Supporting Information

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SI Materials and Methods

Lipids and Source Materials for Lipid Extractions. Use of human tissues and animals was approved by the Brigham and Women's Hospital institutional review board and the Dana–Farber Cancer Institute Animal Care and Use Committee, respectively. C57BL/6 mice were from Jackson Laboratories. Whole-fat cow's milk was from Hood or Nestle. Skim cow's milk was from Nestle. Human milk samples were from Mothers' Milk Bank Northeast. Mouse milk was collected from C57BL/6 mothers of suckling pups aged 5–14 d as described (1). Similac advance was used as a cow's milk-based infant formula. Soy milk was from White Wave Foods. Whey and casein were from Sigma. Calf thymus polar lipids were extracted from three pooled, freshly collected specimens (Research 87). Mouse thymus polar lipids were from 6-wk-old C57BL/6 female mice. FBS was from Gemini Bio (BenchMark) or Thermo Scientific (HyClone). Human, mouse, rat, rabbit, and horse sera were from Atlanta Biologicals. Sheep, pig, and chicken sera were from Sigma. Goat serum was from Atlanta Biologicals or Sigma. Fish serum (SeaGrow) was from East Coast Bio. β-GlcCer d18:1/24:1, β-GlcCer d18:1/18:1, β-GlcCer d18:1/18:0, β-GlcCer d18:1/16:0, ceramide d18:1/24:1, and phosphatidylserine were from Avanti Polar Lipids. Cow's milk GlcCer, Gaucher's spleen GlcCer, Soy GlcCer, and diganglioside GD1a were from Matreya. α-GlcCer d18:1/24:1 and α-GlcCer d18:0/26:0 were synthesized as described (2). In some figure legends, the sphingoid base is not indicated for space considerations, and was d18:1 where omitted.

Polar Lipid Extractions. Lipid extraction methods for lyophilized milk and thymus samples have been described (3). Serum lipids were extracted using a modified Folch method (4). Briefly, 10 volumes of 2:1 $CHCl_3$:CH_3OH were added to serum and vortexed. The aqueous upper phase containing precipitated proteins was removed. To the lower phase, 0.2 volumes of water was added, and the mixture vortexed. The lower layer from the resulting biphase contained the polar lipids including GlcCer, and was dried down. The dried lipids were resuspended in 3.3 volumes 10:1 CH_3OH:water (vol/vol) and biphased against 3.3 volumes petroleum ether three times to remove nonpolar lipids. Six volumes of $CHCl_3$ and 2 volumes water were added to the lower methanolic phase. From the resulting biphase, the lower phase was dried to recover polar lipids.

TLC. HPLC grade water, $CHCl_3$, CH_3OH, isopropanol, and hexane were from Fisher Scientific. 10-cm aluminum or plastic silica gel 60 TLC plates were used (EMD Millipore Chemicals). For normal phase TLC, the mobile phase was 65:25:3.75 $CHCl_3$:CH_3OH:water (vol/vol/vol). For HICMW TLC, the mobile phase was 25:35:0.75:35:5 hexane:isopropanol:$CHCl_3$:CH_3OH:water (vol/vol/vol/vol/vol). For preparative TLC, lipids from the indicated retention time were recovered from the scraped silica by vortexing in 2:1 $CHCl_3$:CH_3OH (vol/vol). Lipids were visualized with α-naphthol/sulfuric acid (Sigma) followed by charring.

GCase Digestion. Recombinant human lysosomal glucocerebrosidase, Velaglucerase Alfa (5), was from Shire. GCase was reconstituted per the manufacturer's instructions and stored at −80 °C. Digestions were carried out as previously described (6), with some modifications, in 50 mM citrate/50 mM phosphate buffer pH 5.5 with 0.25% sodium taurocholate and 0.5 mg/mL BSA (all digestion buffer components from Sigma). The lipid for digestion was dried and resuspended in the reaction mixture at up to 250 μg/mL with a 1:20 molar ratio of phosphatidylserine added (7), and the mixture was sonicated for 30 seconds. GCase was added at 1 U per mg of lipid, and the digestion was carried out at 37 °C for 16 h on an orbital shaker. To recover lipids from the aqueous reaction mixture, 5 volumes of 2:1 $CHCl_3$:CH_3OH were added, followed by vortexing for 15 min. The lower phase was recovered and dried. The GlcCer retention time was recovered from this crude extract by normal phase preparative TLC for assay.

