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Diverse Endogenous Antigens for Mouse Natural Killer T Cells: Self-Antigens That Are Not Glycosphingolipids

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Abstract

Natural killer T cells with an invariant antigen receptor (*i*NKT cells) represent a highly conserved and unique subset of T lymphocytes having properties of innate and adaptive immune cells. They have been reported to regulate a variety of immune responses, including the response to cancers and the development of autoimmunity. The development and activation of *i*NKT cells is dependent on self-antigens presented by the CD1d antigen-presenting molecule. It is widely believed that these self-antigens are glycosphingolipids (GSLs), molecules that contain ceramide as the lipid backbone. Here we used a variety of methods to show that mammalian antigens for mouse *i*NKT cells need not be GSLs, including the use of cell lines deficient in GSL biosynthesis and an inhibitor of GSL biosynthesis. Presentation of these antigens required the expression of CD1d molecules that could traffic to late endosomes, the site where self-antigen is acquired. Extracts of antigen-presenting cells (APCs) contain a self-antigen that could stimulate *i*NKT cells when added to plates coated with soluble, recombinant CD1d molecules. The antigen(s) in these extracts are resistant to sphingolipid-specific hydrolase digestion, consistent with the results using live APCs. Lyosphosphatidylcholine, a potential self-antigen that activated human *i*NKT cell lines, did not activate mouse *i*NKT cell hybridomas. Our data indicate that there may be more than one type of self-antigen for *i*NKT cells, that the self-antigens comparing mouse and human may not be conserved, and that the search to identify these molecules should not be confined to GSLs.

Keywords

endogenous antigen; CD1d; NKT; glycosphingolipid-deficient cells

Introduction

Natural killer cells (NKT) cells are a unique population of innate-like or natural memory T lymphocytes that exhibit the properties of both conventional T cells and NK cells (1, 2). The majority of NKT cells express highly restricted T cell receptors (TCR) with an invariant α

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chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) and limited β chains (Vβ11 in humans and Vβ8, Vβ2, Vβ7 in mice). Therefore they are referred to as invariant NKT cells (*i*NKT) (1-4). Following TCR stimulation, *i*NKT cells can rapidly secrete large amounts of Th1 and Th2 cytokines to activate various immune cells, such as natural killer cells, neutrophils, and macrophages. *i*NKT cells amplify innate immune responses and they are believed to play a central role in bridging innate and adaptive immune responses during microbial infections. They are also reported to be involved in the suppression of tumors and the prevention of autoimmune diseases (1, 2, 5, 6).

*i*NKT cells recognize lipid antigens presented by CD1d, a MHC class I-like antigen (Ag) presenting molecule. After synthesis and transport to the cell surface, mouse CD1d molecules (mCD1d) are internalized and directed to late endosomes and lysosomes by a tyrosine-containing sequence motif in their cytoplasmic tails (7-9). This intracellular trafficking and location are required for the presentation of exogenous Ags with complex oligosaccharides, such as those that have to be processed in lysosomes to remove the terminal sugar(s) (10). The lysosomal environment also assists in the presentation of Ags that do not require processing (11-13).

α-galactosyl ceramide (αGalCer), a synthetic glycosphingolipid (GSL) that has some minor structural modifications from a compound originally isolated from a marine sponge, is the most well-known Ag for *i*NKT cells (14). Several groups have shown that a monosaccharide-containing GSL from *Sphingomonas spp.* bacteria was a natural Ag that could bind CD1d and stimulate the TCR of *i*NKT cells (15-17). Because Sphingomonas are ubiquitous, this suggested that αGalCer has a bacterial origin. Subsequently, further investigation of natural variants of GSL Ags from Sphingomonas with different carbohydrate and lipid structures showed they varied greatly in antigenic potency (18, 19). Regardless, these Sphingomonas bacteria are not highly pathogenic.

Glycodiacylglycerols, found in *Borrelia burgdorferi* which causes Lyme disease, also subsequently were shown to exhibit antigenic activity for *i*NKT cells (20). This established that not only are the glycolipid Ags for *i*NKT cells found in pathogenic organisms, but that they do not need to be GSLs, which are found only in Sphingomonas. All bacterial glycolipid Ags identified so far have in common a hexose sugar in α linkage, but bound to either a ceramide or diacylglycerol lipid. Mammalian GSLs, by contrast, have a β linkage, which is not highly antigenic.

While microbial Ags for *i*NKT cells are now becoming characterized, the nature of the self-antigen(s) that activate these cells is less well understood. There is evidence, however, that the endogenous self-antigens (self-Ags) are loaded onto mCD1d in lysosomes (8, 9). The mammalian GSL isoglobotrihexosyl ceramide (iGb3), formed in lysosomes, can activate mouse and human *i*NKT cells, although its abundance in the thymus and other important sites, and its importance for *i*NKT cells, remain controversial (17, 21, 22). Here, we employed mutant cell lines, which have defects in *de novo* synthesis of GSLs, to study the characteristics of self-Ag. Our results indicate that, similar to microbial Ags, the self-Ags for mouse *i*NKT cells have a diverse nature and they do not need to be GSLs.

Materials and Methods

Reagents and mice

αGalCer (10, 14, 16, 23) and GM-CSF were kindly provided by Kyowa Hakko Kirin Co., Ltd., and Galα(1-2)αGalCer (10) was provided by Dr. Chi-Huey Wong (Scripps Research Institute, La Jolla). Glutaraldehyde, ganglioside GM1 (GM1), ceramide, gentamicin, fatty acid-free BSA, and sodium oleate were purchased from Sigma-Aldrich (St. Louis, MO).

Nutridoma-SP was obtained from Roche (San Francisco, CA). *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) and high performance thin layer chromatography (HPTLC) silica gel 60 were from Calbiochem (San Diego, CA) and VWR (Lutterworth, UK), respectively. LysoPCs (synthetic C16:0, C18:0, C18:1, purified from chicken egg or from soy) were from Avanti Polar Lipids (Alabaster, AL). The hybridoma producing the 3C11 mouse CD1d blocking antibody was kindly provided by Dr. Jonathan Yewdell (NIH, Bethesda, MD). Two lines of Vα14 transgenic mice were kind gifts, one from Dr. Agnes Lehuen (University of Paris, France) (24) and the other from Dr. Albert Bendelac (University of Chicago) (25). C57/B6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions and the experiments were approved by the Clinical Medicine Ethical Review Committee, University of Oxford or the Institutional Animal Care and Use Committee of the La Jolla Institute of Allergy and Immunology.

Cell lines and culture conditions

LY-B cells mutant for GSL biosynthesis and LY-B/cLCB1 cells with biosynthesis restored by gene transfection are derivatives of Chinese hamster ovary (CHO) cells, and were kind gifts from Dr. Kentaro Hanada (National Institute of Infectious Diseases, Tokyo, Japan) and were described previously (26). CHO-K1 cells and B cell lymphoma A20 cells were obtained from the American Type Culture Collection (Rockville, MD). Tail-deleted mouse CD1d was generated by PCR amplification using the following primers: 5′-GAATTCACCATGGCCATGCGGTACCTACCATGGC -3′ (forward) and 5′-GTCGACTCACCAGATATAGTAGACTACAGCACCC -3′ (reverse) with wild type mCD1d as template. The PCR products were digested by EcoRI and SalI, and subcloned to a retroviral plasmid pBABE(puro). Stable transfectants of wild-type or tail-deleted mCD1d were established by retroviral transfection and drug selection, as described before (27). *i*NKT cell hybridomas 1.2, 1.4, 2C12 and 24 (28), 24.7 and 24.9E (29) were characterized previously.

The mCD1d and vector transfectants of LY-B, LY-B/cLCB1 and CHO-K1 cells were maintained in complete medium (Ham's F12 medium supplemented with 2 mM L-glutamine, 100 μg/ml each of penicillin and streptomycin and 10% FBS). To remove accumulated GSLs, 1×10^6 cells were plated in a 100 mm tissue culture plate and cultured in complete medium for 2 days, and then the cells was washed with serum-free medium twice followed by a five-day cultivation in sphingolipid and serum-free medium (Ham's F12 medium with 1% Nutridoma-SP, 10 μM sodium oleate-bovine serum albumin complex and 10 μg/ml gentamicin). A20 and A20/CD1d, 1.2, 1.4 and 2H4 cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 μg/ml each of penicillin and streptomycin, 50 μM β-mercaptoethanol and 10% FBS. 24.7 and 24.9E were cultured in RPMI-1640 with 10 mM HEPES, pH7.2-7.4, 2 mM L-glutamine, 100 μg/ml each of penicillin and streptomycin, 50 μM β-mercaptoethanol, essential amino acids, non-essential amino acids and 10% FBS.

The mouse B16 melanoma cell line, GM95, deficient in ceramide glucosyltransferase (30) was grown in RPMI+10% FBS (R10 medium). The GluCerT cell line, in which the genetic defect has been restored by transfection with a ceramide glucosyltransferase cDNA (31) was grown in R10 medium supplemented with 50 μg/ml hygromycin B. Both cell lines were infected with lentivirus vectors encoding mCD1d (32). Transduced cell lines were then sorted based on mCD1d expression. The GM95 cells were originally obtained from Dr Y. Hirabayashi, RIKEN, Japan and the stable GluCerT line was provided by Dr. Gerrit van Meer (University of Amsterdam, Netherlands) (33). Control experiments were carried out to confirm that GM95 cells were defective in GSL biosynthesis, while GluCerT cells expressed

GSLs, including GM3, confirming the activity of the ceramide glucosyltransferase (data not shown).

For *N*B-DGJ treatment, A20 or A20/CD1d cells were cultured for three days in medium containing 100 μM *N*B-DGJ or the equivalent volume of vehicle (methanol).

Flow cytometry

Cells were washed and blocked in staining buffer (PBS, 2% BSA, 10 mM EDTA and 0.1% sodium azide) containing anti-FcR antibody 2.4G2 for 30 min at 4°C, then stained with fluorophore conjugated antibodies (BD PharMingen). After washing and fixation with PBS, 1% paraformaldehyde and 0.1% sodium azide, cells were analyzed on a FACSCalibur or a LSR II flow cytometer (BD Bioscience), and the data processed using FlowJo software.

