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# Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals

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**Invariant natural killer T cells (*i*NKT cells) have a prominent role during infection and other inflammatory processes, and these cells can be activated through their T cell antigen receptors by microbial lipid antigens. However, increasing evidence shows that they are also activated in situations in which foreign lipid antigens would not be present, which suggests a role for lipid self antigen. We found that an abundant endogenous lipid, β-D-glucopyranosylceramide (β-GlcCer), was a potent *i*NKT cell self antigen in mouse and human and that its activity depended on the composition of the *N*-acyl chain. Furthermore, β-GlcCer accumulated during infection and in response to Toll-like receptor agonists, contributing to *i*NKT cell activation. Thus, we propose that recognition of β-GlcCer by the invariant T cell antigen receptor translates innate danger signals into *i*NKT cell activation.**

Invariant natural killer T (*i*NKT) cells constitute a subset of αβ T cells that recognize lipid antigens presented by the nonpolymorphic CD1d molecule. In contrast to peptide-specific diverse αβ T cell antigen receptor (TCR) major histocompatibility complex (MHC)-restricted T cells, these cells have an invariant TCR α-chain that uses TCR α-chain variable region 14 ($V_{\alpha}14$) and α-chain joining region 18 ($J_{\alpha}18$) paired with a limited $V_{\beta}$ chain repertoire in mouse or $V_{\alpha}24$ and $J_{\alpha}18$ paired with $V_{\beta}11$ in human. How *i*NKT cells, with an invariant TCR, restricted by a nonpolymorphic antigen-presenting molecule are activated in a wide variety of infectious and non-infectious pathological processes is not well understood[1,2].

Much effort has focused on the identification of lipids that are cognate antigens for the *i*NKT cell TCR. The discovery of the pharmacologic antigen α-galactosylceramide (α-GalCer)[3], as well as the discovery of glycolipid antigens from the bacteria *Borrelia burgdorferi*[4] and *Sphingomonas*[5,6], each with a primary α-linked monohexose, suggested the possibility that the major structures recognized by *i*NKT cells might be α-linked glycolipids. However, recognition of such lipids does not explain the role of *i*NKT cells during the majority of infections or during inflammation, as primary α-glycosidic linkages have not been shown to occur in most microbes or in mammalian glycolipids. Further, *i*NKT cells have a major role in situations where foreign lipid antigens would not be present at all, including autoinflammatory conditions, viral infection or stimulation by Toll-like receptors (TLRs)[7–10]. These observations support a central role for lipid self antigen in the activation of *i*NKT cells.

The rapid activation of *i*NKT cells by antigen-presenting cells (APCs) exposed to lipopolysaccharide (LPS) or other TLR agonists is notable[7–10] and provides a robust and salient model for understanding the response of *i*NKT cells to innate signals. It is clear that two signals are likely required for the physiological activation of *i*NKT cells, with the primary signal being provided through the TCR by a CD1d-lipid complex and a second signal being provided by APC-derived cytokines, predominantly interleukin 12 (IL-12)[7,11]. Accumulation of a stimulatory lipid self antigen has been proposed to provide the TCR-mediated signal to *i*NKT cells after TLR-agonist stimulation[9,10], but the specific lipid antigen responsible has not been identified.

Phospholipids, including lyso-phosphatidylcholine (lyso-PC), have been proposed to be self antigens for *i*NKT cells, but their stimulatory activity is weak and has only been shown in a subset of *i*NKT cells[12,13]. Isoglobotrihexosylceramide (iGB3) contains a terminal α-linked carbohydrate and can activate mouse *i*NKT cells through CD1d. Based largely on the observation that *Hexb*$^{-/-}$ (the *Hexb* gene product converts iGB4 to iGB3) mice have defective *i*NKT cell development, this lipid was proposed as a relevant self antigen[14]. However, a subsequent study reported that iGB3-synthase–deficient mice have a normal *i*NKT cell phenotype[15], and it has been suggested that the *i*NKT cell defect in *Hexb*$^{-/-}$ mice might be caused by altered lysosomal function rather than lower iGB3 concentrations[16]. Further, iGB3 is present in almost undetectable concentrations in mouse lymphoid tissues[17,18], and it has also been reported that humans do not express the relevant synthase and are thus unable to synthesize iGB3 (ref. 19).

In this study, we screened a panel of naturally occurring glycosphingolipids (GSLs) for antigenic activity on *i*NKT cells. We found that β-D-glucopyranosylceramide (β-GlcCer), which has been reported as being nonantigenic[20], potently activated *i*NKT cells in both mouse and human through a cognate TCR interaction. In addition, β-GlcCer, the precursor of most GSLs outside of the central nervous

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system, accumulated in APCs after stimulation with LPS and accumulated *in vivo* after bacterial infection. Blocking β-GlcCer synthesis in bone-marrow–derived dendritic cells (BMDCs) resulted in less autoreactivity and *i*NKT cell activation in response to LPS or whole bacteria, whereas blocking the subsequent step in GSL synthesis, the conversion of β-GlcCer to β-lactosylceramide (β-LacCer), had either no effect or an opposite effect. Our data identify β-GlcCer as a potent, physiologically relevant self antigen for *i*NKT cells that is upregulated in response to microbial danger signals.

## RESULTS

### Antigenic activity among a panel of GSLs

To identify self lipids that stimulate *i*NKT cells, we planned to first isolate the lipids present in CD1d on APCs and then screen those lipids for activity. We have determined the dominant GSLs eluted from CD1d on APCs[21] (summary, **Supplementary Table 1**). β-GlcCer would not have been detected by our previous analysis, as carbohydrate head groups were derivatized after GSL digestion with ceramide glycanase, an enzyme that does not cleave monohexose from a ceramide backbone. For this reason, as we transitioned to the activity determination phase of our *i*NKT cell antigen-discovery efforts, we included β-GlcCer. We used two assay systems to screen for lipid activity: a single-TCR–specificity *i*NKT cell hybridoma cultured together with CD1d-trasfected macrophages from the mouse macrophage line RAW and a primary *i*NKT cell line cultured with primary CD11c$^+$ BMDCs. We tested GSLs (**Supplementary Table 1**) as well as phospholipids, including phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, lyso-PC, lyso-phosphatidylethanolamine, lyso-phophatidylserine and lyso-phosphatidylinositol. Among the panel of lipids tested, none of the higher-order GSLs or phospholipids was stimulatory. In contrast, the simplest GSL, β-GlcCer, reproducibly activated *i*NKT cells (**Fig. 1a**,**b** and data not shown). In a primary mouse *i*NKT cell line, we further showed that β-GlcCer elicited the production of both interferon-γ (IFN-γ) and IL-4 (**Fig. 1c**,**d**), which is a characteristic of TCR-mediated, but not cytokine-mediated, *i*NKT cell activation[8]. As has been reported for other signals mediated by the *i*NKT cell TCR, the ability of β-GlcCer to induce *i*NKT cell IFN-γ production could be enhanced by the addition of IL-12 (**Fig. 1e**), an indirect stimulus for *i*NKT cells that we proposed as a crucial second signal during infection[7,11]. We confirmed the chemical composition of bovine milk β-GlcCer by proton nuclear magnetic resonance spectroscopy, two-dimensional correlation spectroscopy and total correlation spectroscopy nuclear magnetic resonance, and we detected no α-anomeric carbohydrate in these analyses (data not shown). From these results, we concluded that an early biosynthetic GSL, β-GlcCer, activates *i*NKT cells in a CD1d-dependent manner.

### β-GlcCer in primary lymphoid tissues

To determine whether β-GlcCer could be detected in tissues that might be sites of *i*NKT cell activation, we analyzed polar lipids extracted from mouse thymus, spleen, liver and BMDCs by thin layer chromatography (TLC). We observed a monohexosyl ceramide in the thymus, spleen and BMDC extracts (**Fig. 2a**). A detectable but lower amount of monohexosyl ceramide was present in the liver polar lipid extracts. By borate-impregnated TLC analysis, we determined that the monohexosyl ceramide band in the thymus, spleen and BMDC extracts was almost exclusively composed of β-GlcCer and not β-GalCer (**Supplementary Fig. 1**). To estimate the β-GlcCer content in these tissues, we used a TLC-based densitometric analysis of the tissue lipid extracts and compared it to a β-GlcCer standard titration.