In Vitro Activity Assays. For mouse assays with the invariant natural killer T (iNKT) cell hybridoma DN32 (8) and CD1d-transfected (RAW-CD1d) or untransfected (RAW-WT) RAW-264.7 cells were used (9). DN32 cells (5×10^4) were cultured with 5×10^4 antigen-presenting cells per well in flat-bottom 96-well plates for 16 h. Where indicated, L363 or mouse IgG2a, κ isotype (eBioscience) was added at 5 μg/mL Anti-mouse CD3ε (145-2C11) was from BD Biosciences. The plate-bound CD1d assay has been described (10). Briefly, lipids were loaded into biotinylated CD1d monomers (NIH Tetramer facility) at a 50:1 molar ratio (using the starting lipid equivalent before digestions) in PBS pH 7.4 (Life Technologies) with 0.05% tween-20 (Sigma) for 16 h at 37 °C. Lipid-loaded CD1d was bound to a streptavidin-coated plate for 30 min in PBS followed by washing. A total of 10^5 cells of a primary mouse iNKT cell line (11) derived from C57BL/6 splenocytes were added for 16 h. For human assays using a primary iNKT cell line (10) or the J3N.5 clone (12), freshly isolated, magnetically sorted (Miltenyi) $CD14^+$ peripheral blood mononuclear cells were cultured with GM-CSF and IL-4 (Peprotech) to derive antigen-presenting cells, as described (12). iNKT cells (5×10^4) were cultured with 5×10^4 antigen-presenting cells per well in flat-bottom 96-well plates for 16 h.

All assays were performed in RPMI-1640 media supplemented with 10% FCS (Gemini Bio), penicillin/streptomycin, L-glutamine and β-mercaptoethanol (Life Technologies) at 37 °C in 5% CO_2. Lipids were dried under nitrogen and sonicated in media for assay. ELISA was performed using high capacity 96-well plates (Costar). Mouse IL-2, mouse IFN-γ, and human IFN-γ for ELISA standards were from Peprotech. For bar graphs and X–Y graphs with error bars shown, SEM is shown.

L363 Flow Cytometry. For flow cytometric analysis of L363 binding, RAW-CD1d cells were cultured overnight with 10 μg/mL α-GlcCer d18:1/24:1 in ultra-low attachment 24-well plates (Corning) at 5×10^5 cells per well. Cells were collected the next day and stained with L363-phycoerythrin (eBioscience) for 30 min at 4 °C in PBS with 0.5% FBS and 2 mM EDTA (Life Technologies), washed, and analyzed using a FACSCanto II system (BD Biosciences) and analyzed using FlowJo (TreeStar).

Mass Spectrometry and NMR Spectroscopy. Collision-induced dissociation tandem mass spectrometry ($CID\text{-}MS^n$) was performed on a Thermo-Finnigan linear ion-trap mass spectrometer with an Xcalibur operating system at the University of Washington, St. Louis. For large-scale fractionation of cow's milk GlcCer, the fractions indicated in Fig. 6*B* and Fig. S8*B* were purified from 50 mg of starting material, and the entire sample was analyzed by direct injection. NMR was performed at the University of Georgia Complex Carbohydrate Research Center. For NMR experiments, samples were dissolved in CD_3OD and dried under a stream of nitrogen and then dissolved in 0.5 mL of 2:1 $CDCl_3$-CD_3OD. One-dimensional proton and zero-quantum filtered

TOCSY (zTOCSY) NMR spectra were acquired on a Varian VNMRS 900 MHz spectrometer at 27 °C. The 1D-proton spectra were signal-averaged from 4,000 scans, and the 1D-zTOCSY spectra from 45,000 scans. The spin-lock time in the zTOCSY experiments was 70 ms. Chemical shifts were measured relative to the residual $CHCl_3$ signal (δ = 7.489 ppm).