GSL purification and analysis

GSL extraction and purification was performed as described previously (34). Purified lipid extracts were subjected to ceramide glycanase digestion, purified from the leech *Hirudo medicinalis*, as described (35). The released GSL-derived oligosaccharides were labeled with anthranilic acid (2AA) as described previously (36). Samples were analyzed by normal phase HPLC (NP-HPLC) using a Waters 2695 separations module and a 2475 fluorescent detector with solvents and gradient conditions as previously described (34). Exoglycosidase digestion was performed with sialidase A and Jack Bean β-hexosaminidase (both from ProZyme, Inc.). GSL-derived 2AA labeled oligosaccharides were dried in a ScanVac and resuspended in the supplied digestion buffer with 50 mU of enzyme at 37°C for 48 h. Samples were made up to 100 μl volume with water and spun for 30 min at 4,000 × *g* through a pre-washed 10,000 MW centrifugal filter (Millipore). The filter was washed with a further 100 μl of water and the sample dried prior to normal phase-HPLC analysis. Quantization of samples was performed as previously described (34).

Mouse iNKT cell sorting

Freshly isolated spleens were mashed and passed through 70 μm cell strainers (Fisher Scientific, Pittsburgh, PA) to give a single cell suspension. Cells from three spleens were pooled and red blood cells were removed by lysis. The splenocytes positive for mCD1d/αGalCer tetramer staining were sorted in FACSAria cell sorter (BD Biosciences).

Antigen presentation assays

GM95 and GluCerT targets were plated at titrated cell numbers/well in 96 well plates. The next day, mouse CD1d/αGalCer tetramer positive splenocytes from Vα14 transgenic mice (24) were added at 2×10^4 cells/well. For the mouse CD1d blocking experiments, 3C11 antibody diluted at 20μg/ml was added to the targets for 1 h prior to the addition of the tetramer positive splenocytes. After 24 h, supernatants were harvested and the concentration of mouse IFNγ was determined by ELISA according to the manufacturer's instructions (Mabtech, Sweden).

For CHO cell self-Ag presentation, transfectants of LY-B, LY-B/cLCB1 and CHO-K1 cells expressing wild-type, tail-deleted mCD1d or vector that were cultured in complete medium or in sphingolipid and serum-free medium for five days were collected, washed and seeded in 96-well plates at the indicated concentrations. The hybridomas were washed in serum-free medium twice, and then mixed with the APCs overnight, while the sorted mouse *i*NKT cells, obtained as described above, were cultured with the APCs for two days. For antibody blockade, 20 μg/ml 1B1 antibody against mCD1d or isotype control (eBiosciences) were added to APCs before they were cultured with the hybridomas. A20 and A20/CD1d cells that were treated with *N*B-DGJ for 3 days were fixed in 0.05 % glutaraldehyde, then

neutralized with 2 mM L-lysine and washed before culture with hybridoma cell line 24.7. Cytokine (IL-2 or IFNγ) release was evaluated in a sandwich ELISA using monoclonal antibodies (BD PharMingen and R&D Systems, respectively).

To assess *i*NKT cell activation by Ag(s) present in CHO cells, LY-B and LY-B/cLCB1 transfectants expressing wild-type mCD1d or vector, which had been cultured in sphingolipid and serum-free medium for five days, were inoculated at 3×10^4 cells/well in 96 well plates and purified mouse spleen *i*NKT cells were added at 3×10^4 cells/well. 24 h later, cells were collected and stained for flow cytometry analysis.

For αGalCer presentation, 1×10^6 CHO-K1 cells expressing wild type, tail-deleted mCD1d or vector alone were pulsed with the indicated amounts of αGalCer or vehicle (56 mg/ml sucrose, 7.5 mg/ml histidine and 5 mg/ml Tween-20, pH7.2) overnight. Then the cells were washed, and 1×10^5 APCs were cultured in the presence of 1×10^5 hybridomas per well in a 96-well plate for 20-24 h. IL-2 production in the supernatants was measured as above. In the lysoPC Ag presentation experiments, 1×10^6 A20/CD1d cells were pulsed with vehicle, αGalCer or indicated amounts of different sources of lysoPC overnight.

For analyzing Galα(1-2)αGalCer presentation by *N*B-DGJ treated A20/CD1d cells, 1×10^6 A20/CD1d cells expressing wild type mCD1d were cultured in complete medium containing 100 μM *N*B-DGJ or vehicle (methanol), in which *N*B-DGJ was dissolved for 3 days. On the second day of culture, the indicated amounts of Galα(1-2)αGalCer or vehicle was added to pulse the cells overnight. On the third day, A20/CD1d were collected, washed thoroughly and mixed with *i*NKT cell hybridomas as described above. Supernatants were collected after 20-24 h for IL-2 ELISA assay.

Cell-free antigen presentation assay

CD1d transfectants of LY-B, LY-B/cLCB1 and CHO-K1 cells, after a five-day cultivation in sphingolipid and serum-free medium, or A20/CD1d from a standard culture, were collected and washed with PBS twice. The cells were resuspended in deionized water at 10×10^6 cells/ml and sonicated (1 min in ice for 3 times). The indicated amounts of αGalCer or crude sonicates diluted in PBS were incubated for 24 h in 96-well plates that had been coated previously with 1.0 μg per well purified mCD1d. After washing, 5×10^4 Vα14*i* NKT cell hybridoma cells were cultured in the plates for 20 h, and IL-2 in the supernatant was measured by ELISA. For CD1d blockade, 20 mg/ml 1B1 antibody or isotype control were mixed with the sonicates before they were plated in the CD1d coated wells.

Chromatography of glycolipids

The indicated amounts of GM1 were incubated with or without 2 mU rEGCase II for 2 hours at 37°C. Ceramide release by the enzyme was analyzed by HPTLC as described before (37). Briefly, the reaction was terminated by adding 5 volumes of chloroform/methanol (2:1, v/v). The mixture was vortexed and centrifuged at 7000 × *g* for 5 min to separate the organic and aqueous phases. The organic phase was evaporated under nitrogen airflow and then dissolved in 5 μl chloroform/methanol (2:1, v/v). The organic phase was analyzed by HPTLC using chloroform/methanol (9:1, v/v) as the developing solvent. The ceramide was revealed by staining the plate with Coomassie Brilliant Blue R-250 (38).

20 mU rEGCase II, 100 mU SCDase or reaction buffer as control was incubated overnight at 37 °C with 5 μg GM1 standard or 500 μl CHO-K1/CD1d cell sonicate, prepared as described above from 5×10^6 cells. Then the enzyme treated sonicates or the control GM1 standard was dried by centrifugal evaporation. GM1 was extracted by the addition of 200 μl chloroform: methanol (2:1, v:v) and vigorous mixing. Protein debris was removed by centrifugation (13,000 × *g*, 5 min) and the lipid containing supernatant was dried under a

stream of nitrogen. The lipids were resuspended in 15 μl chloroform: methanol (2:1, v:v) before application to a HPTLC plate (12 × 20 cm silica gel 60). In addition, standards (neutral and monosialoganglioside TLC qualimix, Mattreya) were applied to the plate. The plate was developed in a solvent system consisting of chloroform: methanol: 0.22% calcium chloride di-hydrate (65:35:8, v/v/v) for 8 cm. The plate was allowed to air dry before spraying with orcinol reagent to specifically stain carbohydrate (0.2% in 2N sulphuric acid). Once dried, the plate was heated to 80 °C until the bands appeared purple. The enzyme treated or control cell sonicates were first purified by C18 solid phase extraction columns as described above. The purified lipids were dried and 1×10^6 cell equivalents were loaded onto HPLTC plates and analyzed as for the GM1 standard (1 μg).

Enzymatic hydrolysis

Vehicle (deionized water), 20 ng αGalCer, 1 μl CHO-K1/CD1d cell sonicate or 2 μl A20/CD1d sonicate were incubated with 1-4 mU rEGCase II or SCDase (Takara Bio Inc., Japan) or reaction buffer overnight at 37°C. After the reaction solutions were boiled for 5 min to inactivate the enzymes, the ability of the sonicates and controls to stimulate *i*NKT cell hybridomas was measured in CD1d coated plate assays.

In vivo iNKT cell activation and response

Lipid antigen pulsed bone marrow derived dendritic cells (BMDCs) were injected to mice as described before (16, 20). Briefly, mouse DCs were collected from femurs and tibias, cultured in RPMI 1640 medium supplemented with glutamine, penicillin and streptomycin, β-mercaptoethanol, 10% FBS, 10 ng/ml GM-CSF for six days, followed by incubation with 10 ng/ml αcGalCer or 20 μg/ml synthetic lysoPC (C18:0) or 20 μg/ml purified lysoPC (from soy) for 24 h. After washing with PBS, these Ag pulsed DCs (5×10^5 per mouse) were injected intravenously into mice. 4 and 14 h post injection, blood was collected and IL-4 and IFNγ in serum were determined by ELISA (BD PharMingen and R&D Systems, respectively). Splenocytes and liver cells positive for mCD1d/αGalCer tetramer were analyzed ex vivo 14 h after injection for intracellular cytokine production.

Results

Endogenous antigen presentation in ceramide glucosyltransferase deficient cells

In the GSL biosynthesis pathway, ceramide glucosyltransferase (EC 2.4.1.80) and galactosyltransferase are two key enzymes that synthesize the two precursors of all complex GSLs, β-glucosylceramide and β-galactosylceramide, respectively. Previous work showed that the galactosyltransferase-deficient mice had normal *i*NKT cell development and frequency, which indicates that β-galactosylceramide could not be the sole or major self-Ag or a non-redundant precursor of the self-Ag (39). In fact, most GSLs are derived from β-glucosylceramide, but due to the lethality of ceramide glucosyltransferase deficiency in mice, *i*NKT cell differentiation in the absence of this enzyme could not be analyzed *in vivo*. The B16 melanoma cell line, GM95, is deficient in ceramide glucosyltransferase, and therefore could be employed as an *in vitro* model for analyzing the effects of deficiency for β-glucosylceramide and its derivatives (30). The defect was rescued in these cells by introducing the wild type ceramide glucosyltransferase cDNA, and the restored GM95 cells, called GluCerT cells herein, were used as controls (31). Additional control experiments were carried out to confirm that GM95 were defective in GSL biosynthesis, while GluCerT cells had a restored content of ganglioside GM3 (GM3), demonstrating the activity of ceramide glucosyltransferase (data not shown). Both cell lines were transfected with lentivirus vectors encoding mCD1d. There is a similar CD1d expression level on the cell surface of the ceramide glucosyltransferase deficient and enzyme restored transfectants (data not shown). Furthermore, there was no significant difference in the ability of mCD1d

transfected GM95 and GluCerT cells to present the synthetic GSL antigen Galα(1-2)αGalCer (data not shown). This compound requires internalization and lysosomal carbohydrate processing to remove the distal galactosyl group in order to generate a stimulatory epitope for *i*NKT cells (10). These data indicate that the absence of ceramide glucosyltransferase has no effect on the surface expression of mCD1d, its intracellular trafficking, or on the internalization and processing of exogenous GSL antigens.