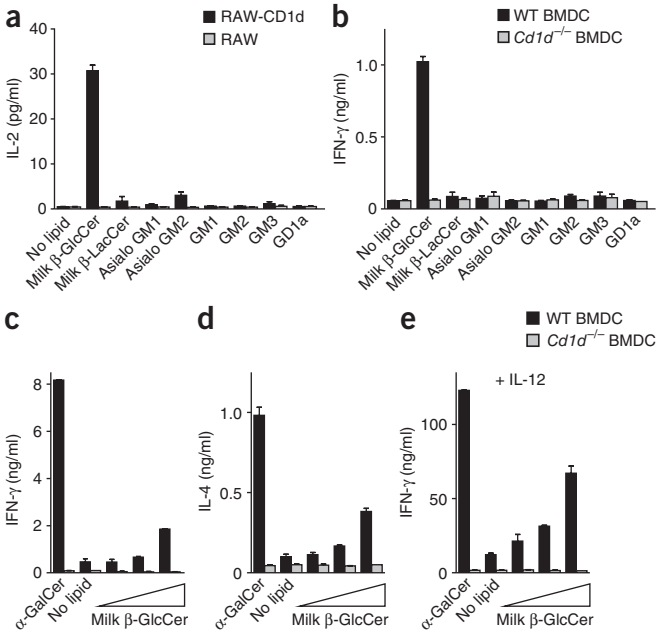


**Figure 1** Reactivity of *i*NKT cells to a panel of GSLs. (**a**) Enzyme-linked immunosorbent assay (ELISA) of the production of IL-2 by the *i*NKT cell hybridoma DN32 cultured with RAW cells or CD1d-transfected RAW cells (RAW-CD1d) in the presence of various lipids (horizontal axis; 10 μg/ml). GM1, GM2, GM3 and GD1a, **Supplementary Table 1**. (**b**) ELISA of the production of IFN-γ by a primary *i*NKT cell line cultured together with wild-type (WT) or *Cd1d*$^{-/-}$ mouse CD11c$^+$ BMDCs in the presence of various lipids (horizontal axis; 10 μg/ml). (**c**–**e**) ELISA of the production of IFN-γ (**c**,**e**) and IL-4 (**d**) by a primary mouse *i*NKT cell line cultured together with CD11c$^+$ BMDCs in the presence of α-GalCer (10 ng/ml) or bovine milk β-GlcCer (fivefold dose titration with a top concentration of 20 μg/ml), in the absence (**c**,**d**) or presence (**e**) of IL-12 (20 pg/ml). Data are representative of at least three independent experiments (mean and range of duplicate wells).

The β-GlcCer content of mouse thymus, spleen and CD11c$^+$ BMDCs was estimated at 4.6 ± 1.1 μg, 3.6 ± 0.8 μg and 3.9 ± 0.1 μg per mg of polar lipids (mean ± s.d.). In order to determine whether the β-GlcCer detected in mammalian lymphoid tissues had stimulatory activity in *i*NKT cells, we used preparative-scale high-performance liquid chromatography coupled with mass spectrometry to purify β-GlcCer from mouse spleen and thymus crude polar lipid extracts (**Supplementary Fig. 2**). We found the purified β-GlcCer–containing fractions to stimulate an *i*NKT cell hybridoma in co-culture with CD1d-transfected RAW cells (**Fig. 2b**). In the assay shown in **Figure 2b**, we estimated the β-GlcCer concentrations at 18.0 ± 4.0 μg/ml and 23.0 ± 5.5 μg/ml for the thymus and spleen β-GlcCer fractions, respectively (mean ± s.d.). For the spleen polar lipid extracts, we found that β-LacCer and phospholipid-containing fractions purified by the same method that we used to purify the β-GlcCer fractions were unable to stimulate the *i*NKT cell hybridoma (**Fig. 2b**). Thus, β-GlcCer is present in mammalian lymphoid tissues, and, when purified from these tissues, activates *i*NKT cells in a CD1d-dependent manner.

Because β-GlcCer has been reported as being nonantigenic for *i*NKT cells[20], we questioned whether the activity of bovine-milk and mammalian β-GlcCer that we observed depends on specific lipid structures attached to the carbohydrate head groups that are present in these purified materials. To address this possibility, we determined the fatty acid compositions of β-GlcCer in bovine milk, mouse spleen and mouse thymus by electrospray ionization mass

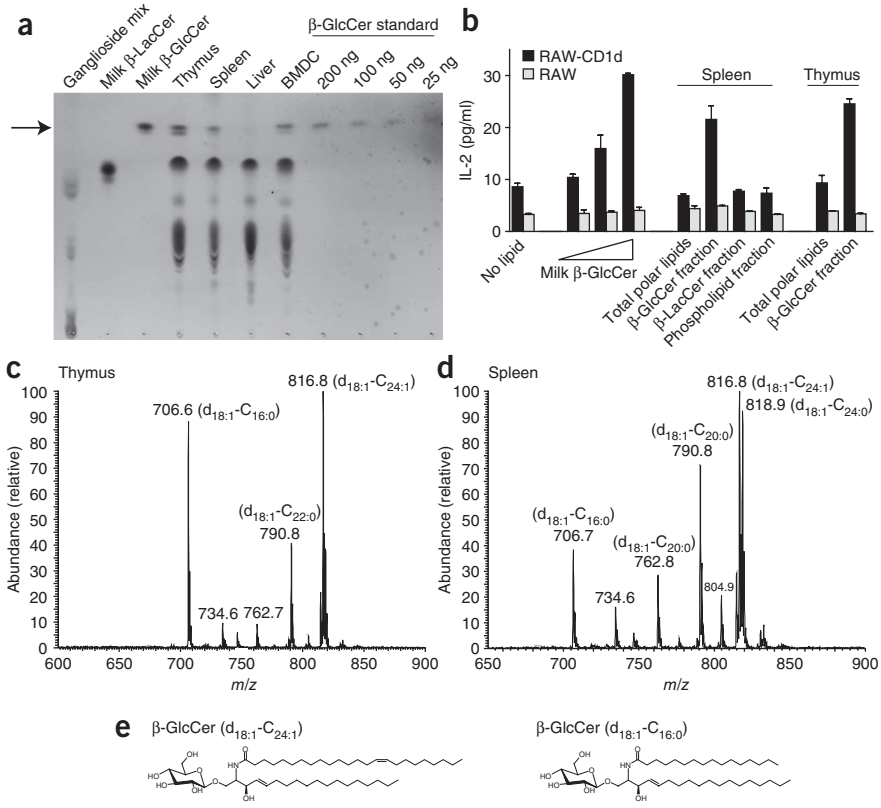


**Figure 2** β-GlcCer is present in primary lymphoid tissues and activates *i*NKT cells. (**a**) TLC analysis of polar lipids extracted from mouse thymus, spleen, whole liver and BMDCs along with GSL standards and bovine milk β-GlcCer dose titration. Arrow indicates mobility of β-GlcCer. (**b**) ELISA of IL-2 production by the *i*NKT hybridoma DN32 cultured together with RAW cells or CD1d-transfected RAW cells as APCs, with lipid fractions from mouse thymus and spleen, or bovine milk β-GlcCer (fivefold dose titration to a top concentration of 20 μg/ml). Data are representative of two separate experiments (mean and range of duplicate wells). (**c**,**d**) ESI-MS analysis of β-GalCer purified from thymus (**c**) and spleen (**d**), assessed in the electrospray-positive mode and presented relative to the most abundant species, set as 100 (*m/z*, mass/charge). Major β-GlcCer ions are presented with a lithium adduct; fatty acid composition (determined by collision-induced dissociation tandem mass spectrometry) is in parentheses. Data are representative of two experiments. (**e**) Structures of two abundant β-GlcCer forms detected by ESI-MS.

spectrometry (ESI-MS) in the positive-ion mode (**Fig. 2c**,**d** and **Supplementary Fig. 3**). We confirmed structural assignments by collision-induced dissociation tandem mass spectrometry as described[22]. In all three samples analyzed, the major ceramide backbone consisted exclusively of sphingenine ($d_{18:1}$). In mouse thymus, the major *N*-acyl chains detected were $C_{24:1}$, $C_{22:0}$ and $C_{16:0}$. In mouse spleen, the major *N*-acyl chains detected were $C_{24:1}$, $C_{24:0}$, $C_{22:0}$, $C_{20:0}$ and $C_{16:0}$. In bovine milk, the major *N*-acyl chains detected were $C_{24:0}$, $C_{23:0}$, $C_{22:0}$, $C_{20:0}$ and $C_{16:0}$. We present here the structures of two abundant β-GlcCer forms in thymus and spleen (**Fig. 2e**). For all samples, we also detected other β-GlcCer molecular species in smaller quantities, confirming the diversity of *N*-acyl chains in mammalian GSLs. These results show that the β-GlcCer present in mammalian tissues contains multiple *N*-acyl chain structures, with $C_{24:1}$ being the most abundant of these chains in lymphoid tissues.

### β-GlcCer $C_{24:1}$ is a potent mouse *i*NKT cell antigen

To functionally test the role of *N*-acyl chain composition on the activity of β-GlcCer, we studied a panel of synthetic β-GlcCer compounds that all contained sphingenine as the ceramide backbone, with the *N*-acyl chains varying from 8 to 24 carbons and being either fully saturated or containing one unsaturation (**Supplementary Fig. 4**). We detected β-GlcCer $C_{24:1}$, $C_{18:1}$, $C_{18:0}$ and $C_{16:0}$ in primary mouse tissue by ESI-MS, but we did not detect the 12- and 8-carbon *N*-acyl chain forms of this lipid (**Fig. 2c**,**d**), and these forms have not, to our knowledge, been reported to occur in mammals. β-GlcCer $C_{24:1}$, $C_{12:0}$ and $C_{18:1}$ activated an *i*NKT hybridoma *in vitro*, whereas β-GlcCer $C_{18:0}$ and $C_{16:0}$ showed no activity (**Fig. 3a**). Lyso–β-GlcCer ($d_{18:1}$) and free ceramide backbones corresponding to each synthetic β-GlcCer were not active (data not shown). We then compared the potency of β-GlcCer $C_{24:1}$ to the microbial antigen GSL-1 from *Sphingomonas*[5,6] and two previously proposed self antigens, iGB3 (ref. 14) and lyso-PC[12]. The iGB3 we used was $d_{18:1}$-$C_{26:0}$, and, although it is not matched to β-GlcCer $C_{24:1}$ in *N*-acyl chain structure, the $C_{26:0}$ acyl chain would be expected to impart maximal activity based on data from α-GalCer analogs[23,24]. As determined by assay with a primary *i*NKT cell line in co-culture with $CD11c^+$ BMDCs, the antigenicity of β-GlcCer $C_{24:1}$ was less than that of GSL-1 but was greater than that of iGB3, and, as reported before[25], we did not detect any activity for lyso-PC in mouse (**Fig. 3b**). Although we did not detect β-GalCer at potential sites of peripheral *i*NKT cell activation (**Supplementary Fig. 1**), and a β-GalCer–deficient mouse was reported not to have a demonstrable *i*NKT cell defect[20], given the structural similarity of β-GalCer to β-GlcCer, we hypothesized that β-GalCer might also activate *i*NKT cells. Purified bovine brain β-GalCer, β-GalCer $d_{18:1}$-$C_{24:1}$, and, as shown before[26,27], the non-physiological lipid β-GalCer $d_{18:1}$-$C_{12:0}$ activated mouse *i*NKT cells in a CD1d-dependent manner, albeit less potently than each corresponding β-GlcCer (**Supplementary Fig. 5**).

