the complexes of α-GalCer with mouse CD1d (**Fig. 6c**), although it did resemble the position of the galactose in BbGL-IIc. Key contacts between Asp153 and the 2′-OH and 3′-OH groups of the galactose of α-GalCer were not conserved but, similar to the structure of other DAG antigens, instead the sugar of Glc-DAG-s2 was slightly more upright in the binding groove and farther from Asp153 (**Fig. 6c**), probably positioned in this way as a result of its different lipid backbone. A 60° counterclockwise rotation of the glucose relative to the position of α-GalCer, when viewed from above the carbohydrate, brought the 2′ OH of the glucose in proximity to Arg79, where it formed the only hydrogen-bond interaction directly with a mouse CD1d amino acid. A further H_2O-mediated hydrogen bond was formed between the 6′ OH of glucose and the backbone oxygen of the *sn*-2-linked fatty acid with Thr159 of mouse CD1d. Overall, these few interactions between the glucose and mouse CD1d gave rise to only a weak electron density for the glucose head group, which suggested a more flexible and dynamic binding for Glc-DAG-s2. Furthermore, from the perspective of the TCR, the glucose moiety was shifted away from Asp153 and toward the α-helix amino acid Arg79, sitting more to the center of the binding groove and similar to the structure of BbGL-IIc, in contrast to the binding of α-GalCer to mouse CD1d, in which the galactose was in more intimate contact with Asp153 of the α2 helix (**Fig. 6c–f**). The position of the hexose sugar should be more unfavorable for

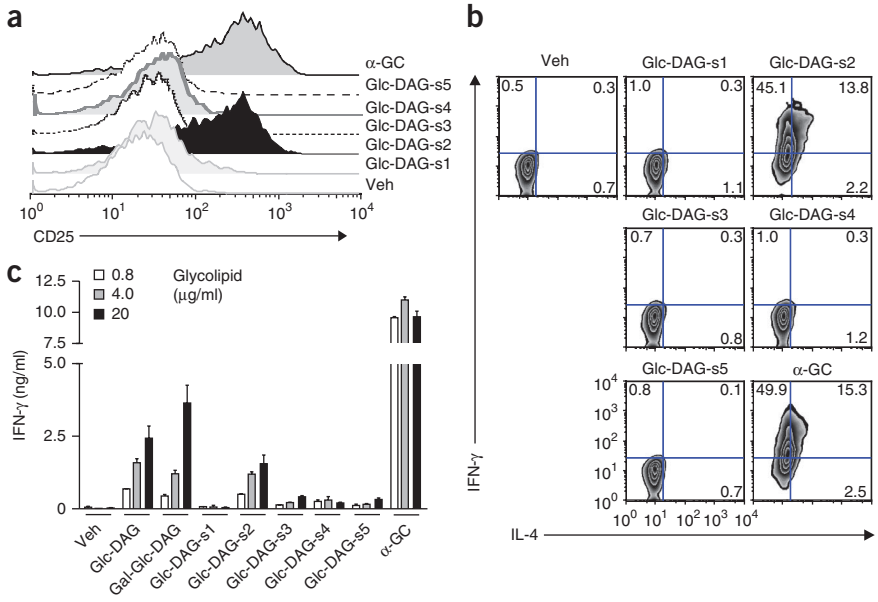

Figure 5 Stringent requirement for vaccenic acid in the stimulation of *i*NKT cells. (**a**,**b**) Expression of CD25 (**a**) and intracellular IFN-γ and IL-4 (**b**) by tetramer-positive liver mononuclear cells obtained from mice ($n = 3$ per group) 14 h after transfer of DCs pulsed with α-GalCer (0.1 μg/ml), synthetic variants of Glc-DAG (Glc-DAG-s1 through Glc-DAG-s5 (fatty acid composition, **Table 1**); 20 μg/ml) or vehicle. (**c**) Secretion of IFN-γ by human $V_\alpha 24i$ NKT cell lines ($n = 5$) cultured for 24 h with human Hela cells transfected to express CD1d, in the presence of vehicle alone, purified glycolipids (Glc-DAG and Gal-Glc-DAG), synthetic glycolipids (Glc-DAG-s1 through Glc-DAG-s5) or α-GalCer. Data are representative of two independent experiments with similar results (**a**,**b**) or two experiments (**c**; mean and s.d. of triplicate wells).