The auto-reactivity of mouse CD1d/αGalCer tetramer positive splenocytes from Vα14 transgenic mice for the transfected GM95 and GluCerT cells was determined by culture with purified *i*NKT cells from spleen and measurement of IFNγ synthesis by ELISA. As expected, in the absence of CD1d expression in either the GM95, or restored GluCerT cell lines, there was no induction of IFNγ production following culture with Vα14 transgenic splenocytes (Fig. 1A). By contrast, expression of mCD1d in these cells was sufficient to activate the *i*NKT cells. Interestingly, compared with the GluCerT transfectants with restored β-glucosylceramide synthesis, titrations of GM95 cells stably expressing mCD1d induced nearly equal amounts of IFNγ synthesis. This indicates that β-glucosylceramide deficiency in GM95 cells did not lead to defective endogenous antigen biosynthesis and presentation. Furthermore, the introduction of a mCD1d specific antibody resulted in a greatly reduced auto-reactivity of mouse *i*NKT cells, clearly showing that the self-Ag presentation is mediated by the mCD1d molecule (Fig. 1B).

Transfection of mouse CD1d to Chinese hamster ovary (CHO) cell lines

Serine palmitoyltransferase (SPT) is a key enzyme for the regulation of cellular sphingolipids by initiating their biosynthesis (40). Two gene products, sphingosine long chain base subunit 1 and 2 (LCB1 and LCB2), are required for the catalytic activity of SPT. LY-B cells, having a mutation in LCB1, were derived previously from CHO-K1 cells (26). The mutation results in dramatically reduced sphingomyelin and GSLs because of defective *de novo* sphingolipid synthesis (26, 41). This defect has been rescued in LY-B/cLCB1 cells by introducing a human LCB1 cDNA to the parental LY-B cell line (26, 41). The restored cells do not have a significant difference in GSLs from those of CHO-K1 cells ((26, 41) and data not shown). In order to see if this biosynthetic deficiency in GSLs has an effect on endogenous Ag presentation, all these three cell lines were transfected with mCD1d cDNA and selected by puromycin to generate stable transfectants. mCD1d expression on the cell surface was determined by flow cytometry. As shown in Fig. 2, the LCB1 mutant LY-B cells, the restored cells (LY-B/cLCB1) and the wild type cells (CHO-K1) expressed an equivalent and high amount of mCD1d, while the control vector transfectants were negative for surface mCD1d expression.

Biochemical analysis of GSL content

The mCD1d transfectants were grown in sphingolipid and serum free medium and analyzed by high performance liquid chromatography (HPLC), as described previously (34), to confirm the reduction in GSL content in the LY-B mutant cells. Fig. 3A showed that the three cell lines (LY-B/CD1d, LY-B/cLCB1/CD1d and CHO-K1/CD1d) had similar GSL profiles, with three prominent peaks corresponding to lactosylceramide (LacCer), GM3 with acetyl sialic acid, and GM3 with glycolyl sialic acid. All these GSLs were dramatically reduced in the LY-B mutant cells, and there were no alterations in the GSLs present in either the LCB1 restored or wild type cells. Because an enzyme, ceramide glycanase, used for GSL analysis contains LacCer contamination (data not shown), this GSL was excluded from the quantitative analysis. The analysis showed that both forms of GM3 in mutant cells were less than 10% the amount found in the control cells after five days in sphingolipid and serum-free medium (Fig. 3B). There was no significant difference in the GSL profile of CHO-K1 cells expressing wild type mCD1d, or a mCD1d molecule with the cytoplasmic tail deleted,

compared to CHO-K1 cells that do not express mCD1d (data not shown), indicating that expression of high amounts of mCD1d does not stabilize or increase the GSL content of these cells.

Mutant cells present endogenous Ag

The mutant cells (LY-B/CD1d) and the restored cells (LY-B/cLCB1/CD1d) were cultured in sphingolipid and serum-free medium and were tested for their ability to stimulate *i*NKT cell hybridomas that were washed with serum-free medium. To assess TCR engagement by mCD1d-presented endogenous antigen, we measured IL-2 production in the culture supernatants by ELISA. While no IL-2 release was induced by either of the two vector transfectants, in a titration of the mCD1d-expressing APCs, unexpectedly, similar amounts of IL-2 were produced by *i*NKT hybridomas incubated with the LY-B mutant cells compared to cultures with the restored cells. Similar results were obtained with *i*NKT cell hybridomas 1.2 (Vα14Vβ8.2), 24.9E (Vα14Vβ8.3) (Fig. 4A) and 1.4 (Vα14Vβ10) (data not shown). To confirm mCD1d mediated stimulation of the *i*NKT cells, a specific anti-mCD1d monoclonal antibody (mAb) was tested for its ability to block T cell activation. Fig. 4B shows that the mAb dramatically decreased IL-2 production by the *i*NKT cell hybridomas incubated with either the mutant LY-B or the restored cells that express mCD1d. Importantly, these data suggest that the CHO cells have a self-Ag that stimulates mouse *i*NKT cells in a mCD1d-dependent fashion, and that the mutant LY-B cells do not have defects in synthesizing and presenting an endogenous Ag to *i*NKT cells.

Interestingly, the non-invariant CD1d-reactive hybridoma 24 (Vα3.2Vβ9) was not activated by the mCD1d-expressing transfectants (Fig. 4A), although it responded to CD1d transfected A20 mouse B lymphoma cells. These data indicate that the self-Ag that activates the 24 hybridoma cells is not present in the CHO cell derivatives, which is consistent with earlier results showing the stimulatory ligand for these cells is not widely expressed, for example it is absent in several wild mouse strains (42).

We also determined if the endogenous lipid Ag from CHO cells elicit autoreactive responses from primary mouse *i*NKT cells. Mutant and restored cells were cultured in sphingolipid and serum-free medium for five days, followed by titration and culture with purified mouse spleen *i*NKT cells. The activation of the primary *i*NKT cells was detected by an ELISA assay to measure IFNγ release. Fig. 4C shows that vector transfectants of both mutant LY-B cells and restored LY-B/cLCB1 cells did not induce significant IFNγ secretion from freshly isolated mouse *i*NKT cells, but that their mCD1d transfectants were capable of stimulating these cells equivalently. The activation of a significant fraction of the primary *i*NKT cells was suggested by the increased expression of CD25 (Fig. 4C).

Stimulation of iNKT cells requires endosomal localization of mCD1d

In mice, presentation of the self-Ag requires lysosomal function and localization of mCD1d to late endosomes/lysosomes (7, 43). If the cytoplasmic tail of mCD1d is deleted or mutated, mCD1d cannot load lipid Ags in lysosomes because of the loss of a tyrosine-containing sequence motif that interacts with AP-3 and directs mCD1d to lysosomes (7-9, 43). We transfected CHO-K1 cells with a plasmid encoding mCD1d with the cytoplasmic tail deleted. Flow cytometry analysis showed that the C-terminal truncation did not affect the cell surface expression of mCD1d compared with wild type mCD1d transfectants (Fig. 2). However, IL-2 production from *i*NKT cell hybridomas induced by CHO-K1 cells expressing the tail-deleted form of mCD1d was much reduced compared with that by the wild type CD1d transfectants (Fig. 5A). The presentation of αGalCer, which can directly stimulate *i*NKT cells without a strict requirement for internalization and lysosomal localization, was comparable for cells expressing the tail deleted or wild type mCD1d molecules (Fig. 5B).

These data indicate that the cytoplasmic tail deletion did not affect the ability of mCD1d to bind exogenous Ags. Also, they indicate that the endogenous Ag for *i*NKT cells in heterologous CHO-K1 cells shares some properties with the self-Ag responsible for positive selection in the mouse thymus, in that both require the cytoplasmic tail of mCD1d and normal mCD1d intracellular trafficking for effective presentation (7).

Cell free assay of the sonicates from different transfectants

We used CD1d-coated plates to stimulate *i*NKT hybridomas, as opposed to APCs, because this format eliminates some of the complexities of interpreting results when APCs are used. For example, it is possible that different APCs, even sublines of CHO cells, could differ in their ability to take up lipid Ags, and to transport them to lysosomes and/or other compartments. In lysosomes, glycolipid Ags likely interact with transport or exchange proteins (44), they may undergo processing or degradation (10), and ultimately loading onto CD1d molecules. By contrast, the cell-free Ag presentation assay, which uses immobilized CD1d to directly present lipid Ags, eliminates these intracellular steps, although it cannot be used to detect Ags that require processing. To prepare fractions to be tested for the presence of *i*NKT cell Ags, the three CHO cell CD1d transfectants were washed, resuspended and sonicated. Dilutions of the sonicates were placed in mCD1d coated wells, the plates were washed and then cultured with three *i*NKT cell hybridomas with different Vβ rearrangements. The sonicates induced comparable, dose dependent IL-2 production (Fig. 6), indicating that the three transfectants contained similar amounts of lipid Ags, despite reduced GSLs caused by the defective sphingolipid synthesis in the LY-B cells. This result is consistent with that obtained using APCs (Fig. 4A). Hybridoma IL-2 release could be blocked with the specific anti-CD1d antibody 1B1 (Fig. 6), confirming the CD1d-dependence of the response.