To examine self antigen activity *in vivo*, we injected mice intravenously with a subset of the β-GlcCer synthetic panel and iGB3. Two hours after injection with β-GlcCer $C_{24:1}$, two-thirds of liver *i*NKT cells were IFN-γ positive and greater than 40% were IL-4 positive, which is a substantially higher percentage than that seen in mice injected with iGB3 (**Fig. 3c**,**d**). In liver mononuclear T cells from CD1d-deficient mice that lack NKT cells, there was no detectable response to β-GlcCer $C_{24:1}$ injection. We also observed potent activation of $CD11c^+$ dendritic cells and B cells *in vivo* 24 h after β-GlcCer $C_{24:1}$ injection, most likely as a result of transactivation (**Supplementary Fig. 6**). We concluded from these studies that the activity of β-GlcCer is dependent on the *N*-acyl chain structure and that the abundant $d_{18:1}$-$C_{24:1}$ form of β-GlcCer is a potent antigen for *i*NKT cells *in vivo*.

In addition to *i*NKT cells with an invariant TCR α-chain, there are CD1d-restricted T cells in the diverse αβ TCR compartment called 'diverse NKT cells'. Because there is no method available at present with which to identify primary diverse NKT cells, we screened a panel of ten individual diverse NKT cell hybridomas for activity by

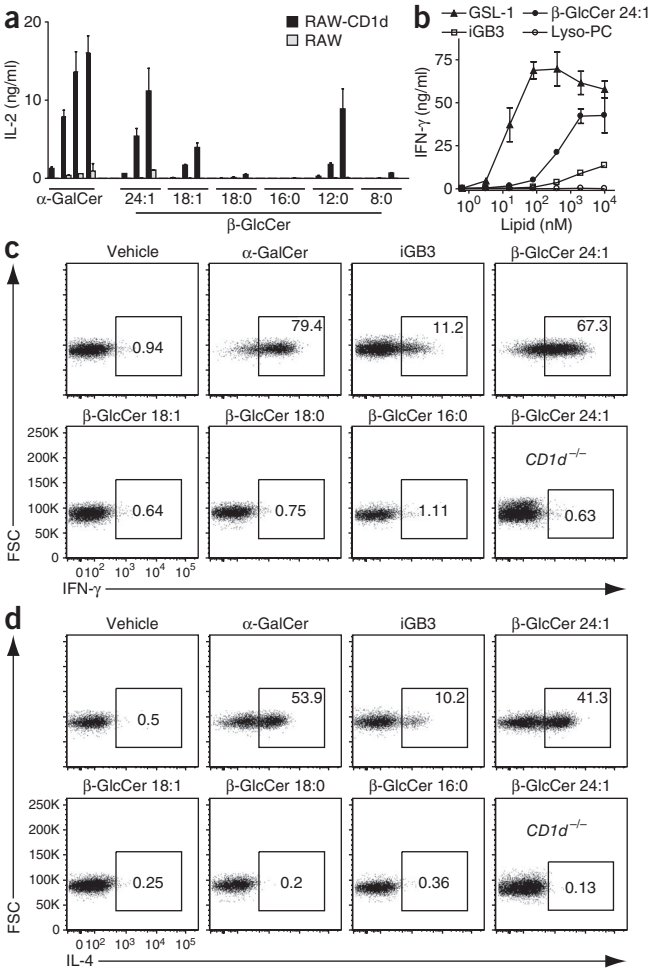


**Figure 3** Reactivity of *i*NKT cells to a β-GlcCer panel with differing *N*-acyl chains. (**a**) ELISA of IL-2 production by the *i*NKT cell hybridoma DN32 cultured together with RAW cells or CD1d-transfected RAW cells, plus β-GlcCer with various *N*-acyl chains (horizontal axis; fivefold-dose titration of with a top concentration of 10 μg/ml) or α-GalCer (10 ng/ml). (**b**) ELISA of IFN-γ production by a primary mouse *i*NKT cell line cultured together with wild-type CD11c$^+$ BMDCs, plus β-GlcCer $C_{24:1}$, reported *i*NKT cell lipid self antigens or a microbial GSL antigen. (**c**,**d**) Cytokine capture assay of IFN-γ (**c**) and IL-4 (**d**) in liver mononuclear cells from mice given intravenous injection of 25 μg lipid (above plots) or 1 μg α-GalCer, presented as the TCRβ$^+$ PBS-57–loaded tetramer–positive gate, except bottom right plot (total TCRβ$^+$ gate is shown for a CD1d-deficient mouse injected with β-GlcCer $C_{24:1}$). Numbers in outlined areas indicate percent *i*NKT cells producing IFN-γ or IL-4. Structures of the synthetic lipids used here are in **Supplementary Figure 4**, and as all structures contained a $d_{18:1}$ shingenine base, they are abbreviated throughout with only the *N*-acyl chain composition listed (for example, 'β-GlcCer 24:1' indicates β-D-glucopyranosylceramide $d_{18:1}$-$C_{24:1}$). FSC, forward scatter. Data are representative of three separate experiments (**a**,**b**; mean and range of duplicate wells) or at least three independent experiments (**c**,**d**).

coculture with primary mouse BMDCs and the synthetic β-GlcCer panel described above. Two of these ten diverse NKT cell hybridomas, VII68 and XV19 (ref. 28), showed reactivity to a subset of β-GlcCer compounds (**Supplementary Fig. 7** and data not shown). We concluded from these data that β-GlcCer also activates diverse NKT cells, but that this reactivity may depend on different *N*-acyl chain structures than do *i*NKT cells.

### β-GlcCer $C_{24:1}$ mediates a cognate TCR interaction

To confirm that β-GlcCer directly mediates *i*NKT cell stimulation through CD1d, we tested a synthetic β-GlcCer panel and bovine milk β-GlcCer in an APC-free system with purified, plate-bound CD1d. In this system, bovine milk β-GlcCer, β-GlcCer $C_{24:1}$ and β-GlcCer $C_{12:0}$ activated a primary *i*NKT cell line (**Fig. 4a**), showing direct, CD1d-dependent activation of *i*NKT cells by β-GlcCer. Next, we asked if CD1d tetramers loaded with β-GlcCer $C_{24:1}$ could bind *i*NKT cells directly. Indeed, tetramers loaded with β-GlcCer $C_{24:1}$ stained a portion of the *i*NKT cells from both C57BL/6 and BALB/c mice, as identified by sequential double staining with a CD1d tetramer loaded with PBS-57 (an α-GalCer analog; **Fig. 4b**). The staining seemed to be limited to the portion of the *i*NKT cells that stained most brightly with the PBS-57–loaded tetramer, suggesting that β-GlcCer $C_{24:1}$–loaded tetramer staining was brightest for the *i*NKT cell population with the highest affinity TCRs. The TCR $V_\beta$-chain repertoire of mouse *i*NKT cells is limited, and those cells with the $V_\beta$-chains $V_\beta2$, $V_\beta7$, $V_\beta8.1$, $V_\beta8.2$ and $V_\beta8.3$ have been found to have a higher affinity for α-GalCer–loaded CD1d tetramers compared with less frequently used $V_\beta$-chains ($V_\beta6$, $V_\beta9$, $V_\beta10$ and $V_\beta14$)[29]. Consistent with the affinity hierarchy described for α-GalCer–loaded tetramers, β-GlcCer $C_{24:1}$–loaded tetramers identified the most frequently used *i*NKT cell TCR $V_\beta$-chains (**Fig. 4c**,**d**).

Although β-GlcCer tetramer staining was limited to a fraction of mouse *i*NKT cells (**Fig. 4b**), the majority of the *i*NKT cells produced cytokines after stimulation with β-GlcCer *in vivo* (**Fig. 3**). Further, *i*NKT cells substantially downregulate their surface TCR expression after activation with a strong antigen but not after indirect activation[30]. Twenty-four hours after intravenous injection of β-GlcCer $C_{24:1}$, *i*NKT cells were almost undetectable, suggesting TCR-dependent activation of almost all mouse *i*NKT cells (**Supplementary Fig. 8**). The difference, then, between *i*NKT cell activation and tetramer binding is likely because of a higher affinity requirement for tetramer binding relative to activation. We concluded from these tetramer studies that β-GlcCer $C_{24:1}$ mediates a cognate interaction between the *i*NKT cell TCR and CD1d in mouse.