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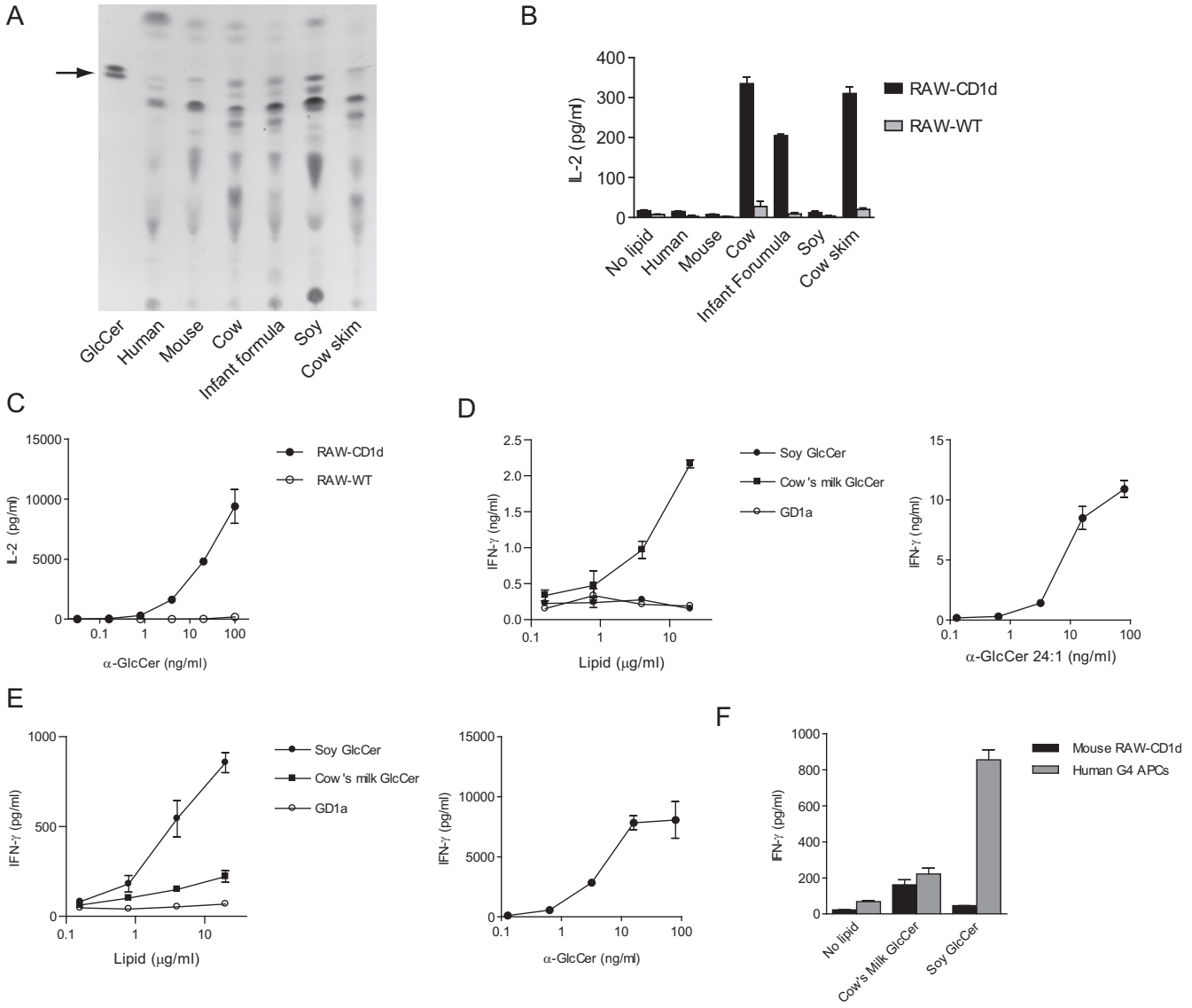