The antigenic activity is resistant to GSL specific hydrolases

We used enzymatic digestion to probe further if the Ag in the eukaryotic CHO cell lines could be a type of GSL. Endoglycoceramidase II (rEGCase II) is an enzyme that can specifically cleave the β-linked glycosidic bonds between the glucoses of the GSL oligosaccharide and the ceramide backbone (45, 46). The function of the enzyme was demonstrated by incubating increasing amounts of a GM1 standard with rEGCase II. Fig. 7A shows the enzyme completely degraded up to 10 μg GM1 to ceramide in two hours. The degradation of GSLs in the CHO cell sonicates also was tested. After overnight digestion, rEGCase II completely degraded LacCer in the CHO cell sonicate (Supplementary Fig. 1). Despite this, even overnight rEGCase II digestion did not affect the antigenic activity of CHO-K1/CD1d sonicates (Fig. 7B).

We also used a sphingolipid-specific hydrolase, sphingolipid ceramide N-deacylase (SCDase), which specifically cleaves the N-acyl linkage between the fatty acid and the sphingoid base in the ceramide of various sphingolipids (47). The capacity of SCDase to degrade GSLs in CHO cell sonicates was demonstrated after enzyme treatment. After digestion, bands migrating in the positions of LacCer and GM3 shifted lower, corresponding to the lyso derivatives of these GSLs (Supplementary Fig. 1). Fig. 7C shows SCDase digestion also significantly reduced the antigenic activity of the highly potent synthetic Ag αGalCer. There was no significant decrease in the antigenic activity of the CHO-K1/CD1d sonicates, however, indicating that the endogenous Ag in CHO-K1 cells is not a GSL.

An inhibitor of GSL biosynthesis does not affect endogenous Ag presentation by mouse cells

A20/CD1d, a mouse B lymphoma cell line with stable mCD1d expression, was employed to determine if the endogenous Ag in mouse cells has the same characteristics of the Ag in

CHO hamster cells. As mentioned above, ceramide glucosyltransferase is a key enzyme in the GSL biosynthesis pathway. It catalyzes the transfer of UDP-glucose to ceramide to form a glycosylceramide (β-linkage). *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) is a selective inhibitor of ceramide glucosyltransferase (32, 48, 49), and therefore was used to reduce the GSL content in A20/CD1d cells. Control A20 cells and A20/CD1d transfectants were cultured in medium with methanol vehicle or 100 μM *N*B-DGJ for 3 days to reduce the GSL levels. Because *N*B-DGJ has been reported to interfere with intracellular lipid trafficking (50), we first tested if the concentration of *N*B-DGJ used interfered with the response to Galα(1-2)αGalCer, an antigen that requires normal mCD1d and lipid Ag intracellular trafficking (10). However, the response to this Ag presented by A20/CD1 cells treated with 100 μM *N*B-DGJ was comparable to that of methanol vehicle treated A20/CD1 cells (Supplementary Fig. 2), indicating that such interference was not occurring. For the self-antigen response, the APCs were fixed with 0.05% glutaraldehyde and titrated for incubation with auto-reactive *i*NKT hybridoma cells. Fig. 8A shows that A20 cells did not induce IL-2 production, but A20/CD1d transfectants, treated with *N*B-DGJ or the control, both stimulated *i*NKT cells to produce similar amounts of IL-2.

The antigenic activity in mouse cells is resistant to GSL specific hydrolases

Because the inhibitor may not have been capable of a sufficient depletion of endogenous Ag, we used enzymatic hydrolysis to digest a sonicate of A20/CD1d cells. In an APC-free assay using CD1d coated plates, we found that sonicates of A20/CD1d cells stimulated a dose-dependent release of IL-2 by three *i*NKT cell hybridomas (Fig. 8B). In addition, a CD1d-specific mAb, but not the isotype control, eliminated IL-2 production, demonstrating that as for the CHO-K1 cell extracts, hybridoma activation was CD1d-dependent. Also similar to extracts from the CHO-K1 cells, the activity in the sonicates of A20/CD1d cells was also resistant to rEGCase (Fig. 8C) and SCDase (Fig. 8D) digestion. These results indicate that the A20 mouse B cell line also has an endogenous lipid Ag, but one that also is not likely to be a GSL.

Divergent self-Ags for mouse and human iNKT cells

It was recently reported that the majority of human *i*NKT cell clones react to lysophosphatidylcholine (lysoPC) (51). Because the antigenic activity of some antigens can be highly dependent on the fatty acid composition (52), we tested several sources of lysoPC, including purified and synthetic materials, for their ability to activate mouse *i*NKT cell hybridomas. As shown in Fig. 9A, different sources of lysoPC, at concentrations up to 20 μg/ml, did not stimulate any of the four hybridoma *i*NKT cell hybridomas tested. We have found that pulsing of BMDCs with a glycolipid Ag, followed by injection into mice, is a highly sensitive method for detecting the activation of *i*NKT cells in vivo. As shown in Supplementary Fig, 3, incubation of BMDCs with concentrations of αGalCer as low as 10 ng/ml was able to elicit *i*NKT cell activation as measured by intracellular cytokine staining. Injection of the antigen-pulsed DC also led to increased serum cytokines (Fig. 9B). By contrast, when BMDCs were pulsed with either synthetic or purified lysoPC, they failed to stimulate any detectable response by populations of *i*NKT cells. These data indicate that the lysoPC is not a universal *i*NKT cell self-Ag.

Discussion

*i*NKT cells differentiate in the thymus where they are positively selected by self-Ags presented by CD1d expressed on the cell surface of double positive thymocytes (1, 2, 4). The endogenous Ags have a critical role in the development and maturation of *i*NKT cells (21, 53, 54). Malfunctions in CD1d expression (55, 56), intracellular trafficking (7, 8, 43), lipid Ag binding (11, 57-59), or lipid synthesis and metabolism (21, 53, 54), which affect the

presentation of endogenous Ags to the invariant TCR, will lead to significantly reduced populations of *i*NKT cells. Because these cells acquire an antigen-experienced phenotype in the thymus, with transcriptionally active *Ifng* and *Il4* gene loci (60, 61), it has been generally assumed that the positively selecting glycolipids are in fact true agonists for the invariant TCR (1, 62). Autoreactivity of mature, peripheral *i*NKT cells is also manifested in a variety of situations (5, 17, 49, 63). It is possible that a single type of agonist Ag is responsible for both positive selection and activation of mature *i*NKT cells, but this remains unproven and in fact, the structure of the endogenous Ag(s) for *i*NKT cells remains a controversial issue (22).

A consensus in this field of research has been that the endogenous Ags are GSLs, with a ceramide lipid moiety broadly similar in structure to αGalCer, the first *i*NKT cell Ag identified. This conclusion has rested primarily upon three findings. First was the report that CD1d transfectants of GM95 could not stimulate *i*NKT cell autoreactivity, backed up by the results from the use of inhibitors that interfere with GSL biosynthesis (39). In this original report, however, stable as opposed to transient transfectants of GM95 could in fact stimulate autoreactivity. The ability of the stable transfectants to activate *i*NKT cells is in agreement with the results we report here. Secondly iGb3 was reported to be essential for both *i*NKT cell differentiation in the thymus and for activation of these cells in the periphery (17, 21). While iGb3 is a mammalian GSL that can activate *i*NKT cells, the results from several types of experiments argue that it cannot be the essential or unique physiological antigen for *i*NKT cells. For example, it has been reported that α-galactosidase A deficient mice, which accumulate iGb3, also have a reduced *i*NKT cell frequency (10, 54), as do some mouse strains with GSL biosynthetic deficiencies unrelated to iGb3 synthesis (53, 54). This finding suggests that GSL storage diseases affect *i*NKT cell differentiation independent of a direct involvement in Ag biosynthesis. Also, in one study iGb3 could not be detected in mouse thymus or DCs (34), although this was not the case in another report (64), and questions have been raised as to whether humans have a functional iGb3 synthase (65). Furthermore, and perhaps most important, mice deficient in iGb3 synthase had no defect in *i*NKT cell number or function (66). A third set of findings in support of GSLs, although not iGb3, as the major self-Ags come from studies of *i*NKT cell activation by CpG ODN, a TLR9 ligand. CpG ODN induced DCs to synthesize a charged GSL that then activated *i*NKT cells in a CD1d-dependent fashion to produce IFNγ (49). The structure of this novel GSL ligand has not been determined. Recently it was reported that the enzyme α-galactosidase A limits the presentation of the self-antigen, implicating some type of GSL antigen, perhaps even iGb3, but in this case as well, a structure for the antigen was not determined (67).

It is possible that insight into the nature of the self-Ag(s) recognized by *i*NKT cells can be obtained from the analysis of the structures of microbial Ags that activate the invariant TCR. A striking characteristic of these bacterial Ags is that they have hexose sugars, mainly galactose, glucose, or closely related sugars, in α-linkage to lipid backbones that have two hydrophobic carbon chains. The first microbial Ags, obtained from *Sphingomonas spp.*, have a GSL structure similar to αGalCer (16, 17), and it is widely believed, although not proven, that Sphingomonas bacteria in the marine sponge were the actual source of the GSL Ag. Subsequently, it was shown that the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease, has monoglycosylated diacylglycerols (DAGs) that activate *i*NKT cells (20). This finding established the principle that Ags for *i*NKT cells need not be GSLs.

Herein, we used a variety of methods to show that, like the microbial Ags, the self-Ags for *i*NKT cells do not have to be GSLs. An important tool for these studies was the use of CD1d transfectants of cell lines defective for GSL biosynthesis, including the GM95 cell line, with a mutant ceramide glucosyltransferase, and LY-B cells, with a more than 90% reduction in GSLs due to an upstream defect in the GSL biosynthetic pathway. The findings were

confirmed in a cell-free Ag presentation assay comparing sonicates from the mutant LY-B cells, LY-B cells with GSL biosynthetic activity restored, and parental CHO-K1 cells. Specific enzymatic digestions provided further evidence that the endogenous Ag from these cells was not a GSL. The endoglycoceramidase, rEGCase II, hydrolyzes β-linked glycosphingolipids with complex sugar head groups, such as iGb3, to ceramides and oligosaccharides, but the enzyme did not affect the antigenic activity of the sonicates from wild type CHO-K1 cells. This result was confirmed by digestion with SCDase. This enzyme degraded αGalCer to its lyso- form, dramatically decreasing the ability of this potent compound to activate *i*NKT cell hybridomas, but it had no significant effect on the antigenic activity of the sonicates.