### β-GlcCer is a self antigen for human *i*NKT cells

Given the high degree of evolutionary conservation seen for both CD1d and *i*NKT cells[1], we would expect a physiologically relevant self antigen to activate both mouse and human *i*NKT cells. We asked whether β-GlcCer was a self antigen in humans by testing the synthetic β-GlcCer panel described above for activity on human *i*NKT cells. Similar to mouse *i*NKT cells, β-GlcCer $C_{24:1}$, $C_{12:0}$ and, to a lesser degree, $C_{18:1}$, activated three independent human *i*NKT cell clones[31] when presented by human peripheral blood mononuclear cell (PBMC)-derived APCs, and this activation was efficiently inhibited by a monoclonal antibody against CD1d (**Fig. 5a**,**b**). In this system, iGB3 did not activate *i*NKT cells. In a primary human *i*NKT cell line, we compared the antigenic potency of *Sphingomonas* GSL-1, β-GlcCer $C_{24:1}$, iGB3 and lyso-PC. In humans, β-GlcCer $C_{24:1}$ was less potent than GSL-1 but was far more potent than iGB3 or lyso-PC. In fact, in contrast to mouse *i*NKT cells, we did not detect activity for iGB3 in primary human *i*NKT cells. Lyso-PC did not stimulate *i*NKT cells to produce detectable IFN-γ or IL-4, but, as has been reported[12], we did detect a small amount of granulocyte macrophage colony-stimulating factor (**Fig. 5c** and data not shown).

Having shown the activity of β-GlcCer on human *i*NKT cell clones and a primary human *i*NKT cell line, we next cultured freshly isolated PBMCs overnight with β-GlcCer without the addition of exogenous APCs and measured *i*NKT cell intracellular cytokine production.

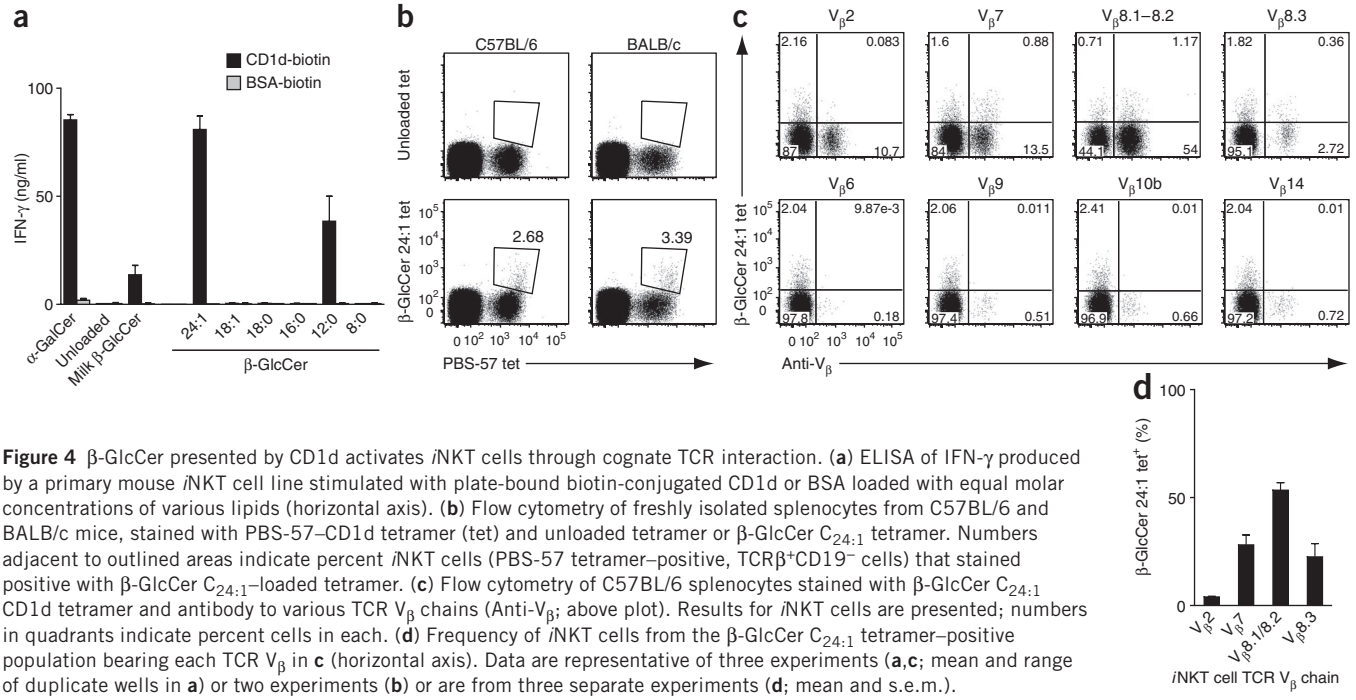


**Figure 4** β-GlcCer presented by CD1d activates *i*NKT cells through cognate TCR interaction. (**a**) ELISA of IFN-γ produced by a primary mouse *i*NKT cell line stimulated with plate-bound biotin-conjugated CD1d or BSA loaded with equal molar concentrations of various lipids (horizontal axis). (**b**) Flow cytometry of freshly isolated splenocytes from C57BL/6 and BALB/c mice, stained with PBS-57–CD1d tetramer (tet) and unloaded tetramer or β-GlcCer $C_{24:1}$ tetramer. Numbers adjacent to outlined areas indicate percent *i*NKT cells (PBS-57 tetramer–positive, $TCR\beta^+CD19^-$ cells) that stained positive with β-GlcCer $C_{24:1}$–loaded tetramer. (**c**) Flow cytometry of C57BL/6 splenocytes stained with β-GlcCer $C_{24:1}$ CD1d tetramer and antibody to various TCR $V_\beta$ chains (Anti-$V_\beta$; above plot). Results for *i*NKT cells are presented; numbers in quadrants indicate percent cells in each. (**d**) Frequency of *i*NKT cells from the β-GlcCer $C_{24:1}$ tetramer–positive population bearing each TCR $V_\beta$ in **c** (horizontal axis). Data are representative of three experiments (**a**,**c**; mean and range of duplicate wells in **a**) or two experiments (**b**) or are from three separate experiments (**d**; mean and s.e.m.).

β-GlcCer $C_{24:1}$ was able to stimulate cytokine production by this assay, and this effect was completely blocked by monoclonal antibody to CD1d (**Supplementary Fig. 9a**,**b**). We also used freshly isolated human PBMCs to assay *i*NKT cell proliferation in response to various β-GlcCer *N*-acyl chain variants. β-GlcCer $C_{24:1}$, $C_{12:0}$, and $C_{18:1}$ in co-culture led to a marked expansion of *i*NKT cells over an 8-day period (**Supplementary Fig. 9c**).

To confirm the cognate interaction between β-GlcCer–loaded CD1d and the *i*NKT cell TCR in humans, we made tetramers with β-GlcCer-loaded human CD1d. Staining with PBS-57–loaded tetramers allowed for unambiguous identification of *i*NKT cells, and we confirmed the *i*NKT cells to be $V_\alpha24^+V_\beta11^+$ (**Fig. 5d**). Double staining of freshly isolated PBMCs with β-GlcCer–loaded CD1d tetramers and PBS-57–loaded tetramers showed that a substantial portion of human *i*NKT cells