Fig. S1. Activity of milk polar lipids and GlcCer fractions on human and mouse iNKT cells. (*A*) Normal phase TLC of polar lipids extracted from various milks. For each sample, 100 μg of polar lipids were loaded, and retention time was visualized with α-naphthol stain. An arrow marks the retention time of a GlcCer standard (cow's milk GlcCer). (*B*) Polar lipids from various milks were cocultured with the DN32 mouse iNKT cell hybridoma and CD1d transfected (RAW-CD1d) or untransfected (RAW-WT) antigen-presenting cells. (*C*) For comparison of the response magnitudes in Fig. 1*A*, the activity of α-GlcCer d18:1/24:1 cocultured with the DN32 iNKT cell hybridoma and RAW-CD1d or RAW-WT cells is shown. (*D*) A primary human iNKT cell line was cocultured with soy GlcCer or cow's milk GlcCer and human GM-CSF/IL-4-induced antigen-presenting cells. Diganglioside GD1a shown as a nonstimulatory lipid control. In the right panel, the response of a primary human iNKT cell line to α-GlcCer d18:1/24:1 is shown. (*E*) The J3N.5 iNKT cell clone was cocultured with cow's milk GlcCer or soy GlcCer and human GM-CSF/IL-4-induced antigen-presenting cells. Diganglioside GD1a shown as a nonstimulatory lipid control. In the right panel, the activity of α-GlcCer d18:1/24:1 in this system for comparison of response magnitude. (*F*) The J3N.5 human iNKT cell clone was cocultured with cow's milk GlcCer or soy GlcCer and human GM-CSF/IL-4-induced antigen-presenting cells or mouse RAW-CD1d cells to determine whether species-specific CD1d variations might contribute to soy GlcCer recognition.

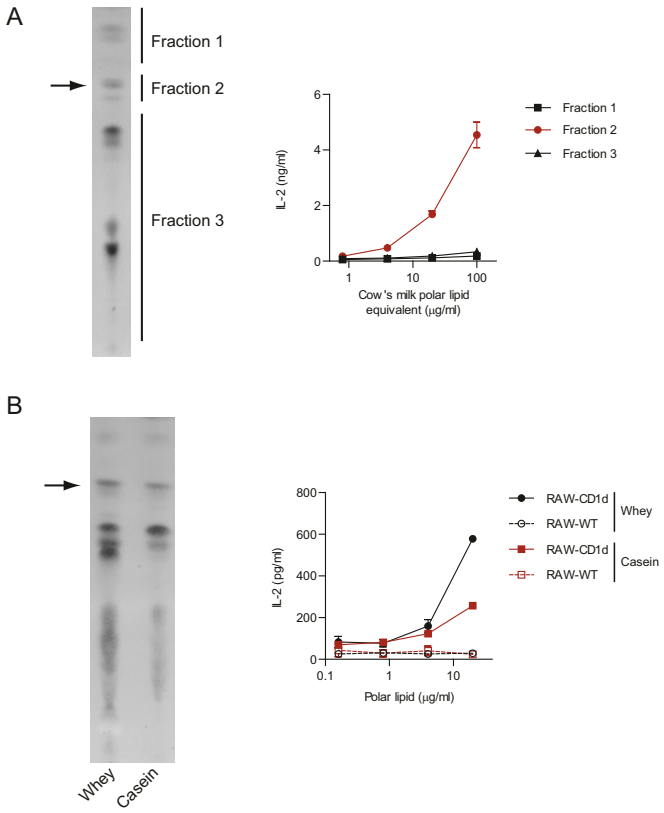


Fig. S2. Fractionation of lipid antigen activity in cow's milk. (*A*) Cow's milk polar lipids were analyzed by TLC and fractionated as indicated by preparative TLC. An arrow marks the retention time of a GlcCer standard. The resulting fractions were assayed for activity by coculture with DN32 and RAW-CD1d or RAW-WT cells. The x-axis values indicate the equivalent amount of lipid added based on the starting material before fractionation. (*B*) Polar lipids were extracted from casein and whey and analyzed by TLC. An arrow marks the retention time of a GlcCer standard. Polar lipids were assayed for activity by coculture with DN32 and RAW-CD1d or RAW-WT cells. Data presented are release of IL-2 by DN32 as measured by ELISA.