While these findings, summarized above, establish that self-Ags for *i*NKT cells need not be GSLs, the physiologic relevance of glycolipid Ag presentation to mouse *i*NKT cells by a transformed hamster cell line is uncertain. However, two additional sets of results suggest that the results are reflective of the properties of the normal self-Ags. First we showed that endogenous Ag presentation by the CHO cells required mCD1d translocation to lysosomes. A similar requirement has been shown for the self-Ag that selects these T lymphocytes in the mouse thymus (7, 8, 43). Second, analyses of Ag presentation by the mouse B lymphoma cell line A20 indicated that the self-Ag from these cells also is unlikely to be a GSL. Consistent results were obtained with A20 cells treated with the GSL biosynthetic inhibitor *N*B-DGJ, and when cell-free A20 extracts were treated with degradative enzymes. In both cases, treatments that would disrupt GSL biosynthesis or degrade GLS directly had no effect on self-Ag presentation.

Our results do not contradict the finding that some self-Ags for *i*NKT cells are in fact GSLs, with iGb3 providing one example, or even that the predominant self-Ags are GSLs. Consistent with the known foreign Ags, however, our results demonstrate that the self-Ags for mouse *i*NKT cells also are likely to be diverse in structure. In fact, the simple and appealing hypothesis that there is a single self-agonist that serves as the selecting ligand in the thymus and as an agonist in the periphery needs to be re-examined. While it is true that *i*NKT cells acquire an Ag-experienced phenotype in the thymus, this still could involve the recognition of a self ligand or a collection of self ligands that interact only weakly with the TCR, analogous to the situation for conventional CD4 and CD8 T cell positive selection by peptides. The antigen-experienced phenotype of *i*NKT cells in the thymus instead could be caused by their positive selection by other thymocytes and/or unique aspects of the signaling pathway that leads to the differentiation of these cells, rather than by interaction with a true agonist ligand. Diacylglycerols provide a model for possibly weaker, positively selecting Ags, because unlike GSLs, their binding to CD1d is stabilized by fewer hydrogen bonds, and they can in fact bind to CD1d in more than one orientation (52).

Besides glycolipids, mammalian phospholipids have also been shown to be CD1d ligands that stimulate a subset of mouse *i*NKT cells (68), but these were clearly not universal Ags for these cells. More recently, Gumperz et al. made the surprising finding that lysoPC, a phospholipid with only a single hydrophobic acyl chain can be eluted from human CD1d, and could stimulate the majority of human *i*NKT cell clones tested (51, 69). It is uncertain how the same TCR can recognize structures with α linked hexose sugars and phospholipids, or why in this case, an antigen with a single acyl chain is more potent than one with two acyl chains, while the opposite is true for GSLs. There is a surprising degree of flexibility in Ag recognition by the invariant TCR, however, as evidenced by recent findings showing a threitol-containing compound, without a complete sugar, also activated *i*NKT cells (70). In contrast to the results of Gumperz et al., we were not able to stimulate mouse *i*NKT cells with lysoPC. The self-Ags for mouse and human *i*NKT cells may differ, however, despite

TCR conservation. The requirement for CD1d localization in lysosomes for presentation of mouse but not human self-Ags is consistent with such a structural difference (71).

We propose that depending on conditions, different types of APCs may employ different lipids as the self-Ags to activate *i*NKT cells. Upon TLR ligand activation, DCs could selectively induce the synthesis of some GSLs (32, 49). In fact, we reported earlier that CpG ODN treatment of APC led to the synthesis of a charged GSL self-Ag for *i*NKT cells. The self-Ag(s) found in our investigation was present constitutively, however, in transformed cultured cell lines including CHO cells and A20 B lymphoma cells. There is no contradiction between these findings, as not only are there differences in the mode of antigen induction or expression, but also differences in the antigen presenting cell types. We speculate that diverse endogenous Ags could play different roles in the development and maturation of *i*NKT cells, and they could even influence the character of the cytokine response elicited by these cells, analogous to the different cytokine responses induced by synthetic GSL Ags with only subtle differences in structure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

αGalCer	α-galactosylceramide
Ag	antigen
APC	antigen-presenting cell
BMDCs	Bone marrow derived dendritic cells
CHO	Chinese hamster ovary cell
GM1	ganglioside GM1
GM2	ganglioside GM2
GM3	ganglioside GM3
GSL	glycosphingolipid
i	invariant
iGb3	isoglobotrihexosyl ceramide
LacCer	lactosylceramide
lysoPC	lysophosphatidylcholine
mCD1d	mouse CD1d
***N*B-DGJ**	*N*-butyldeoxygalactonojirimycin

NKT	natural killer T
rEGCase II	endoglycoceramidase II
SCDase	sphingolipid ceramide N-deacylase
self-Ag	self-antigen

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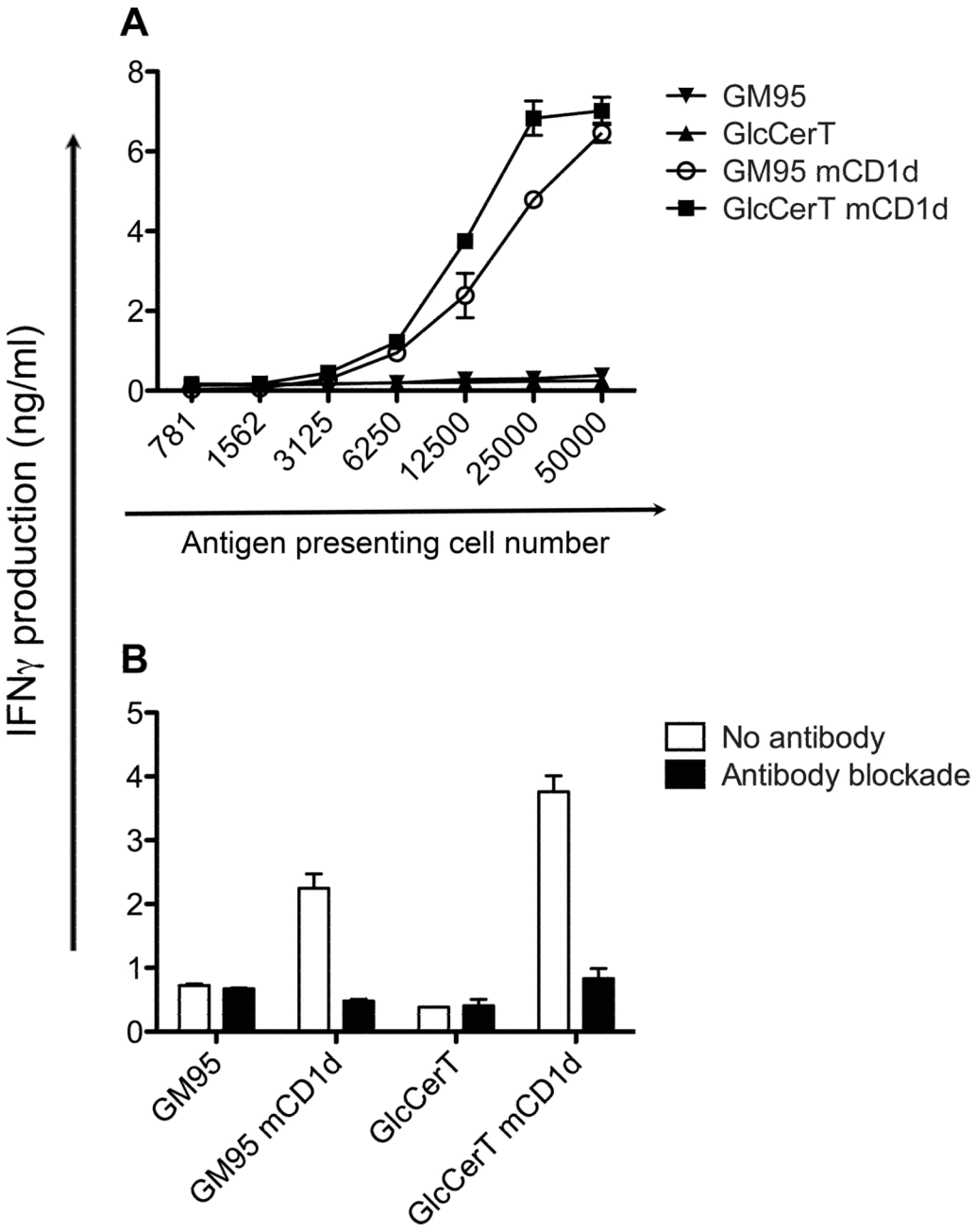

Figure 1. Comparable endogenous Ag presentation by ceramide glucosyltransferase deficient and restored cells

(A) Mutant GM95 and restored GluCerT cell lines were used to stimulate mouse splenocytes from Vα14 transgenic mice. GM95 and GluCerT and their transfectants expressing mCD1d were titrated as indicated and supernatants were assayed for IFNγ by ELISA. (B) Blockade of mouse *i*NKT cell activation by mCD1d specific antibody. 20 μg/ml of specific antibody was added before APCs were mixed with purified mouse splenocytes. APCs without antibody addition were used as controls. Standard deviations from the mean of two duplicate assays are shown. Representative data are shown from one of two experiments.