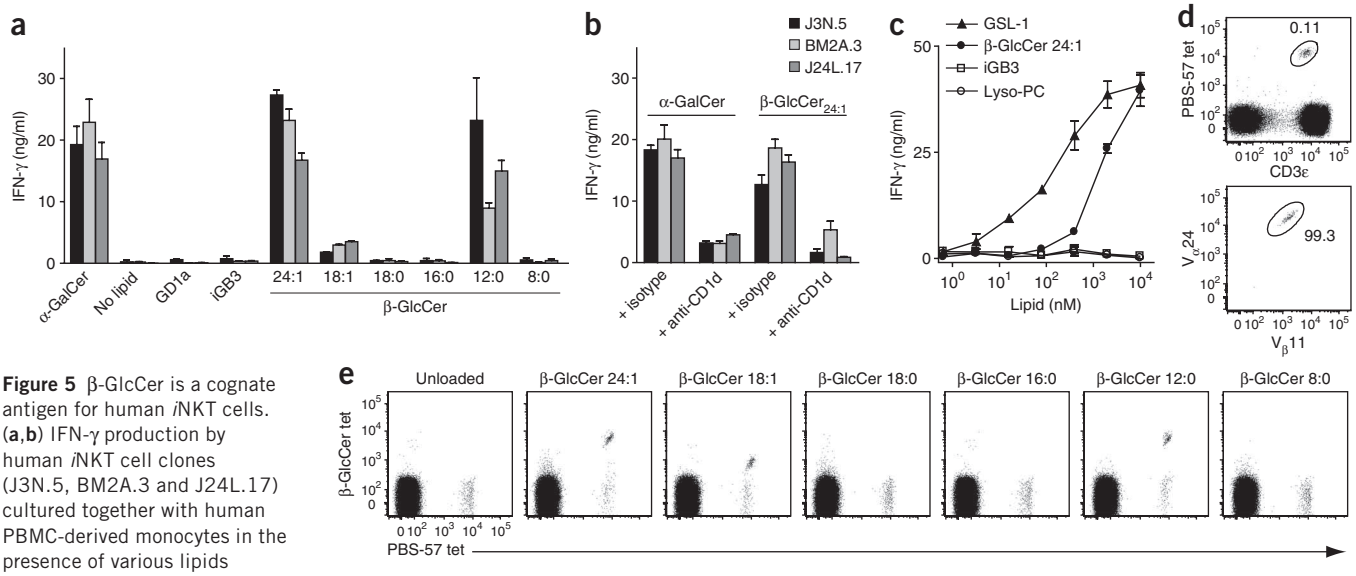


**Figure 5** β-GlcCer is a cognate antigen for human *i*NKT cells. (**a**,**b**) IFN-γ production by human *i*NKT cell clones (J3N.5, BM2A.3 and J24L.17) cultured together with human PBMC-derived monocytes in the presence of various lipids (10 μg/ml; horizontal axis; **a**) or α-GalCer (10 ng/ml; **a**) and with monoclonal antibody to CD1d (anti-CD1d) or isotype-matched control antibody (**b**). (**c**) ELISA of IFN-γ production by a primary human *i*NKT cell line cultured together with PBMC-derived monocytes, plus β-GlcCer $C_{24:1}$, reported *i*NKT cell lipid self antigens or a microbial GSL antigen. (**d**) Identification of *i*NKT cells with anti-CD3ε and PBS-57 tetramers (top) and staining of $CD3\varepsilon^+$ PBS-57 tetramer–positive gated cells with anti-$V_\alpha24$ and anti-$V_\beta11$ to confirm invariant TCR chain use (bottom). Numbers adjacent to outlined areas indicate percent positive cells in each. (**e**) Flow cytometry of PBMCs costained with PBS-57–loaded CD1d tetramer and CD1d tetramers loaded with β-GlcCer *N*-acyl chain variants (above plots), presented as the $CD3\varepsilon^+$ gate. CD1d tetramers loaded with β-GlcCer $C_{24:1}$, $C_{18:1}$ and $C_{12:0}$ stain human *i*NKT cells. Data are representative of three experiments (**a**–**c**; mean and range of duplicate wells), two experiments (**d**) or least three separate experiments (**e**).

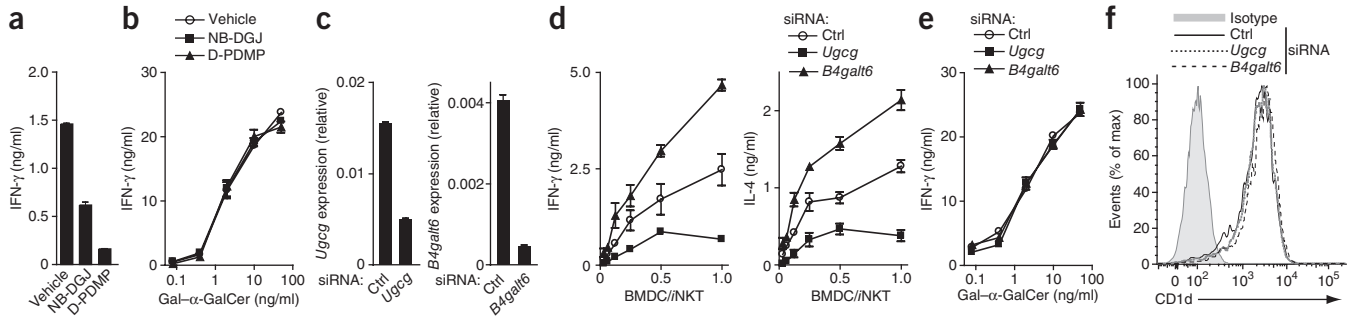


**Figure 6** β-GlcCer contributes to *i*NKT cell self-reactivity. (**a**) ELISA of IFN-γ production by an *i*NKT cell line cultured together with CD11c$^+$ BMDCs at a ratio of 5:1, with vehicle alone or with NB-DGJ or D-PDMP (inhibitors of β-GlcCer synthesis). (**b**) IFN-γ production by *i*NKT cells cultured together with CD11c$^+$ BMDCs and Gal–α-GalCer, in the presence of vehicle alone or NB-DGJ or D-PDMP. (**c**) Quantitative PCR analysis of *Ugcg* and *B4galt6* in CD11c$^+$ BMDCs cultured for 48 h with control siRNA (Ctrl) or *Ugcg*- or *B4galt6*-specific siRNA, presented relative to *Gapdh* expression (encoding glyceraldehyde phosphate dehydrogenase). (**d**) ELISA of the production of IFN-γ and IL-4 by a primary *i*NKT cell line cultured together with the BMDCs in **c** (ratio, horizontal axes), assessing autoreactivity. (**e**) IFN-γ production by an *i*NKT cell line cultured together with CD11c$^+$ BMDCs treated with siRNA as in **c**, assessing the presentation of Gal–α-GalCer. (**f**) Flow cytometry analysis of CD1d surface expression on CD11c$^+$ BMDCs treated with siRNA as in **c**. Data are representative of three separate experiments (mean and range in duplicate wells in **a**,**b**,**d**,**e**; mean and s.e.m. of triplicates in **c**).



bind tetramers loaded with β-GlcCer $C_{24:1}$, $C_{12:0}$ and $C_{18:1}$ (**Fig. 5e**). In complementary experiments, we examined if *i*NKT cell TCR tetramers[32] could recognize CD1d loaded with β-GlcCer. We again found an interaction dependent on the *N*-acyl chain, with β-GlcCer $C_{24:1}$ and $C_{12:0}$ mediating TCR tetramer binding (**Supplementary Fig. 10a**). β-GlcCer–loaded CD1d tetramer staining was positive but was of variable intensity between subjects (**Supplementary Fig. 10b**); for clarity, a subject with a high percentage of β-GlcCer CD1d tetramer–positive *i*NKT cells is shown in **Figure 5**.

As seen in the mouse, β-GlcCer tetramers recognized only a portion of the human *i*NKT cell population identified by α-GalCer-loaded tetramers. The percentage of cells recognized by β-GlcCer tetramers did not decrease after double staining with α-GalCer tetramers under the staining conditions used, suggesting that tetramer competition was not a factor (data not shown). We hypothesized that, as in the mouse, β-GlcCer tetramers might identify human *i*NKT cells with high-affinity TCRs. To address this possibility, we used OCH, an α-GalCer analog that, when loaded in human CD1d tetramers, has been reported to bind a population of human *i*NKT cells with high-affinity TCRs[32]. Double staining with CD1d tetramers loaded with OCH and β-GlcCer $C_{24:1}$ identified the same population (**Supplementary Fig. 10c**), which suggested that β-GlcCer tetramers identified *i*NKT cells with high-affinity TCRs. We saw robust *i*NKT cell activation by β-GlcCer in all human subjects and in all *i*NKT cell clones tested irrespective of the intensity of β-GlcCer CD1d tetramer staining, which suggested, as in our mouse studies, that tetramer binding may have a more demanding threshold than does *i*NKT cell activation.

### β-GlcCer contributes to *i*NKT cell self reactivity

GSL self lipid antigens have been proposed to contribute to *i*NKT cell activation in the absence of foreign lipid antigens[33]. Because β-GlcCer is relatively abundant in lymphoid tissues, and because we found that it potently activates *i*NKT cells from both mice and humans, we postulated that it might contribute to *i*NKT cell self reactivity. We assessed the contribution of β-GlcCer to *i*NKT cell autoreactivity by perturbing the pathways involved in the synthesis of this lipid. We present the pathways involved in the synthesis and degradation of β-GlcCer for reference (**Supplementary Fig. 11a**). In a primary mouse *i*NKT cell line, we found that, as has been shown before[10,20], inhibition of GSL synthesis by either N-butyldeoxygalactonojirimycin (NB-DGJ) or D-Threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) resulted in less *i*NKT cell autoreactivity (**Fig. 6a**). To assess antigen processing and presentation in BMDCs after treatment with GSL synthesis inhibitors, we used Gal–α-GalCer, an α-GalCer analog that requires lysosomal uptake and processing for antigenicity[34] (**Fig. 6b**). NB-DGJ and D-PDMP did not diminish the activation of *i*NKT cells by Gal–α-GalCer. Because both NB-DGJ and D-PDMP inhibit β-GlcCer synthesis, and, consequently, the synthesis of all higher order ceramides that are based on β-GlcCer, we used small interfering RNA (siRNA) silencing of either glucosylceramide synthase (encoded by *Ugcg*) or the downstream enzyme that converts β-GlcCer to β-LacCer, lactosylceramide synthase (encoded by *B4galt6*), in BMDCs to isolate the β-GlcCer–dependent signal (**Fig. 6c**). siRNA silencing of *Ugcg* in BMDCs resulted in less *i*NKT cell autoreactivity, whereas *B4galt6* silencing increased autoreactivity (**Fig. 6d**). Silencing through the use of siRNA did not alter the ability of BMDCs to present Gal–α-GalCer (**Fig. 6e**) and did not alter CD1d surface concentrations as determined by flow cytometry (**Fig. 6f**). Thus, modulation of β-GlcCer concentrations determines *i*NKT cell self reactivity to BMDCs.