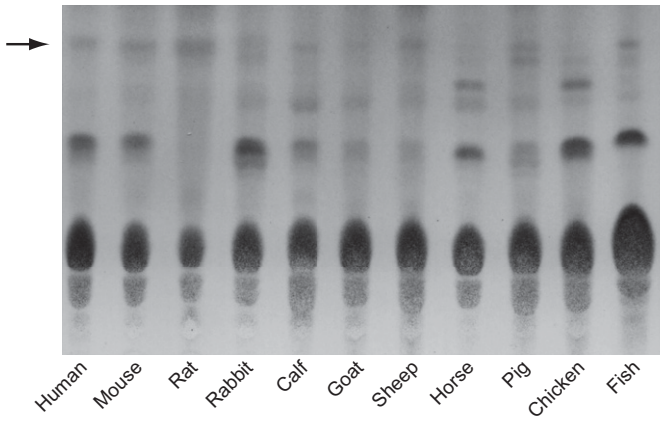


Fig. S3. Polar lipids from the sera of 11 animal species. Polar lipids were extracted from the sera of the species indicated. Polar lipids (200 μg) were spotted on a silica plate and analyzed by normal phase TLC. Retention time was visualized with α-naphthol stain. An arrow marks the retention time of a GlcCer standard.

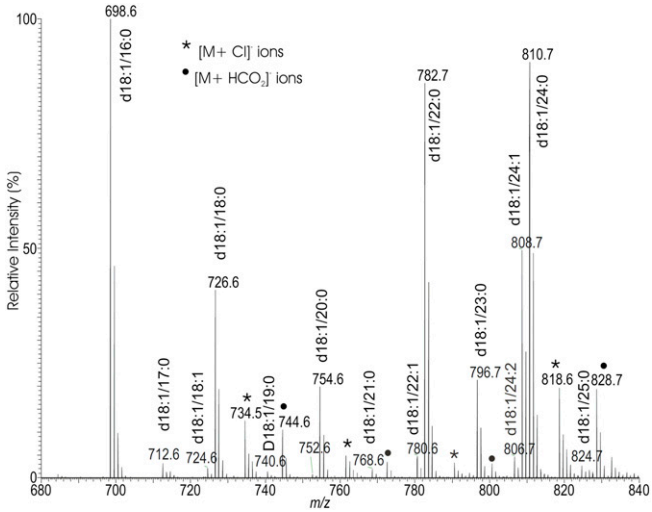
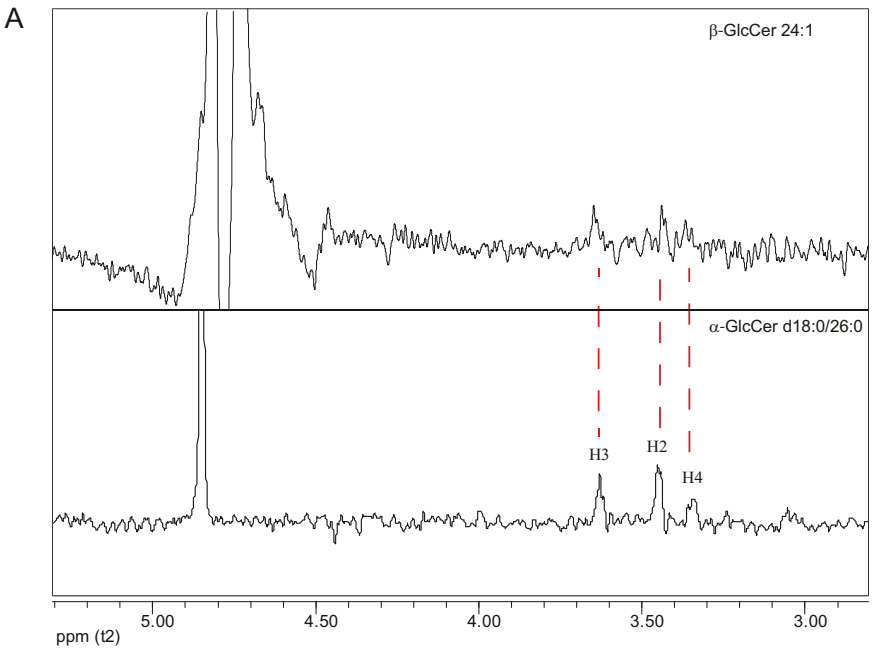