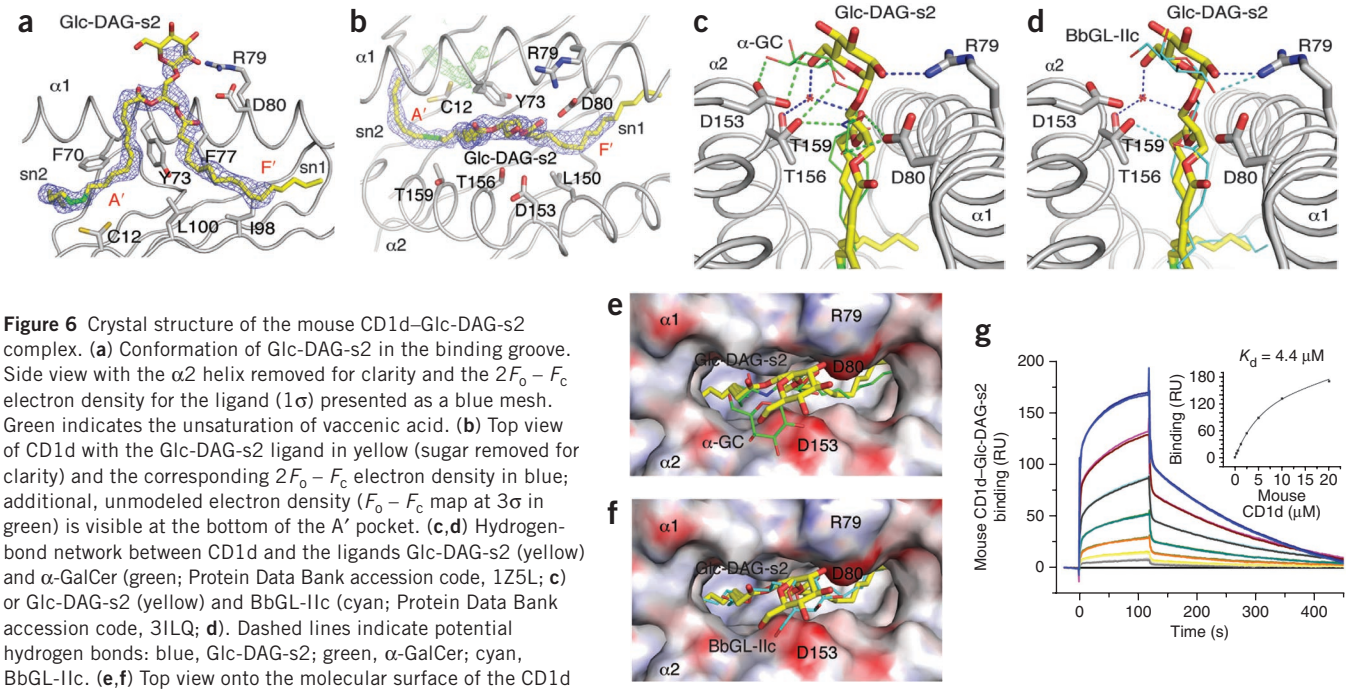

Figure 6 Crystal structure of the mouse CD1d–Glc-DAG-s2 complex. (**a**) Conformation of Glc-DAG-s2 in the binding groove. Side view with the α2 helix removed for clarity and the $2F_o - F_c$ electron density for the ligand (1σ) presented as a blue mesh. Green indicates the unsaturation of vaccenic acid. (**b**) Top view of CD1d with the Glc-DAG-s2 ligand in yellow (sugar removed for clarity) and the corresponding $2F_o - F_c$ electron density in blue; additional, unmodeled electron density ($F_o - F_c$ map at 3σ in green) is visible at the bottom of the A′ pocket. (**c**,**d**) Hydrogen-bond network between CD1d and the ligands Glc-DAG-s2 (yellow) and α-GalCer (green; Protein Data Bank accession code, 1Z5L; **c**) or Glc-DAG-s2 (yellow) and BbGL-IIc (cyan; Protein Data Bank accession code, 3ILQ; **d**). Dashed lines indicate potential hydrogen bonds: blue, Glc-DAG-s2; green, α-GalCer; cyan, BbGL-IIc. (**e**,**f**) Top view onto the molecular surface of the CD1d binding pocket, including its electrostatic potentials: yellow, Glc-DAG-s2 ligand; green, α-GalCer (**e**); cyan, BbGL-IIc (**f**). (**g**) Binding response of mouse CD1d–Glc-DAG-s2 to immobilized $V_\alpha14V_\beta8.2$ TCR, measured by surface plasmon resonance and presented as response units (RU). Inset, binding of increasing concentrations (0.3125–20 μM) of the CD1d–DAG antigen complex.

the TCR for structures with a galactose sugar in α-linkage to the same *S. pneumoniae* DAG lipid, in which case the 4′ OH in the axial position would be tilted even more from the optimal position.

Avid binding of TCRs to *S. pneumoniae* complexes with CD1d

Surface plasmon resonance binding studies with a refolded $V_\alpha14i$ NKT cell TCR supported the idea that the glucose sugar for this class of antigens gives rise to a relatively strong antigenic response similar to that of the *Borrelia* antigens with galactose sugars. The equilibrium dissociation constant for Glc-DAG-s2 was 4.4 ± 0.4 μM (mean ± s.d.), which was slightly better than that of the galactose-containing BbGL-IIc (6.2 μM)[26]. Binding to the TCR was slower (association rate constant, 1,380 ± 60 $M^{-1}s^{-1}$), and the dissociation was also much slower (dissociation rate constant, 0.00605 ± 0.0003 s^{-1}) than the binding and dissociation of BbGL-IIc (**Fig. 6g**). We conclude that the unique hydrophobic chains of the *S. pneumoniae* antigens contribute to the TCR epitope, despite being buried in the CD1d groove, because the orientation of lipid binding to the two pockets of mouse CD1d defines the position of the exposed sugar. Additionally, the altered orientation of the sugar probably permits glucose-containing antigens to be 'preferred' over galactose, unlike the use of either GSL antigens with ceramide lipids or *the B. burgdorferi* DAG antigens with $C_{18:1}$ oleic acids.

DISCUSSION

Here we have shown that the TCR expressed by mouse and human *i*NKT cells recognized unique glycolipid antigens from *S. pneumoniae* and group B *Streptococcus*, which are among the most serious and widespread bacterial pathogens. Notably, the *i*NKT cell response to these glycolipids was conserved in humans. Published work has demonstrated that $V_\alpha14i$ NKT cells are important for host protection[15], and we have now demonstrated that $V_\alpha14i$ NKT cells produced IFN-γ and IL-17 rapidly *in vivo* in the lung after infection with *S. pneumoniae*. This *in vivo* cytokine synthesis was diminished by treatment with mAb to CD1d, and APCs from infected mice activated $V_\alpha14i$ NKT cell hybridomas to produce IL-2, a response that required TCR engagement. Together these results suggest that *i*NKT cells produce cytokines *in vivo* after infection with *S. pneumoniae* because of recognition of an antigen (or antigens) presented by CD1d. Additionally, in agreement with the greater susceptibility of Jα18-deficient mice[15] and CD1d-deficient mice[9] to infection with *S. pneumoniae*, treatment with mAb to CD1d resulted in a greater *S. pneumoniae* colony count in the lung. Therefore, these data suggest that not only is cytokine production by $V_\alpha14i$ NKT cells *in vivo* dependent on TCR engagement but the host-protective effects of $V_\alpha14i$ NKT cell activation are as well.