### β-GlcCer mediates *i*NKT cell activation during infection

The *i*NKT response to LPS-exposed BMDCs requires both a signal through CD1d and APC-derived IL-12 (refs. 7,9,10). We hypothesized that β-GlcCer might be a prominent component of an LPS-induced, CD1d-mediated signal and investigated the pathways involved in the synthesis and degradation of β-GlcCer after TLR agonist exposure. Using a published gene-expression data set[35], we examined the regulation of *Ugcg* and *B4galt6* in mouse BMDCs in response to five TLR agonists. Four of five of the stimuli led to the upregulation of *Ugcg*, with a peak expression occurring at 2–6 h after exposure to the TLR agonists. All stimuli resulted in a decrease in the expression of *B4galt6* mRNA over the first 8 h of exposure to the TLR agonists (**Supplementary Fig. 11b**). We saw only minimal changes in the expression of *Gba* and *Glb1*, which encode the molecules responsible for the degradation of β-GlcCer and β-LacCer, respectively (data not shown). The LPS concentration used for generation of the dataset above was 100 ng/ml, which is substantially higher than we found to be required to stimulate *i*NKT cells in co-culture with BMDCs. With 1 ng/ml of LPS, we determined the expression of the genes involved in β-GlcCer metabolism by quantitative PCR and

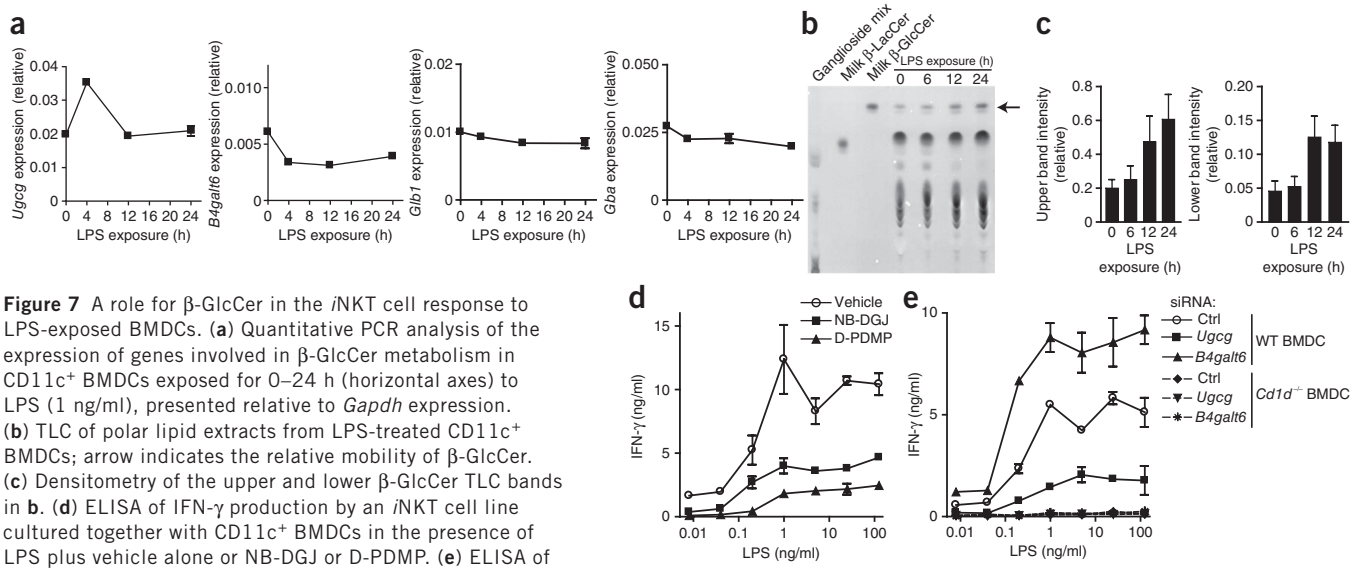


**Figure 7** A role for β-GlcCer in the *i*NKT cell response to LPS-exposed BMDCs. (**a**) Quantitative PCR analysis of the expression of genes involved in β-GlcCer metabolism in CD11c$^+$ BMDCs exposed for 0–24 h (horizontal axes) to LPS (1 ng/ml), presented relative to *Gapdh* expression. (**b**) TLC of polar lipid extracts from LPS-treated CD11c$^+$ BMDCs; arrow indicates the relative mobility of β-GlcCer. (**c**) Densitometry of the upper and lower β-GlcCer TLC bands in **b**. (**d**) ELISA of IFN-γ production by an *i*NKT cell line cultured together with CD11c$^+$ BMDCs in the presence of LPS plus vehicle alone or NB-DGJ or D-PDMP. (**e**) ELISA of IFN-γ production by a primary *i*NKT cell line cultured together with siRNA-treated CD11c$^+$ BMDCs (as in **Fig. 6c**), in the presence of LPS. Data are representative of three independent experiments (error bars, s.e.m. of triplicates (**a**), s.d. (**c**) or mean and range of duplicate wells (**d**,**e**)).

again observed rapid upregulation of *Ugcg* and concomitant down-regulation of *B4galt6* (**Fig. 7a**). TLC analysis of polar lipid extracts from CD11c$^+$ BMDCs after treatment with LPS showed an increase in β-GlcCer (**Fig. 7b**) as quantified by densitometry (**Fig. 7c**; additional controls, **Supplementary Fig. 12a**,**b**). Naturally occurring β-GlcCer migrated as a doublet by TLC in the solvent system used, and the potent antigen β-GlcCer $C_{24:1}$ migrated in the accumulating upper band (**Supplementary Fig. 12c**).

Having shown that β-GlcCer accumulated in BMDCs after TLR agonist stimulation, we next asked whether this lipid contributes to *i*NKT cell activation. We confirmed that, as has been shown for TLR agonists[9,10], inhibition of GSL synthesis resulted in a lower response of *i*NKT cells to LPS-exposed BMDCs (**Fig. 7d**). To isolate the β-GlcCer contribution to this GSL-dependent activation, we used siRNA silencing. We found that *Ugcg* silencing limited the *i*NKT cell response to LPS-treated BMDCs, whereas *B4galt6* silencing enhanced this response (**Fig. 7e**). By TLC analysis, we observed lower β-GlcCer concentrations after treatment with NB-DGJ or D-PDMP and after silencing of *Ugcg*, whereas we saw higher β-GlcCer concentrations after silencing of *B4galt6* (**Supplementary Fig. 13**). We did not detect changes in LPS-mediated IL-12 production or BMDC maturation as assessed by flow cytometry after NB-DGJ, D-PDMP or siRNA targeting of *Ugcg* or *B4galt6* compared with controls. D-PDMP did, however, lead to a slight decrease in CD1d surface concentrations on CD11c$^+$ BMDCs, potentially contributing to the decreased *i*NKT cell activation seen after treatment of BMDCs with this inhibitor

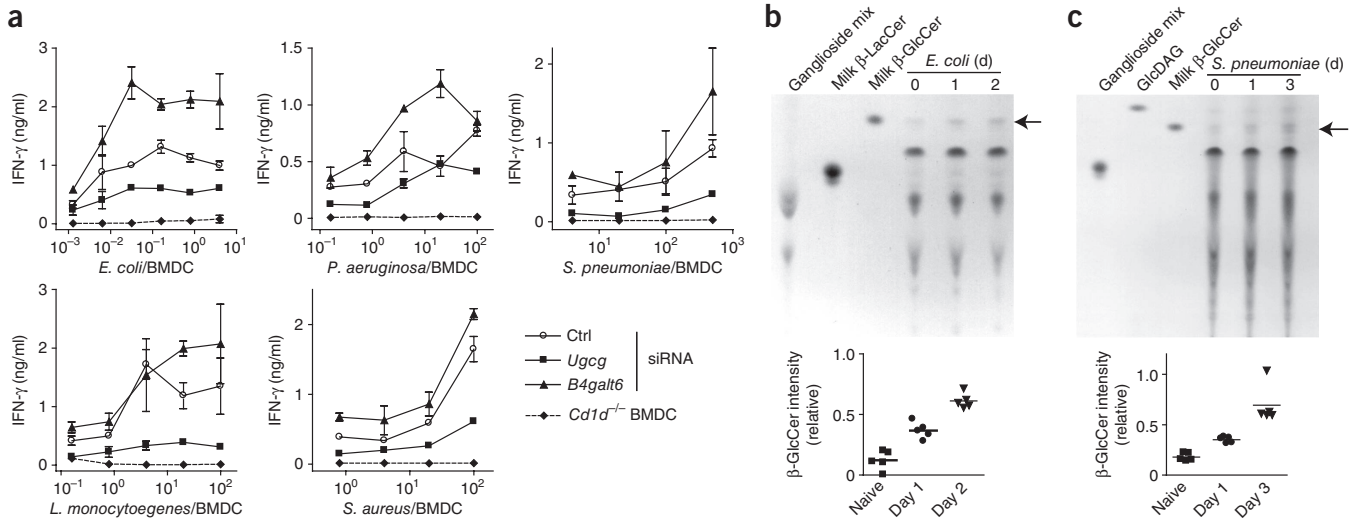


**Figure 8** β-GlcCer contributes to microbial activation of *i*NKT cells. (**a**) ELISA of IFN-γ production by a primary *i*NKT cell line cultured together with siRNA-treated CD11c$^+$ BMDCs (as in **Fig. 6c**) in the presence of heat-killed bacteria (horizontal axes), assessing activation. (**b**) TLC of polar lipid extracts from spleens collected on days 0–2 after intravenous infection of mice with *E. coli*. (**c**) TLC analysis of whole-lung lipid extracts on days 0,1 and 3 after intranasal infection with *S. pneumoniae*, with a solvent system that allowed separation of bacterial GlcDAG from β-GlcCer. Arrows indicate mobility of β-GlcCer. Below (**b**,**c**), densitometry of β-GlcCer bands; each symbol represents an individual mouse, and small horizontal lines indicate the mean. Data are representative of three separate experiments (**a**; mean and range of duplicate wells) or two experiments (**b**,**c**).