Fig. S4. CID-MS^n analysis of GlcCer from the spleen of a patient with Gaucher's disease.



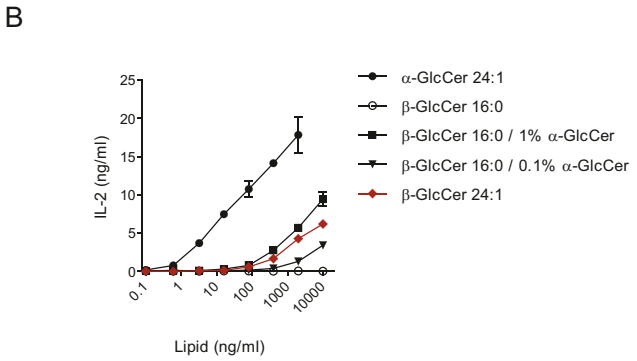


Fig. S5. (*A*) 1D-zTOCSY NMR of candidate α-glucose H1 peaks was performed for a synthetic β-GlcCer d18:1/24:1 or α-GlcCer d18:0/26:0 (phytosphingosine base). Resonant peaks corresponding the H1, H2, H3, and H4 of α-glucose were detected. (*B*) Titration of a highly active α-GlcCer d18:1/24:1 with an inactive β-GlcCer d18:1/C16:0. Lipids were cocultured with DN32 and RAW-CD1d or RAW-WT cells.