We cannot exclude the possibility, however, that some of the CD1d-dependent *in vivo* activation of *i*NKT cells was due to self antigens presented by CD1d. A published study has provided evidence suggesting that the predominant response of $V_\alpha14i$ NKT cells to bacterial infection, including infection with *S. pneumoniae*, is stimulated by IL-12 from activated APCs, leading to the secretion of IFN-γ but not of IL-4 by the $V_\alpha14i$ NKT cells[9]. Microbes known to have antigens and those probably lacking an antigen produce similar responses. This has led to the suggestion that antigen-independent or self antigen–dependent responses are probably dominant over responses to foreign antigen. IL-12 also can act in synergy with responses to relatively weak foreign antigens, and the TCR affinity for the DAG antigens is in the low micromolar range, whereas for α-GalCer it is in the low nanomolar range[26]. We also found that the *in vivo* response to *S. pneumoniae* was dominated by IFN-γ, although we detected IL-17 synthesis by lung $V_\alpha14i$ NKT cells, which probably reflected the greater presence of $V_\alpha14i$ NKT cells committed to IL-17 production there[31]. This is potentially important, given the reported role for IL-17 in the host response to *S. pneumoniae*[32].

It should be possible to distinguish the relative contributions of self and foreign antigens to the activation of $V_\alpha14i$ NKT cells by removing

the expression of either. However, the structures of the predominant self antigens remain controversial, and some published results suggest that they may be diverse[33]. On the microbial side, the results of targeted mutagenesis of the *S. pneumoniae* gene encoding the enzyme that links the glucose sugar to DAG suggest that inactivation of this gene is a lethal mutation. Similarly, group B *Streptococcus* strains with mutated genes encoding molecules involved in the synthesis of unsaturated fatty acids required supplementation with more than one type of fatty acid for their growth, which also apparently allowed the formation of antigens (J.L.V., S.D., S.U. and V.N., data not shown). Therefore, although we cannot unambiguously distinguish the contributions of self and microbial antigens to the CD1d-mediated activation of $V_\alpha 14i$ *i*NKT cells *in vivo*, it is likely that the antigens defined here, which had micromolar affinity for the invariant TCR once bound to CD1d, do make a contribution to the production of cytokines by $V_\alpha 14i$ NKT cells.

Published studies have identified glycosylated DAG antigens in *S. pneumoniae*[9,21], although the complete structures and their antigenic activity have not been determined[9]. The bacterial DAG lipid antigens we have defined here had an unusual *sn*-2-linked fatty acid. Because the aliphatic hydrocarbon chains were buried in the groove, their structure might be expected to be largely irrelevant in determining antigenic potency. However, our data have demonstrated that not only did the exposed sugar contribute to activation but also the microbial lipid made a critical contribution. We found that the position of a single unsaturated bond in vaccenic acid linked to the *sn*-2 position of the glycerol was an important feature for defining the potency of these glucose-containing antigens. Notably, vaccenic acid is uncommon in mammalian cells[22]. The carbohydrate portion, linked to the *sn*-3 position of DAG, is also of interest. It is either a glucose monosaccharide or a disaccharide with glucose linked to the DAG moiety. It was unexpected that glucose was favored as the sugar linked to DAG rather than galactose because this was not true in the context of other glycolipid antigens. We propose that because of the presence of vaccenic acid positioning the DAG antigen, SPN-Glc-DAG-s2 is presented by mouse CD1d in a position more tilted up away from CD1d and toward the TCR, so that the initial interaction of the TCR with a galactose-containing antigen, which contains an axial 4′ OH, may be disfavored. This is consistent with the slower TCR association we found for binding to Glc-DAG-s2 complexes with CD1d. It is likely, however, that after binding the antigen-CD1d complex, the TCR would flatten the orientation of the Glc-DAG-s2 sugar to maintain the conserved binding mode found for other antigens[25,28,33]. Regardless of the mechanism of antigen recognition, our work has established that there are interactions among the lipid, sugar and CD1d in forming an epitope, with stringent requirements for both the lipid and sugar structures. These stringent requirements suggest that a microbe could avoid activation of *i*NKT cells through subtle changes in the biosynthesis of either glycolipid component.