(**Supplementary Fig. 14**). Alteration in some lipid synthesis pathways has been shown to alter endosomal or lysosomal function and subsequently diminish *i*NKT cell activation[16]. For that reason, we used confocal fluorescence microscopy to assess the endosomal and lysosomal systems in CD11c$^+$ BMDCs after treatment with β-GlcCer synthesis inhibitor or siRNA. We saw no morphological abnormalities in the endosomal or lysosomal systems (**Supplementary Fig. 15**). We concluded from these studies that the presentation of β-GlcCer by CD1d is a substantial component of the TCR-mediated activation signal provided to *i*NKT cells by BMDCs after TLR agonist exposure.

Because bacteria might contain *i*NKT cell lipid antigens in addition to TLR agonists, we investigated the relative functional contribution of β-GlcCer as a self lipid antigen in the activation of *i*NKT cells by whole bacteria. *Ugcg* silencing resulted in less activation of *i*NKT cells in response to BMDCs cultured together with *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae* or *Listeria monocytogenes* (**Fig. 8a**). As we noted after LPS exposure in BMDCs, *B4galt6* silencing did not diminish *i*NKT cell activation. These results suggested that β-GlcCer contributes to *i*NKT cell activation during bacterial infection.

To extend the physiological importance of β-GlcCer accumulation in BMDCs after LPS exposure, we examined β-GlcCer concentrations *in vivo* during infection. As a model of Gram-negative bacterial sepsis, we transferred *E. coli* intravenously and assessed β-GlcCer in the spleen. At 24 h after *E. coli* injection, β-GlcCer increased as a portion of the total polar lipids and remained elevated at 48 h after injection (**Fig. 8b**). As a model of Gram-positive bacterial infection, we investigated *S. pneumoniae* pulmonary infection, a model in which *i*NKT cells have been shown to have a prominent role[11,36]. Because *S. pneumoniae* produces α-glucosyldiacylglycerol (GlcDAG), a lipid that co-migrates with β-GlcCer by TLC in some solvent systems, we modified our solvent system to separate these two lipids and included GlcDAG as a lipid standard. As we saw in the spleen after *E. coli* infection, we observed an increase in β-GlcCer concentrations in the total lung polar lipid extracts of mice infected with *S. pneumoniae* (**Fig. 8c**). This increase was prominent by day 3 after infection, corresponding to *i*NKT cell activation in this model[11]. We concluded from these studies that β-GlcCer accumulates in involved organs after bacterial infection.

## DISCUSSION

Although *i*NKT cells are considered to be innate lymphocytes, they use the machinery of the adaptive immune system to express TCRs of limited diversity. The nature of the specificity of these invariant TCRs, and how this specificity might regulate activation of an innate lymphocyte population, are fundamental in understanding the increasingly appreciated role of innate lymphocytes in immunity. Because *i*NKT cells are activated in contexts where foreign lipid antigens may not always be present to provide cognate TCR-mediated signals, self lipid antigens have been proposed to participate in this activation. Identification of the involved self lipid antigens has remained a central issue in the field[33]. We now find that β-GlcCer, the simplest GSL, acts as a physiologically relevant self lipid antigen for *i*NKT cells.

Here we have shown activity for both naturally occurring and synthetic β-GlcCer *in vitro* with mouse *i*NKT cell hybridomas, mouse and human primary *i*NKT cell lines and freshly isolated human *i*NKT cells. By injecting β-GlcCer intravenously, we showed activity *in vivo* on unperturbed mouse *i*NKT cells. β-GlcCer also activates in an APC-free system, as shown with plate-bound, lipid-loaded CD1d. As an additional important proof of principle, β-GlcCer–loaded CD1d tetramers specifically stain a subset of *i*NKT cells from both mouse and human directly *ex vivo*, providing strong evidence that the *i*NKT cell TCR can directly bind CD1d complexes loaded with β-GlcCer $C_{24:1}$. Based on these observations, we conclude that β-GlcCer activates both mouse and human *i*NKT cells through a direct cognate interaction between the *i*NKT cell TCR and β-GlcCer–loaded CD1d.

In mammals, GSLs have diverse structures, varying in both the fatty acyl chain and the carbohydrate head group. Examination of each of these structural features for β-GlcCer provides important insight into the crucial determinants of *i*NKT cell self antigenicity. We found evidence that the activity of β-GlcCer varies with *N*-acyl chain structure. Such differences also have been observed for α-GalCer variants and have been attributed to the kinetics, stability and subcellular location of antigen loading[23,24,37]. Notably, β-GlcCer $C_{24:1}$, the most potent β-GlcCer variant tested for *i*NKT cells, is the specific form found in the highest abundance in mammalian lymphoid tissues.

Various approaches have been used to clarify the contribution of the lipid head group in *i*NKT cell activation. Two informative studies looked at the role of the *i*NKT TCR complementary-determining region 3 β-loop (CDR3β) in *i*NKT cell autoreactivity. Research from one study has shown that naturally occurring, high-affinity human *i*NKT cell TCR interactions with CD1d are dependent on TCR CDR3β and that β-GlcCer–loaded tetramers, similar to OCH-loaded tetramers, could discriminate between high- and low-affinity *i*NKT cell TCRs[32]. Research from a second study examined a particularly autoreactive $V_\alpha14$-$V_\beta6$ *i*NKT TCR generated by random mutagenesis of mouse CDR3β and showed that multiple cellular lipids can influence the binding of this TCR to CD1d, either positively or negatively[29]. Although several higher-order GSLs resulted in less binding relative to unloaded tetramer, β-GlcCer and β-GalCer did not have this effect[29]. These studies, although not specifically implicating monohexosyl ceramides as *i*NKT self antigens, are consistent with the idea that β-GlcCer might support *i*NKT cell TCR binding to CD1d for autoreactive *i*NKT cells.

Comparison of co-crystal structures of CD1d–β-GSL–TCR with the reported CD1d–α-GalCer–TCR structure[38] has shown that the primary β-linked sugar is 'molded' by the TCR into a configuration similar to that seen with α-GalCer, providing a structural explanation for the antigenic activity of β-linked lipids[39,40]. In addition to iGB3, other antigenic primary β-linked GSLs have been described, including β-mannosylceramide and β-GalCer $C_{12:0}$ (refs. 26,27,41). Together with our data, such reports suggest that CD1d-bound, β-linked monohexosyl ceramides can fulfill the structural requirements of a self antigen for *i*NKT cell TCR. The 'energetic penalty' incurred with altering the conformation of a monohexosyl ceramide bound to CD1d to adopt a topology similar to that of α-GalCer is probably lower than that required for more complex GSLs, and our data suggest that the acyl chain composition of the lipid may also have a role in this process.

In addition to fulfilling structural requirements for antigenicity, a physiologically relevant self antigen must be present at sites of *i*NKT cell activation. As with iGB3, β-GalCer and β-mannosylceramide would not be found in any substantial amounts at most sites of peripheral *i*NKT cell activation. We show that β-GlcCer $C_{24:1}$, however, is detectable in lymphoid tissues, accumulates during infection and, therefore, is likely to have a physiological role. This is of particular importance, as elution studies have shown that CD1d presents lipids representative of the total cellular lipid profile or compartment surveyed[21,42,43].

Several lines of evidence have suggested the possibility that GSLs contribute to *i*NKT cell self reactivity, although GSLs may not be the only lipid class contributing to *i*NKT cell autoreactivity[25]. Notably, an *i*NKT-cell–dependent immunomodulatory or inhibitory role

has been reported for β-GlcCer *in vivo*[44]. Through the use of GSL synthesis inhibitors, published studies have indirectly indicated that GSLs are important *i*NKT cell antigens in BMDCs after TLR agonist stimulation[9,10,20]. Our identification of a specific antigenic β-GSL, β-GlcCer, allowed us to target the pathways involved in the synthesis of that lipid. Silencing of *Ugcg*, the gene that encoded β-GlcCer synthase, resulted in less self-reactivity and lower response of *i*NKT cells to BMDCs in the presence of LPS or whole bacteria. Targeting *B4galt6*, which encodes the main enzyme involved in the conversion of β-GlcCer to β-LacCer, increased self reactivity as well as the response of *i*NKT cells to LPS and some bacteria, likely as a result of the observed accumulation of β-GlcCer. Our siRNA silencing results, controlled for antigen presentation, IL-12 production, APC activation status and endosomal morphology, strongly suggest that β-GlcCer has an important role in the CD1d-dependent signal in APCs during many infections. Additionally, β-GlcCer has been reported to accumulate in spleen, serum and liver of LPS-exposed rodents[45], suggesting that endosomal uptake of systemically circulating β-GlcCer by APCs could provide another source of antigen. Thus, the induction of GSL biosynthesis, both in antigen-presenting cells and systemically, provides a mechanism for danger sensing by *i*NKT cells, which is mediated by β-GlcCer.