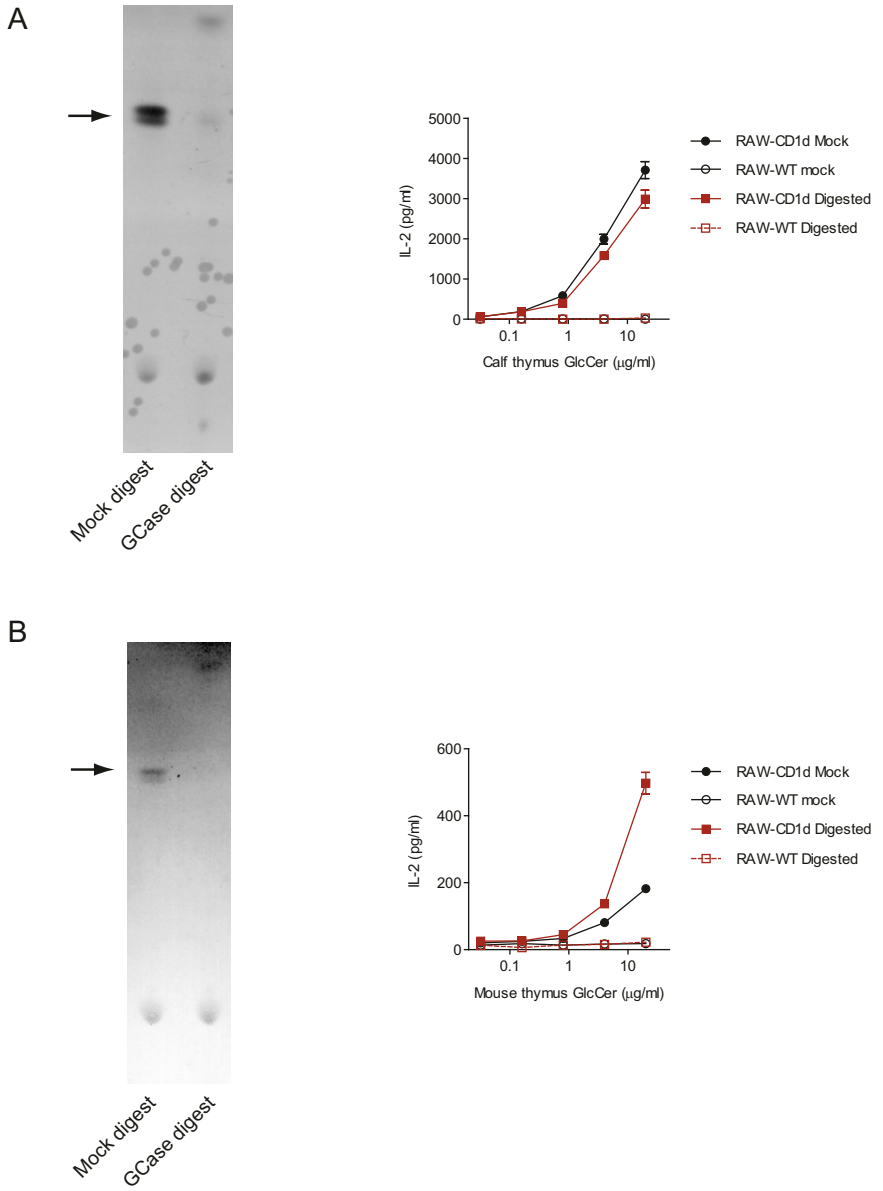


Fig. S6. GCase digestion of thymus GlcCers. Normal phase TLC of calf (*A*) or mouse (*B*) thymus GlcCer either GCase digested, or mock-digested (no enzyme). For each sample, 2 μg of lipid (or the equivalent amount of lipid based on the amount added to the enzymatic digestion) was loaded and retention time was visualized with α-naphthol stain. An arrow marks the retention time of a GlcCer standard. Antigenic activities (*Right*) of thymus GlcCers, either digested or mock-digested was measured by coculture with DN32 and RAW-CD1d or RAW-WT cells after repurification by preparative TLC.

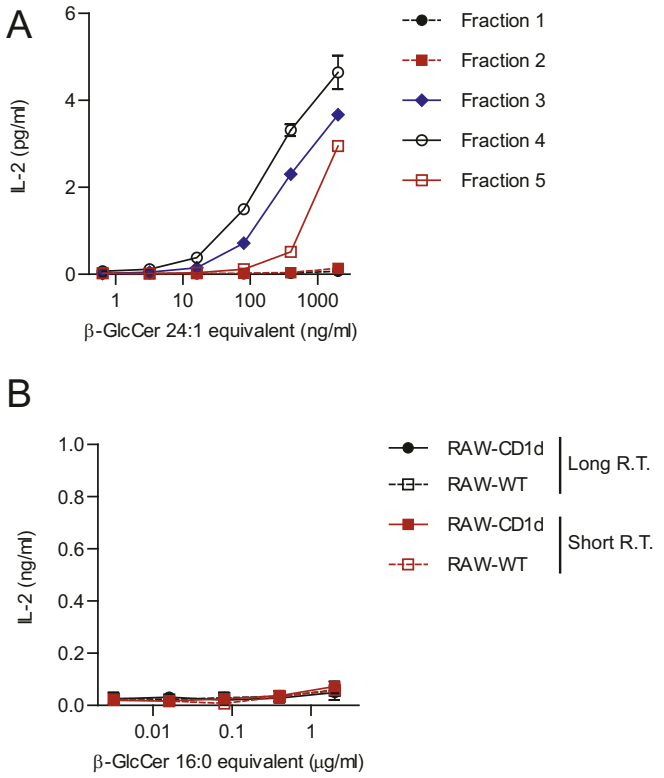


Fig. S7. HICMW TLC fractionation of synthetic GlcCers. (*A*) β-GlcCer d18:1/24:1 synthetic was fractionated in the same manner as was performed for cow's milk GlcCer in Fig. 5C. The indicated fractions were tested for activity by cocultured with the DN32 and RAW-CD1d or RAW-WT cells. (*B*) β-GlcCer d18:1/16:0 synthetic was fractionated in the same manner as was performed for β-GlcCer d18:1/24:1 in Fig. 5*A*. The indicated fractions were tested for activity by coculture with the DN32 and RAW-CD1d or RAW-WT cells. Data presented are release of IL-2 by DN32 as measured by ELISA.