The ability of the *i*NKT cell TCR to recognize diverse antigens in a conserved manner has been referred to as 'pattern recognition'[34]. The original idea of pattern recognition referred to a microbe-associated molecular pattern, which is a structural feature of fundamental importance to microbes that is not found in the responding mammalian host[35]. We propose that the invariant TCR expressed by *i*NKT cells recognizes a previously unknown type of microbe-associated molecular pattern defined by a hexose sugar α-linked to a lipid, usually one with two hydrophobic tails, such as in ceramide or DAG. Furthermore, the abundance of these antigens, and the likely requirement for microbial viability, demonstrate their fundamental importance, similar to that of other microbe-associated molecular patterns.

In summary, we have reported here that the invariant TCR expressed by *i*NKT cells recognized glycolipids from clinically important pathogens with worldwide distributions that cause invasive diseases with high lethality in the absence of antibiotic therapy. The specificity of these responses was conserved in mice and humans, and in mice, the CD1d-presented antigens were required for the activation of $V_\alpha 14i$ NKT cells and host protection. Therefore, we propose that the invariant V_α TCR is a particularly useful one and is evolutionarily conserved, in part because of its ability to recognize a set of widely distributed glycolipids that are an essential part of many microbes, including pathogens.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. Protein Data Bank: mouse CD1d-Glc-DAG-s2 structure, 3T1F.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

Y. Kinjo and M.K. designed most the study, except D.M.Z. designed the crystal structure study and the Biacore assay; Y. Kinjo, P.I., J.L.V., E.G., V.N., D.M.Z. and M.K. prepared the manuscript; Y. Kinjo, J.L.V. and B.P. did most of the immunology experiments; P.I., K. Kawahara and A.G.-V. analyzed bacterial glycolipids; P.I., M.I. and C.-H.W. synthesized glycolipids; G.S.B. provided informational support; E.G., Y.L. and D.M.Z. determined the crystal structure of the CD1d-Glc-DAG-s2 complex and did the Biacore assay; X.L., P.R. and M.T. did the human NKT cell experiments; Y. Kinjo, J.L.V., Y. Kaneko, A.O., Y.M. and K. Kawakami did *S. pneumoniae* infection experiments; S.D., S.U. and V.N. prepared bacterial sonicates and provided advice on bacterial culture and infection; A.K. made the mouse CD1d protein; H.Y. and P.W.A. prepared bacteria for glycolipid analysis; and M.K. provided overall supervision.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Reagents. Granulocyte-macrophage colony-stimulating factor and α-GalCer were provided by Kyowa Hakko Kirin. *Sphingomonas* GalA-GSL and *B. burgdorferi* BbGL-IIc were synthesized as described[12,36]. Antibodies for staining included anti-CD19 (1D3), anti-CD25 (3C7), anti-CD44 (IM7), anti-CD69 (H1.2F3) and anti-TCRβ (H57-597; all from BD Biosciences); anti-IFN-γ (XMG1.2; BD Biosciences and BioLegend); rat immunoglobulin G1 κ-chain isotype-matched control antibody (R3-34 (BD Biosciences) and RTK2071 (BioLegend)); anti–tumor necrosis factor (MP6-XT22; eBioscience); anti-IL-17a (TC11-18H10.1; BioLegend); and anti-IL-4 (BVD4-1D11; Invitrogen).