Two signals have an important role in *i*NKT cell activation during microbial infection: the first is a lipid antigen presented by CD1d to the *i*NKT cell TCR, and the second is an inflammatory cytokine, such as IL-12. We propose that β-GlcCer, a self antigen that accumulates after APC activation, provides a major TCR signal for *i*NKT cells. The ability of an *i*NKT cell to be activated by the integration of APC-dependent innate signals explains how these cells, with an invariant TCR, can be activated in multiple pathologic contexts in the absence of foreign lipid antigens. The recognition of the self antigen β-GlcCer by an invariant TCR is a clear example of the translation of an innate danger signal with the machinery of the adaptive immune system, a mechanism that may apply to other innate T lymphocytes.

## METHODS

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

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

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### AUTHOR CONTRIBUTIONS

P.J.B. and R.V.V.T. conceived of, did and interpreted data from the experiments; P.J.B. was the main author of the manuscript; M.B., A.T., F.-F.H., J.P.S., S.D.G. and E.Y.K. assisted with the experimental design and data interpretation, did experiments and edited the manuscript; G.S.B. assisted with the design of the experiments and synthesized key materials; and M.B.B. assisted with the design of the experiments and data interpretation, supervised the research and substantially contributed to the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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## ONLINE METHODS

**Mouse and human subjects.** C57BL/6 and BALB/c mice were from Jackson Laboratories. *Cd1d*$^{-/-}$ mice on a C57BL/6 background were provided by M. Exley. Animal studies were approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Human peripheral blood was obtained from healthy donors in accordance with approval by the Brigham and Women's Hospital institutional review board.

**Mouse *in vitro* NKT cell assay.** For all *in vitro* coculture assays, cells were incubated for 14–18 h in 96-well flat-bottomed plates. The *i*NKT cell hybridoma DN32 and CD1d-transfected RAW macrophage cells have been described[21,46] and were used at a density of $5 \times 10^4$ cells per well. The generation of CD11c$^+$ BMDCs and primary *i*NKT cell lines have also been described[11,47]. The *i*NKT cells were used at a density of $2.5–5 \times 10^4$ per well at a ratio of 5:1 with BMDCs unless otherwise noted. IL-12 was from PeproTech. LPS from *Salmonella abortus equi* was from Sigma-Aldrich. The preparation of heat-killed bacteria and the strains used have been described[11]. NB-DGJ (Calbiochem) was used at a concentration of 50 μM. D-PDMP (Matreya) was used at a concentration of 10 μM.

**Human *in vitro* *i*NKT cell assays.** Human *i*NKT cell clones have been described[31]. Human primary *i*NKT cell lines were generated by population expansion from freshly isolated PBMCs in IL-2 (50 U/ml; Novartis) and IL-15 (5 ng/ml; PeproTech) in culture for 14 d with α-GalCer (10 ng/ml). The *i*NKT cells were purified by selection with magnetic beads (Miltenyi Biotec) and were >99% positive for binding to the PBS-57–tetramer. The generation of human PBMC-derived monocytes has been described[31]. The *i*NKT cells ($5 \times 10^4$) were cultured with PBMC-derived monocytes ($5 \times 10^4$ per well).

**Intravenous injection of lipids.** Lipids for injection were dried under nitrogen and sonicated in PBS at pH 7.4 and 0.05% (vol/vol) Tween-20, and 200 μl of this mixture was injected into the tail veins of mice. For analysis of cytokine elaboration, mice were killed 1 h after injection of α-GalCer and 2 h after injection of all other lipids. Livers were collected after perfusion with ice-cold PBS, mechanically dissociated and passed through a 70-μm filter before isolation of mononuclear cells by density centrifugation with Histopaque (Sigma-Aldrich).

***In vivo* infection.** *E. coli* (American Type Culture collection stain 25922) was injected intravenously at a dose of $6 \times 10^3$ colony-forming units per mouse as determined by limiting dilution on lysogeny broth agar plates. *S. pneumoniae* (stain URF918) was provided by K. Kawakami; growth and intranasal infection used have been described[11]. For *S.pneumoniae* infection, $1.5 \times 10^3$ colony forming units per mouse were used, as determined by limiting dilution on Todd-Hewitt agar plates containing 5% (vol/vol) sheep red blood cells.

**Antibodies and flow cytometry.** Data were acquired with a FACSCanto II system (BD Biosciences) and analyzed with FlowJo (TreeStar). Doublets were excluded with FSC-A and FSC-H linearity. Mouse antibodies were as follows (all from BD Biosciences): anti-TCRβ (H57-597), anti-CD3 molecular complex (17A2), anti-CD1d (1B1), anti-$V_\beta$2 (B20.6), anti-$V_\beta$6 (RR4-7), anti-$V_\beta$7 (TR310), anti-$V_\beta$8.1-8.2 (MR5-2), anti-$V_\beta$8.3 (1B3.3), anti-$V_\beta$9 (MR10-2), anti-$V_\beta$10b (B21.5), anti-$V_\beta$14 (14-2), anti-IFN-γ (XMG1.2) and anti-CD3ε (145-2C11). Human antibodies were as follows: anti-CD3ε (UCHT1; BD Biosciences), anti-$V_\alpha$24 (C15; Immunotech) and anti-$V_\beta$11 (C21; Immunotech). For ELISA, mouse anti-IFN-γ and anti-IL-4 sets (BD Biosciences) and human anti-IFN-γ sets (Pierce) were used. Monoclonal anti-CD1d (42.1) has been described[48], and mouse IgG1 isotype-matched control antibody (554121) was from BD Biosciences. Human and mouse PBS-57-loaded CD1d tetramers were from the NIH tetramer facility. Mouse IFN-γ and IL-4 cytokine-capture assays were done according to the manufacturer's instructions (Miltenyi Biotec). β-GlcCer tetramer binding was done at 25 °C for 30 min in PBS, 0.5% FBS and 2 mM EDTA, followed by the addition of PBS-57-loaded tetramer and lineage markers for 10 min and washing before sample acquisition.

**CD1d loading and tetramer assembly.** Mouse and human biotinylated CD1d were from the NIH tetramer facility. For loading, lipids were dried under nitrogen, sonicated in 0.05% (vol/vol) Tween-20 for mouse CD1d or 0.025% (vol/vol) Triton X-100 for human CD1d and incubated overnight at 37 °C with CD1d. Mock-loaded, biotinylated bovine serum albumin (Sigma-Aldrich) was used as a control for plate-bound CD1d assays. A molar loading ratio (lipid to CD1d) of 50:1 (plate-bound CD1d assays) or 200:1 (tetramerization studies) was used. For tetramerization, streptavidin-phycoerythrin (Invitrogen) was added to lipid-loaded CD1d (at a molar ratio of 1:4.5). For plate-bound CD1d assays, 0.25 μg of loaded CD1d was added to each well of a 96-well streptavidin-coated plate (Thermo Scientific), bound at 25 °C for 30 min and washed extensively before the addition of *i*NKT cells.

**Lipids.** The production of α-GalCer, OCH, Gal–α-GalCer and GlcDAG has been described[24,49]. β–D-GlcCer $d_{18:1}$-$C_{24:1}$(15Z), $C_{18:1}$ (9Z), $C_{18:0}$, $C_{16:0}$, $C_{12:0}$ and $C_{8:0}$ as well as β–D-GalCer $d_{18:1}$-$C_{24:1}$(15Z), $C_{12:0}$ and phospholipids were from Avanti Polar Lipids. iGB3 ($d_{18:1}$-$C_{26:0}$) was from Enzo Life Sciences. *Sphingomonas* GSL-1 was provided by the NIH tetramer facility. All other lipids were from Matreya.

**TLC and mass spectrometry.** Lipid extraction and analysis by TLC has been described[50]. Lipid fractions were separated on silica TLC plates (EMD Chemicals) and visualized with α-naphthol. For most TLC, the mobile phase was 60:30:6 $CHCl_3$:$CH_3OH$:$H_2O$ (vol/vol/vol). For discrimination of GlcDAG and β-GlcCer, the mobile phase was 65:25:3.7 $CHCl_3$:$CH_3OH$: $H_2O$ (vol/vol/vol). Adobe Photoshop was used for densitometry analysis of TLC plates. The relative intensity value for each query band was calculated compared with that a standard band and was normalized to the total intensity of each lane. Densitometric quantification of β-GlcCer in spleen, thymus and BMDC polar lipid extracts was done by extrapolation from a best-fit line to a β-GlcCer standard curve. Mass spectrometry analysis, including low-energy collisionally activated dissociation multiple mass spectrometry, was done on a linear ion-trap mass spectrometer (Thermo Finnigan) with an Xcalibur operating system as described[11].

**Quantitative PCR analysis.** RNA was extracted with the RNeasy system (QIAGEN), and cDNA was synthesized with the QuantiTect system (QIAGEN). Brilliant SYBR Green qPCR Master Mix (Agilent Technologies) and the Stratagene MX3000P system were used for quantitative PCR (primer sequences, **Supplementary Methods**).

**Silencing mediated by siRNA.** Pooled siRNA targeting *Ugcg* or *B4galt6* and control siRNA were from Thermo Scientific. CD11c$^+$ sorted BMDCs ($1 \times 10^6$) were transfected with 1 μM siRNA through the use of a Nucleofector II (program Y-001; Lonza). Then, BMDCs were allowed to 'rest' for 24–48 h in low-adherence Sumilon Celltight culture dishes before use (Sumitomo Bakelite).


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