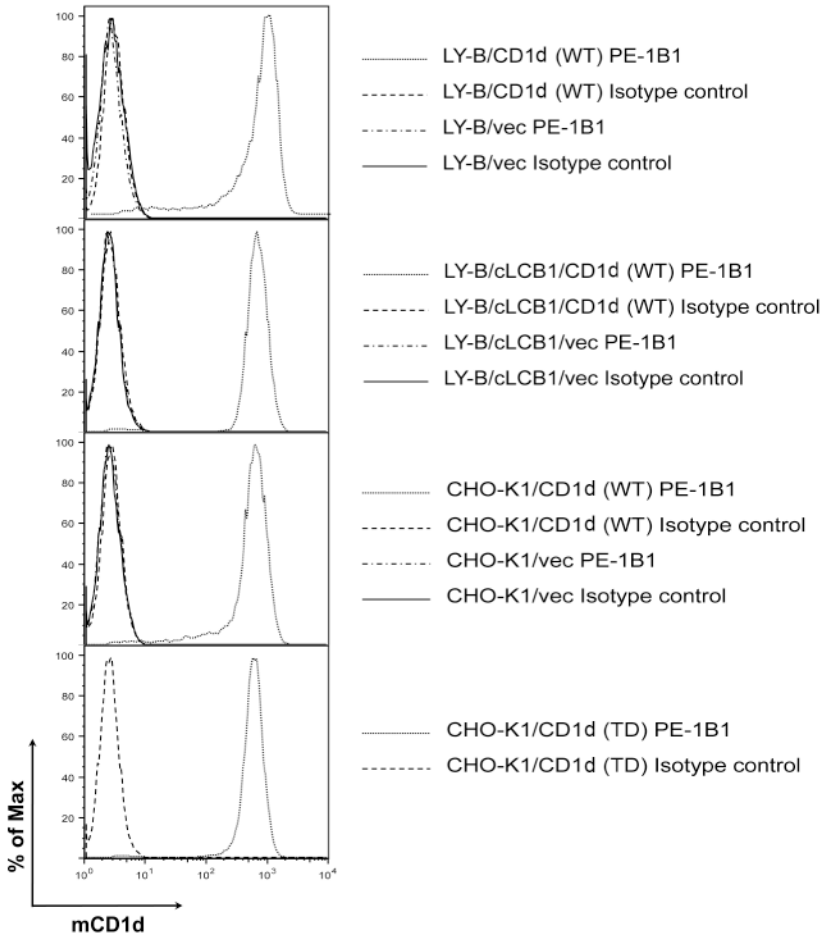

Figure 2. Surface mCD1d expression on transfected cell lines

mCD1d transfectants or empty vector controls were stained with PE-conjugated rat anti-mCD1d mAb or an isotype control, and the expression levels of surface mCD1d, wild type or tail-deleted mutant, were determined by flow cytometry.

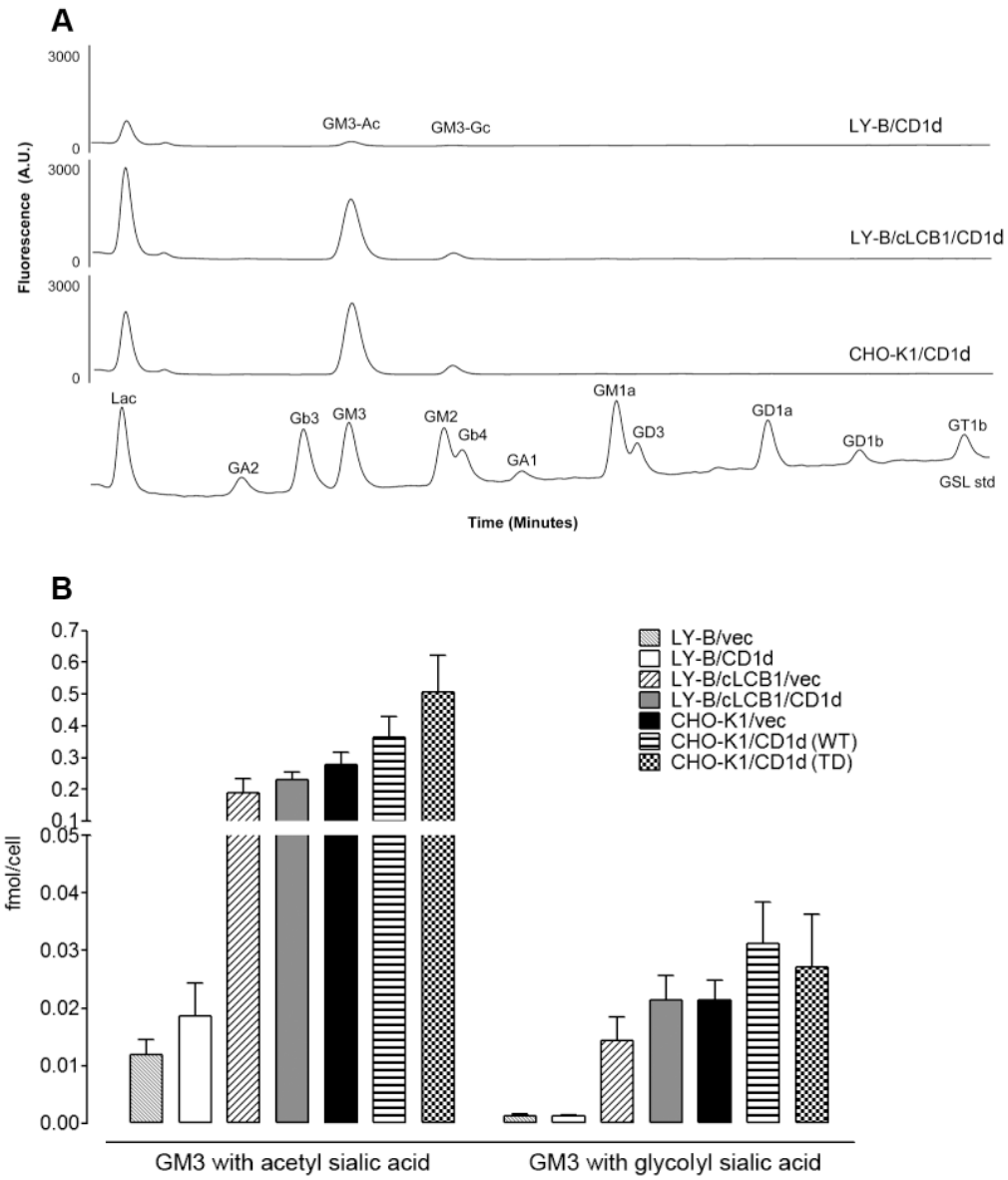

Figure 3. Reduced GSLs in mutant CHO cell transfectants

Cells were cultured in sphingolipid and serum-free medium, GSLs extracted and analyzed by NP-HPLC. (A) Representative NP-HPLC profiles of CHO cell GSL-derived oligosaccharides and GSL standards. Profiles of the LY-B mutant, LY-B/cLCB1 restored and wild type CHO-K1 cells transfected with mCD1d are shown. The fluorescence axis was constant and the cell numbers injected were the same, allowing for the direct comparison of profiles. The identity of the smaller peak migrating near ganglioside GM2 (GM2) as GM3-glycoyl sialic acid (GM3-Gc) was confirmed by exoglycosidase digestion, as this peak was susceptible to sialidase A digestion but not to β-hexosaminidase, confirming that it is not GM2. The standard mix of GSLs is abbreviated according to IUPAC recommendations (72). (B) Quantization of GSLs from CHO cells. GSLs from the samples were calculated as fmol/cell and then normalized to CHO-K1/vec. Data are shown as mean ± SEM, with n = 3 for all samples except n = 2 for CHO-K1/CD1d (TD, tail deleted)) and n = 4 for LY-B/cLCB1/

CD1d. The peak that co-migrates with lactose in the samples contains contamination from the ceramide glycanase and has not been quantitated.

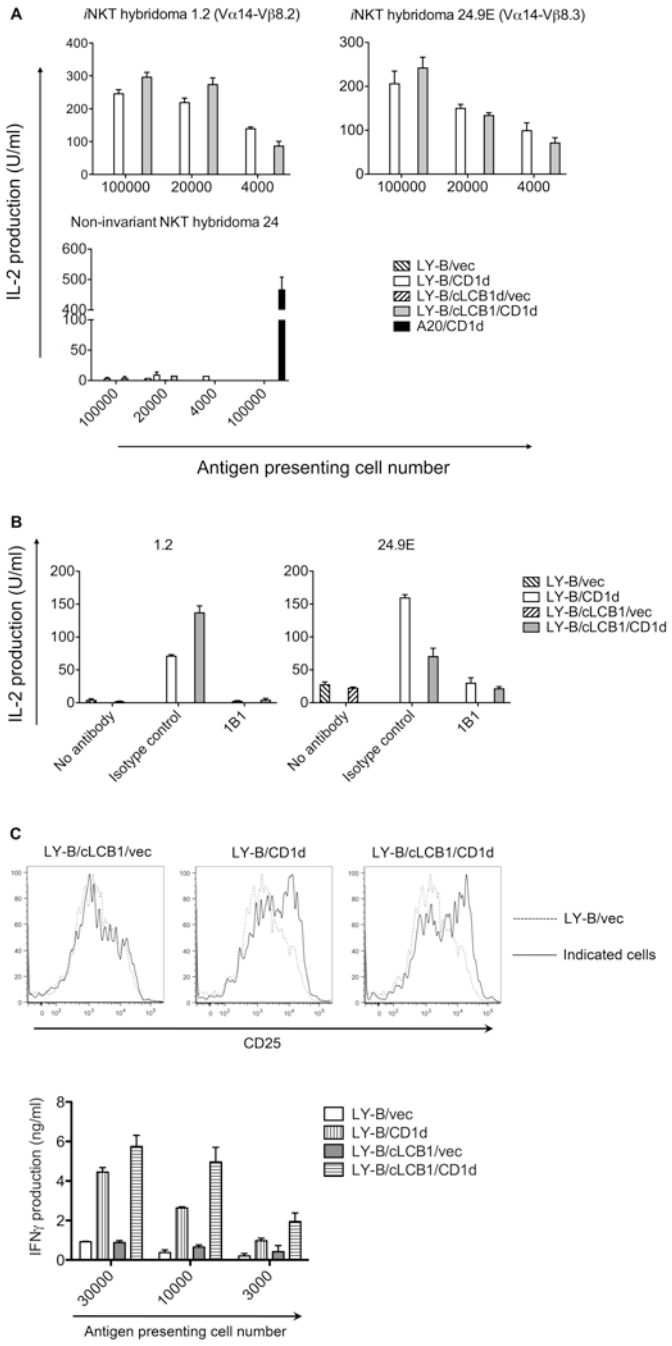

Figure 4. Mutant CHO cell CD1d transfectants present self-Ag

(A) The activation of *i*NKT cell hybridomas by endogenous antigen from mutant cells (LY-B) and restored cells (LY-B/cLCB1) expressing mCD1d or vector. APCs cultured in sphingolipid and serum-free medium for five days were titrated followed by culture with hybridomas. Hybridoma 24 is reactive to an undefined self-Ag presented by mCD1d, but it does not express the Vα14*i* TCR. The results shown are representative of three independent experiments. (B) Inhibition of *i*NKT cell activation by mCD1d specific antibody blockade. 20 μg/ml of specific antibody or isotype control were added to APCs cultured with hybridomas as described in Materials and Methods. The results shown are from one of two independent experiments. (C) Stimulation of mouse *i*NKT cells by self-Ag from mutant and

restored CHO cells. Top: mouse spleen *i*NKT cells and LY-B, LY-B/cLCB1 CD1d transfectants were seeded at 3×10^4/well in sphingolipid and serum-free medium. Cells positive for mCD1d/αGalCer tetramer staining were analyzed for expression of CD25. Grey dotted line: CD25 surface expression on LY-B/vec cells; black solid line: CD25 expression on the indicated vector or CD1d transfected cells. Bottom: purified mouse spleen *i*NKT cells (3×10^4/well) were exposed to titrated LY-B and LY-B/cLCB1 cells expressing either wild type mCD1d or vector control, which had been cultured in sphingolipid and serum-free medium for five days. Representative data from one of two experiments are shown.