Mice. C57BL/6 mice and IL-12p35-deficient mice on the C57BL/6 background were from the Jackson Laboratory. *Myd88*$^{-/-}$ mice[37] and *Myd88*$^{-/-}$*Trif*$^{Lps2/Lps2}$ mice[38] on the C57BL/6 background were gifts from S. Akira (Osaka University) and Bruce Beutler (Scripps Research Institute), respectively. All mice were housed under specific pathogen–free conditions and experiments were approved by the Institutional Animal Care and Use Committee of the La Jolla Institute of Allergy & Immunology and the National Institute of Infectious Diseases, Japan.

Bacterial strains. For preparation of sonicates for immune assays, bacterial strains *S. pneumoniae* URF918 (clinical isolate, serotype 3)[15], *S. pneumoniae* D39, group B *Streptococcus* COH, group A *Streptococcus* M1, *S. typhimurium* and *E.* coli MC-1061 were used. Bacterial sonicates were generated by the addition of 70% ethanol, followed by washing of the bacteria three times with PBS, then resuspension in PBS at a concentration equivalent to 1×10^9 colony-forming units per ml before use. For preparation and analysis of glycolipids, bacterial strains *S. pneumoniae* R6 (ref. 39), group B *Streptococcus* A909 (from M. Antony), group B *Streptococcus* (701346; NCIMB), *S. suis* (702644; NCIMB), two group B *Streptococcus* clinical strains belonging to signature types 12 and 17, and *L. casei* (393; American Type Culture Collection) were used. For glycolipid analysis, bacteria were grown at 37 °C in brain-heart–infusion broth (Oxoid) or on agar (Oxoid) supplemented with 5% (vol/vol) defibrinated horse blood. *L. casei* were grown for 16 h at 37 °C in MRS broth (Oxoid).

***S. pneumoniae* infection.** *S. pneumoniae* URF918 cultured in Todd-Hewitt broth (BD) at 37 °C in an incubator at 5% CO_2 were collected at a mid-log phase and then washed twice in PBS. For induction of pulmonary infection, mice were anesthetized with isoflurane and restrained on a small board. Mice were inoculated with *S. pneumoniae* (1×10^6 to 1×10^7 colony-forming units in a volume of 50 μl per mouse) by insertion of a 24-gauge catheter into the trachea. For induction of systemic infection, *S. pneumoniae* (1×10^7 colony-forming units) were injected intravenously into mice. For blockade of CD1d, mice were treated intraperitoneally with 200 μg anti-CD1d (1B1; BD Biosciences and eBioscience) or rat immunoglobulin G2b isotype-matched control antibody (EB149; 10H5; eBioscience) 6–24 h before and just before infection. At 6 h (spleen) or 13 h (lung), lung mononuclear cells or spleen cells were collected as reported[15,40], and CD19$^-$ cells positive for the α-GalCer-loaded CD1d tetramer were analyzed immediately after isolation for intracellular cytokines without restimulation or pretreatment with brefeldin. For measurement of the lung bacterial burden, tissues were collected at day 3 after infection and were homogenized in PBS by being 'teased' with a stainless steel mesh. Homogenates were inoculated in a volume of 100 μl on 5% (vol/vol) sheep blood Mueller-Hinton agar plates and cultured for 18 h, followed by colony counting. For isolation of CD11c$^+$ cells, spleens were collected at 16–18 h after infection with *S. pneumoniae* or injection of GalA-GSL (20 μg) or α-GalCer (1 μg), and CD11$^+$ cells were purified from spleen cells with an EasySep Mouse CD11c Positive Selection Kit according to the company's instructions (StemCell Technologies). CD11c$^+$ cells (1×10^5) were cultured for 16–18 h with CD1d-reactive hybridomas (5×10^4 cells), and IL-2 in supernatants was measured by enzyme-linked immunosorbent assay (BD Bioscience).