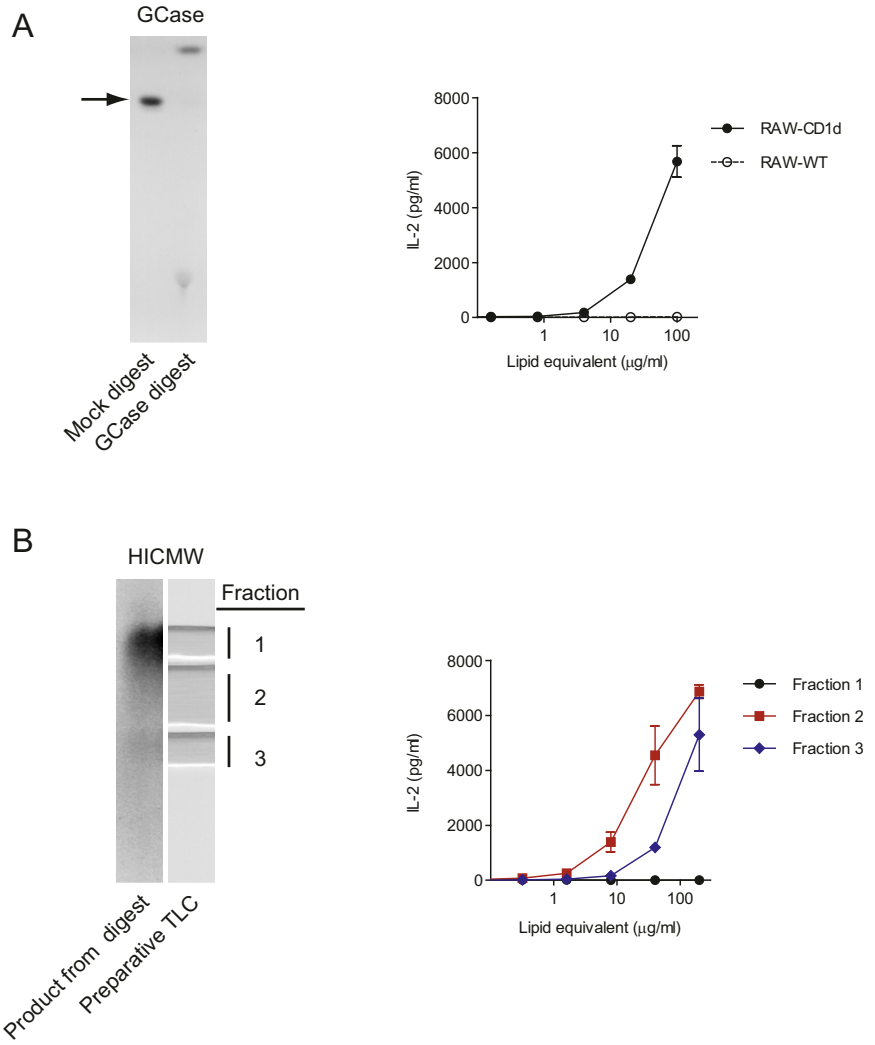


Fig. S8. Large-scale enrichment of the active fraction in cow's milk GlcCer. Activity data for the samples used in Fig. 6 are shown. (*A*) Normal phase TLC of cow's milk GlcCer either GCase digested, or mock-digested. An arrow marks the retention time of a GlcCer standard. Antigenic activity of cow's milk GlcCer following digestion was measured by coculture with the DN32 and RAW-CD1d or RAW-WT cells after repurification by preparative TLC. (*B*) The digested product was fractionated by preparative HICMW TLC as shown. HICMW preparative TLC fractions were assayed for activity by coculture with DN32 and RAW-CD1d or RAW-WT cells. Data presented are release of IL-2 by DN32 as measured by ELISA.