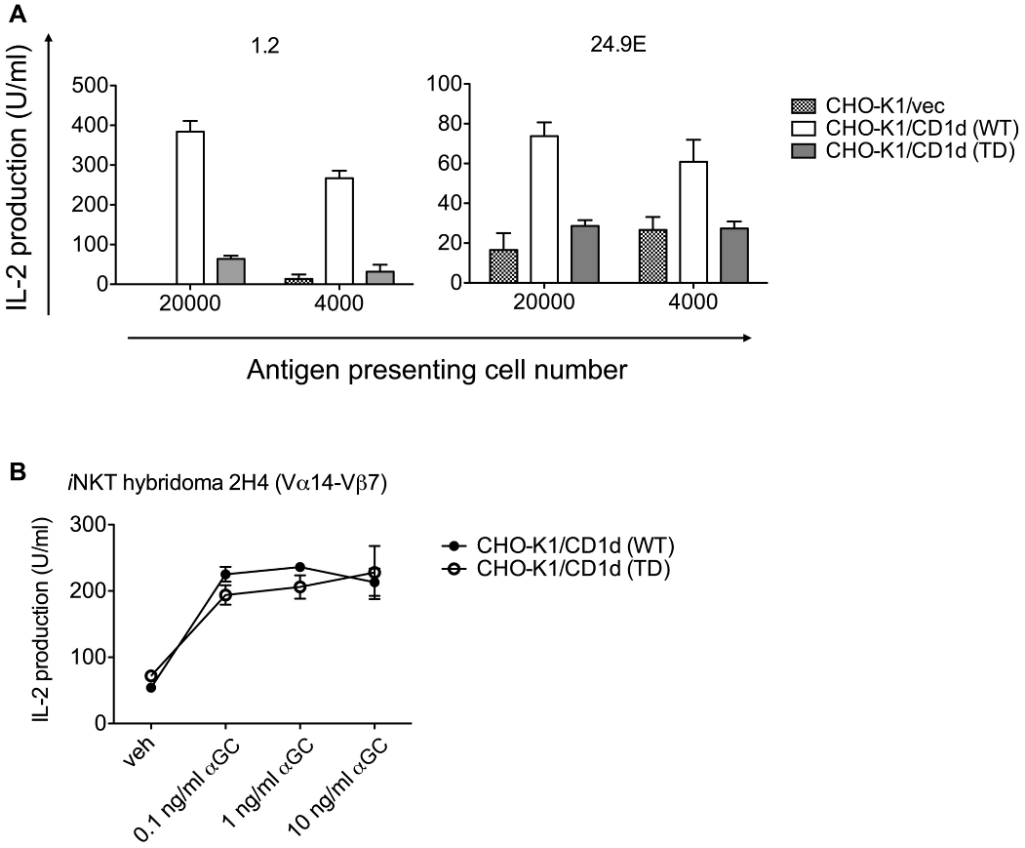

Figure 5. Normal intracellular trafficking of mCD1d is required for self-Ag presentation

(A) Defective intracellular trafficking impaired endogenous Ag presentation by CD1d. Titrated CHO-K1 cells expressing wild type or tail-deleted mCD1d were cultured as described above with *i*NKT cell hybridomas. The data shown are representative of four independent experiments. (B) Cytoplasmic tail deletion of mCD1d did not affect αGalCer presentation and stimulation of *i*NKT cell hybridoma 2H4. This hybridoma did not respond to the endogenous antigen in CHO cells. The APCs were cultured in complete medium, pulsed with indicated concentrations of αGalCer, and then mixed with *i*NKT hybridomas. Veh: vehicle; αGC: αGalCer. Representative data from one of two independent experiments are shown.

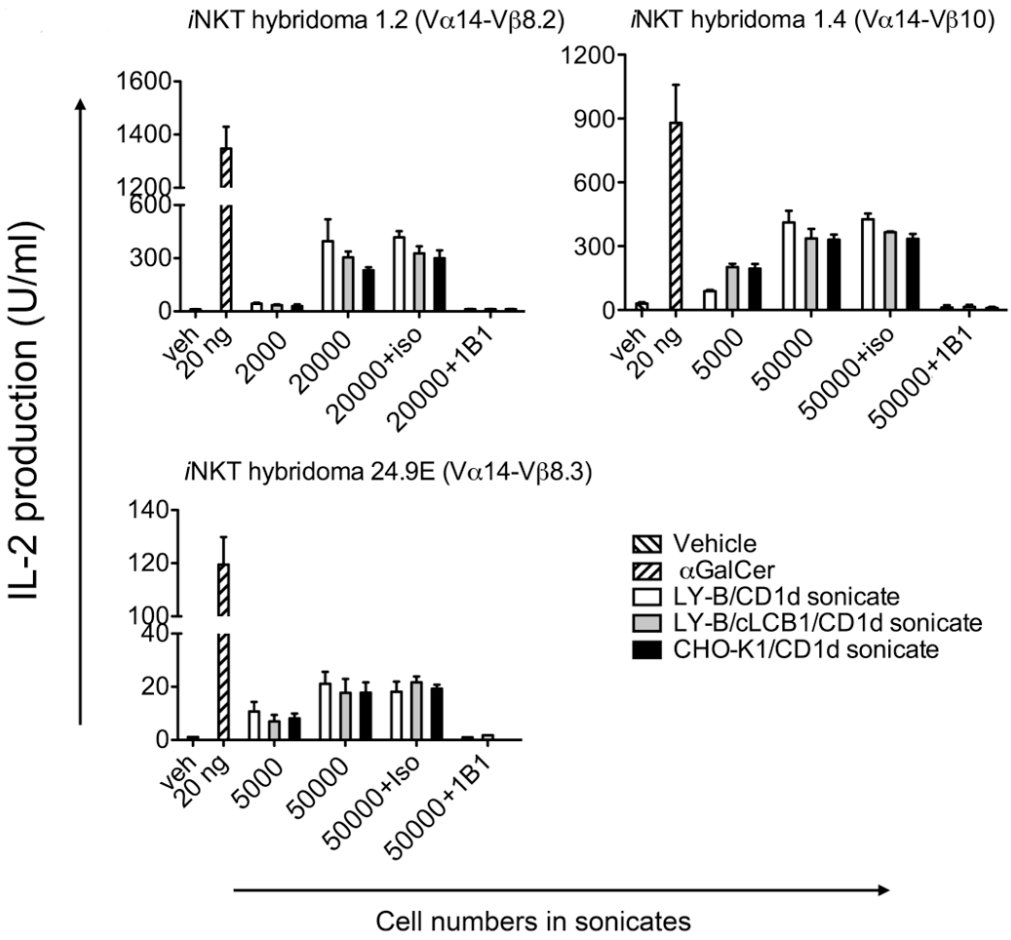

Figure 6. Endogenous Ag activity is found in sonicates of mutant cells

CHO-derived cell lines were cultured in sphingolipid-deficient medium and sonicated. The sonicates prepared from the indicated numbers of cells were diluted in PBS and plated on CD1d coated wells of a 96 well plate with/without 20 μg anti-CD1d or isotype antibody. Vehicle and 20 ng/well αGalCer were used as negative and positive controls, respectively. The result for each *i*NKT cell hybridoma is representative of one of three independent experiments.

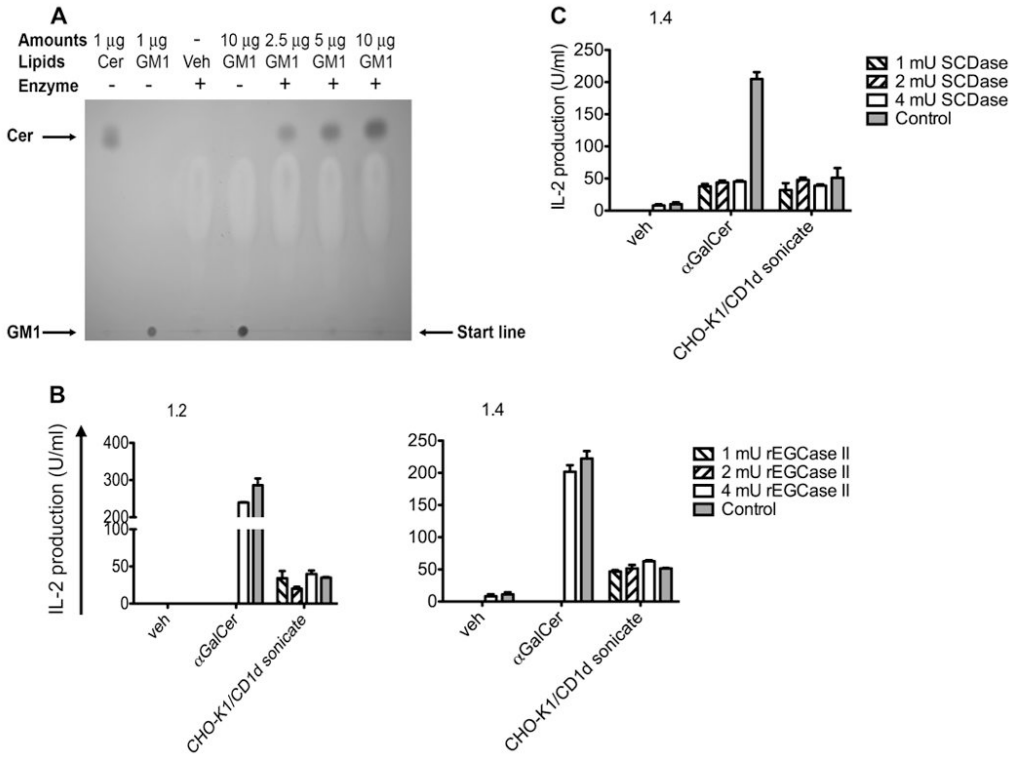

Figure 7. Sphingolipid hydrolysis did not affect antigenic activity

(A) Degradation of GM1 to ceramide by an endoglycoceramidase, rEGCase II. The indicated amounts of GM1 or methanol vehicle were incubated with 2 mU rEGCase II and analyzed by TLC. Cer: ceramide; GM1: ganglioside GM1; Veh: vehicle. (B) Stimulation of *i*NKT cell hybridomas by the CHO-K1/CD1d cell sonicate digested by the endoglycoceramidase, rEGCase II. Vehicle, 20 ng αGalCer or 1 µl sonicate was incubated with titrated rEGCase II overnight at 37°C. The antigenic activity of the digests was determined by the cell free antigen presentation assay using CD1d coated microwells. The data are representative of two independent experiments. (C) Sphingolipid ceramide N-deacylase (SCDase) degradation of the CHO-K1/CD1d cell sonicate. Vehicle, 20 ng αGalCer or 1 µl sonicate was treated with SCDase and analyzed in cell free antigen-presenting assays in cultures with *i*NKT cell hybridomas after addition to CD1d coated plates. The data shown are from one of two independent experiments.