Lipid extraction and purification from bacteria. For lipid purification, bacteria were grown to late exponential phase and were collected by centrifugation at 1,800*g* for 15 min. Lipids were extracted from washed cells and purified as described[41]. Lipid extracts were assessed by thin-layer chromatography on aluminum-backed plates of silica gel 60 F_{254} (5554; Merck), with $CHCl_3$, CH_3OH and H_2O (65:25:4 (vol/vol/vol)). Glycolipids were visualized by spraying of the plates with α-naphthol–sulfuric acid, followed by gentle charring of plates. Other types of lipids were visualized by spraying of the plates with 5% (vol/vol) ethanolic molybdophosphoric acid followed by charring, or by spraying of the plates with a Dittmer and Lester reagent specific for phospholipids. *L. casei* lipids were fractionated on a column of DEAE-cellulose. The chloroform-methanol fraction (2:1 (vol/vol)) was collected and concentrated.

Cell-free antigen-presentation assay. CD1d-reactive hybridomas have been described[10,12]. T cell hybridomas on CD1d-coated plates were stimulated according to published protocols[10,12,40]. Bacterial sonicates or compounds were incubated for 24 h in microwells coated with 1.0 μg mouse CD1d. After being washed, 5×10^4 to 1×10^5 $V_\alpha 14i$ NKT cell hybridoma cells or control cells were cultured in the plates for 16–20 h, and IL-2 in the supernatant was measured by enzyme-linked immunosorbent assay (BD Pharmingen).

***In vivo* responses to microbial glycolipids and flow cytometry.** Activation-marker expression and intracellular cytokine production by cells positive for the α-GalCer–CD1d tetramer were analyzed according to published protocols[10]. Mouse DCs were prepared by culture of bone marrow progenitor cells for 7 d with mouse granulocyte-macrophage colony-stimulating factor. Mouse DCs were incubated for 24 h with *S. pneumoniae* glycolipids (20 μg/ml), group B *Streptococcus* glycolipids (20 μg/ml), GalA-GSL (10 μg/ml) or α-GalCer (0.1 μg/ml). After being washed with PBS, glycolipid-pulsed DCs (5×10^5) were injected intravenously into mice. Liver mononuclear cells positive for the α-GalCer–CD1d tetramer were analyzed (without further treatment) 4 h or 14 h later for activation markers and intracellular cytokines. Cells were analyzed with a FACSCalibur or LSR II (BD Bioscience) and FlowJo software.

$V_\alpha 24i$ NKT cell response. Human $V_\alpha 24i$ NKT cell lines were generated with modifications to a published protocol[10]. $V_\alpha 24i^+$ T cells were isolated from LeukoPak cells with magnetic beads (Miltenyi Biotec) coupled to mAb to $V_\alpha 24$ (6B11; eBioscience), and were cultured for 10 d with irradiated autologous immature DCs (3,000 rads) in the presence of α-GalCer (100 ng/ml) and human recombinant IL-2 (10 IU/ml; R&D Systems). After a second stimulation with α-GalCer-pulsed irradiated autologous immature DCs, cell lines were 95% $V_\alpha 24^+$. $V_\alpha 24i$ NKT cells (3×10^4) were cultured with 3×10^4 irradiated HeLa cells (3×10^4; 10,000 rads) transfected to express human CD1d, in the presence of glycolipids. IFN-γ or IL-4 in supernatants was measured by enzyme-linked immunosorbent assay (eBioscience) after 24 h.

Additional methods. Information on nuclear magnetic resonance analysis, fatty acid analysis, double-bond localization, mass spectrometric analysis, and mouse CD1d-Glc-DAG-s2 crystallization and structure determination is available in the **Supplementary Results**, **Supplementary Methods** and **Supplementary References**.

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