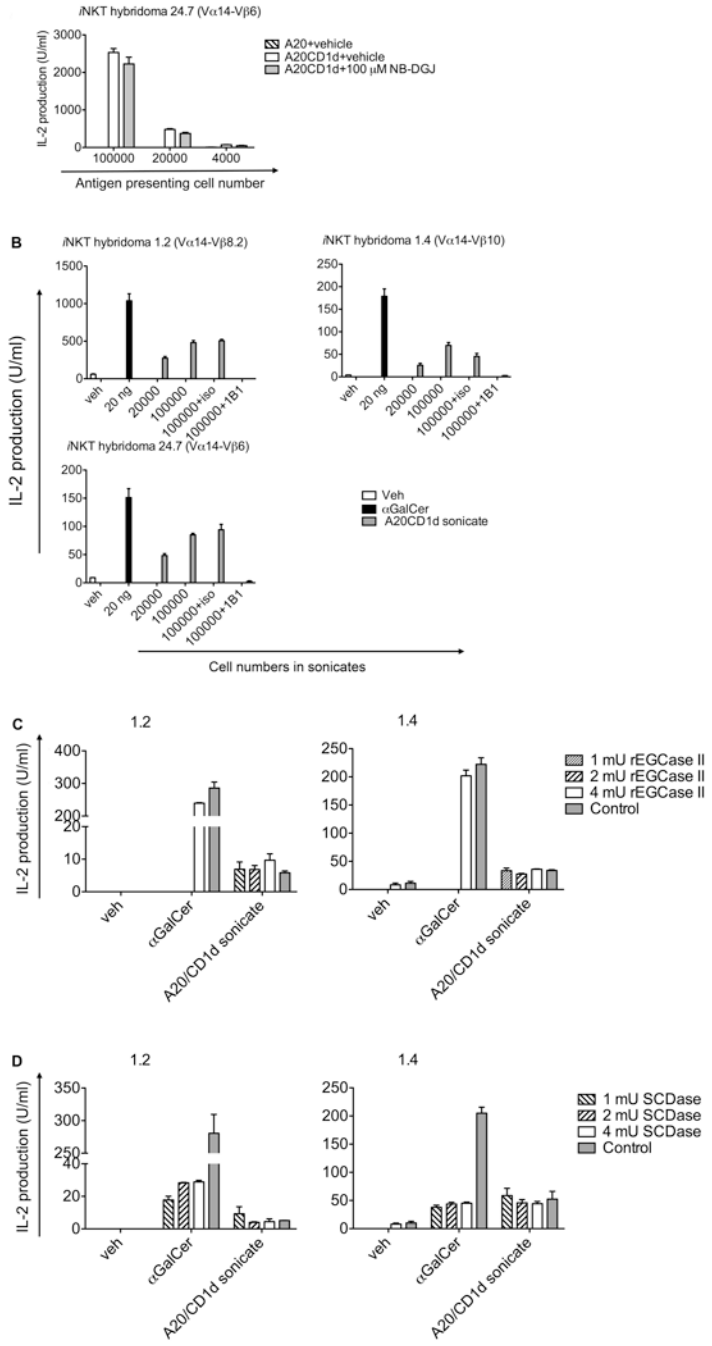

Figure 8. Characterization of the endogenous antigen in A20/CD1d cells

(A) A20 and A20/CD1d cells were cultured in medium with 100 μM *N*B-DGJ or methanol vehicle, the cells were fixed and titrated amounts added to cultures with *i*NKT cell hybridoma 24.7 for measurement of IL-2 release. Shown are the data from one of two independent experiments. (B) Cell free assay of antigenic activity of the A20/CD1d sonicate. Sonicates prepared from the indicated numbers of cells, diluted in PBS, and added to CD1d coated wells, with/without 20 μg anti-CD1d or isotype control antibody. Vehicle and 20 ng/well αGalCer were used as negative and positive controls, respectively. Data shown are representative of three independent experiments. (C) rEGCase II and (D) SCDase hydrolysis of A20/CD1d sonicates. Vehicle, 20 ng αGalCer or 2 μl sonicate was incubated

with titrated amounts of enzyme overnight at 37°C. The stimulation of *i*NKT cell hybridomas by the digests was determined by cell free assays and ELISA. Data are representative of two independent experiments.

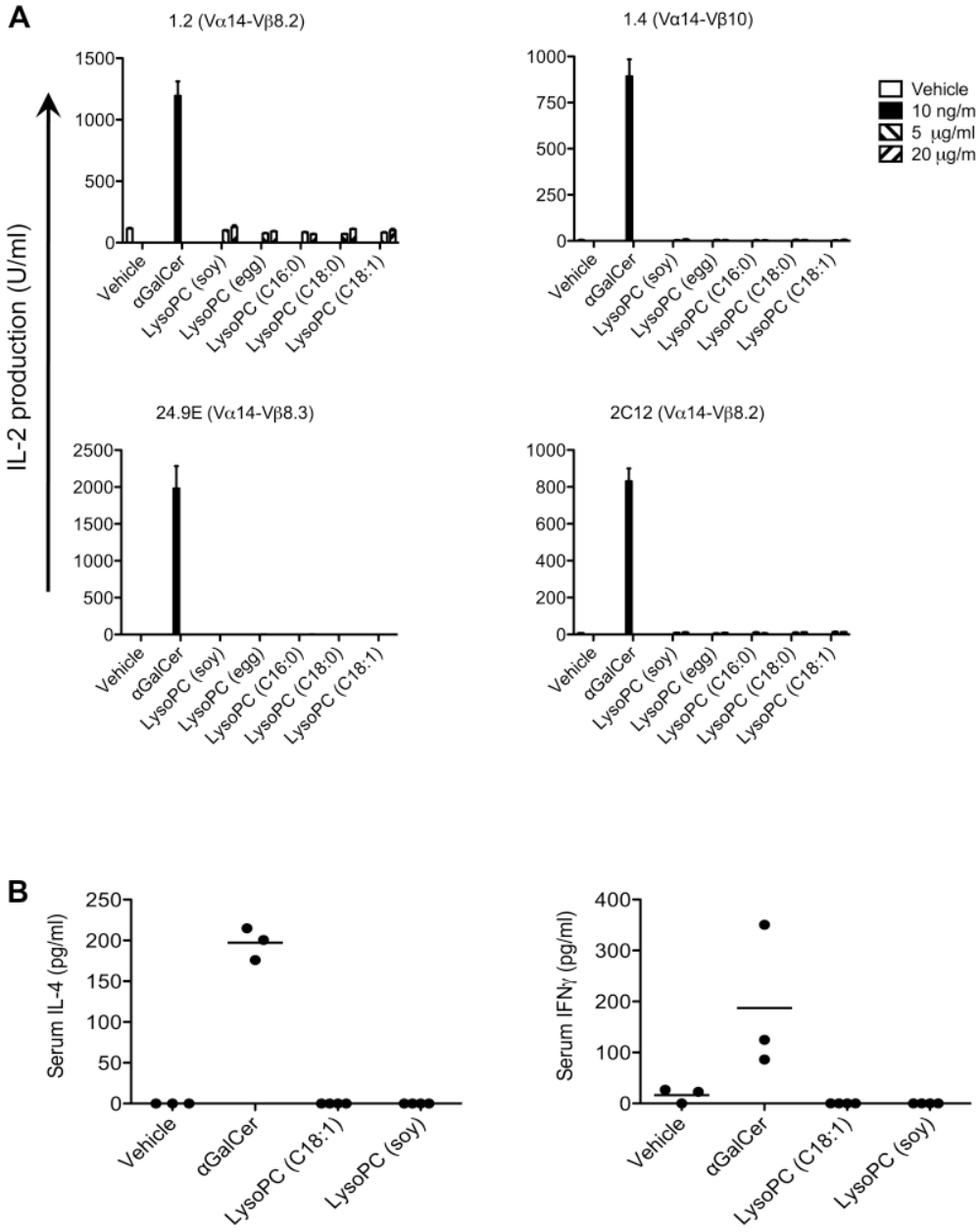

Figure 9. LysoPC did not stimulate mouse *i*NKT cell hybridomas

(A) *i*NKT cell hybridomas 1.2, 1.4, 24.9E and 2C12 were cultured with A20/CD1d APCs incubated with the indicated lipid Ags, including synthetic (C16:0, C18:0, C18:1) and purified (from egg and from soy) sources of lysoPC, vehicle negative control and αGalCer positive control. Hybridoma activation was measured by ELISA for IL-2. (B) Mouse BMDCs pulsed with vehicle, 10 ng/ml αGalCer, 20 μg/ml synthetic lysoPC (C18:0) or purified lysoPC (from soy) were injected to mice intravenously. Blood was collected at 4 h and 14 h after immunization, and IL-4 (4 h, left) and IFNγ (14 h, right) in the serum were determined by ELISA.