Supporting Information

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SI Materials and Methods

Glycolipid Analogues. We have synthesized a focused library of 25 α-GalCer analogues, whose structures are listed in Fig. 1 in the main text. The synthesis of these analogues is summarized in Fig. S1. The analogues C10, 6DW116C7, C11, 6DW116C9, C15, C16, C27, C28, and C29 possess a terminal benzene ring after various lengths at a fatty acyl chain. Three groups of analogues—group 1 (C18–C21), group 2 (C22–C25), and group 3 (C26, 7DW8-4, 7DW8-5, and 7DW8-6)—have modified phenyl groups at positions C6, C8, and C11, respectively. Analogues 6DW135, 6DW138, C12, and C13 have a terminal benzene ring at the end of a truncated phytosphingosine chain.

Animals. Six- to 8-wk-old BALB/c and C57BL/6 mice were purchased from Taconic Farms. C.129S2-Cd1^{tm1Gru}/J mice, a homozygous mutation mouse strain that lacks both *Cd1d1* and *Cd1d2* genes, were purchased from Jackson Laboratory. All mice were maintained under standard conditions in the Laboratory Animal Research Center at Rockefeller University.

Quantification of the Level of Cytokines Produced by Human *i*NKT Cell Lines by ELISA. The levels of IFN-γ, IL-4, GM-CSF, and IL-12 produced by human *i*NKT cell lines were determined by ELISA (eBiosciences). Twenty thousand human *i*NKT cells were cocultured with 2×10^4 HeLa cells transfected with the human CD1d gene, HeLa-hCD1d (provided by Steven Porcelli, Albert Einstein College of Medicine, Bronx, NY), or immature DCs in the presence of the indicated amount of each glycolipid. In some experiments, nM was used whereas μg/mL was used in other experiments. One nM is equal to 7.4×10^{-4} μg/mL of 7DW8-5 and 8.6×10^{-4} μg/mL of α-GalCer, respectively. After 24 h incubation, the cultured supernatants were collected, and concentrations of IFN-γ, IL-12, GM-CSF, and IL-4 were determined by ELISA.

Quantification of the Level of IL-2 Produced by a Mouse *i*NKT Hybridoma by ELISA. Twenty thousand cells of a mouse *i*NKT hybridoma, 1.2, were cocultured with 2×10^4 A20 cells transfected with mouse CD1d gene in the presence of indicated amounts of each glycolipid. After 24 h incubation, the culture supernatants were collected and the concentrations of IL-2 in the supernatants were determined by ELISA (R&D Systems).

Quantification of the Relative Number of Human PBMCs Secreting IFN-γ in an ELISPOT Assay. The relative number of human PBMCs that secrete IFN-γ were determined by an ELISPOT assay. Briefly, after coating 96-well Multiscreen-HA plate (Millipore) with anti–IFN-γ capture antibody (Mabtech), 5×10^5 CD14$^-$ PBMCs were cocultured with 5×10^4 autologous immature DCs in the presence of 0.1 μg/mL of each glycolipid. After 24 h incubation, the plate was washed and incubated with biotinylated anti–IFN-γ antibody and developed by adding ACE substrate (BD Bioscience). Numbers of spot-forming cells were counted under a stereomicroscope.

A Competitive ELISA with 18:1 PE Lipid as an Indicator. Affinities between glycolipid analogues and human or mouse CD1d molecules were measured by a competitive ELISA, using 18:1 Biotinyl PE [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Biotinyl); Avanti Polar Lipids], as an indicator, as previously described (1, 2). After coating 96-well ELISA plates with 3 μg/mL goat antimouse IgG1 antibody, 1 μg/mL of hCD1d:mIgG dimer or mCD1d:mIgG dimer (BD Biosciences) was added and incubated for 2 h. Glycolipid analogues were serially diluted with PBS solution in the presence of 1μg/mL 18:1 Biotinyl PE lipid and then added into wells, followed by overnight incubation at 37 °C. Caproylamine PE lipid [18:1; 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(hexanoylamine); Avanti Polar Lipids] was used as a positive control. After incubation for 1 h and washing, Biotinyl PE-CD1d complex was detected with HRP-labeled Avidin (eBioscience). Inhibition curves were constructed using GraphPad Prism software (version 4.0; GraphPad).

Loading hCD1d:Ig Dimer with Glycolipids and Staining *i*NKT cells. hCD1d: mIgG dimer molecules were conjugated with phycoerythrin with a Lynx Rapid RPE antibody conjugation kit (ABD SeroTec). Phycoerythrin-labeled hCD1d:Ig dimer molecule was then loaded with 1000-fold molar ratio excess of each glycolipid overnight at 37 °C, which was then used to stain human *i*NKT cells as previously described (3, 4). Briefly, various doses, ranging from $10^{-1.5}$ to $10^{2.5}$ nM, of phycoerythrin-labeled hCD1d dimer loaded with each glycolipid and 10 μg/mL of FITC-labeled anti-CD3ε mAb were incubated with 2×10^5 human *i*NKT cells on ice for 30 min. After washing the cells, the stained cells were gated with CD3 and analyzed with FACS LSRII instrument (Becton Dickinson). The level of dimer fluorescence was plotted against dimer concentration. Binding curves were fitted by Graphpad Prism software.

Mouse Immunization. Mice were immunized with a suboptimal dose, 5×10^7 pfu or 5×10^6 pfu, of a recombinant adenovirus expressing HIV p24 antigen, Ad-p24, or that expressing a circumsporozoite protein of a rodent malaria parasite *P. yoelii*, AdPyCS. Mice were also immunized twice with 50 μg of a recombinant plasmid DNA expressing HIV p24 antigen, DNA-p24, at a 3-wk interval.

Human Subjects. Human PBMCs from anonymous blood donors were obtained from leukopacks provided by the New York Blood Center. New York Blood Center does not select donors on the basis of sex or race but ensures that all donors are at least 18 y of age. The work we performed, therefore, did not require an approval from the institutional review board.

1. Li XGC, Garcia-Navarro R, Franck RW, Tsuji M (2009) Identification of C-glycoside analogues that display a potent biological activity against murine and human invariant natural killer T cells. *Immunology* 127:216–225.
2. Shiratsuchi T, Schneck J, Kawamura A, Tsuji M (2009) Human CD1 dimeric proteins as indispensable tools for research on CD1-binding lipids and CD1-restricted T cells. *J Immunol Methods* 345:49–59.
3. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M (2008) Methods for detection, isolation and culture of mouse and human invariant NKT cells. *Nat Protoc* 3:70–78.
4. Sidobre S, et al. (2002) The V alpha 14 NKT cell TCR exhibits high-affinity binding to a glycolipid/CD1d complex. *J Immunol* 169:1340–1348.
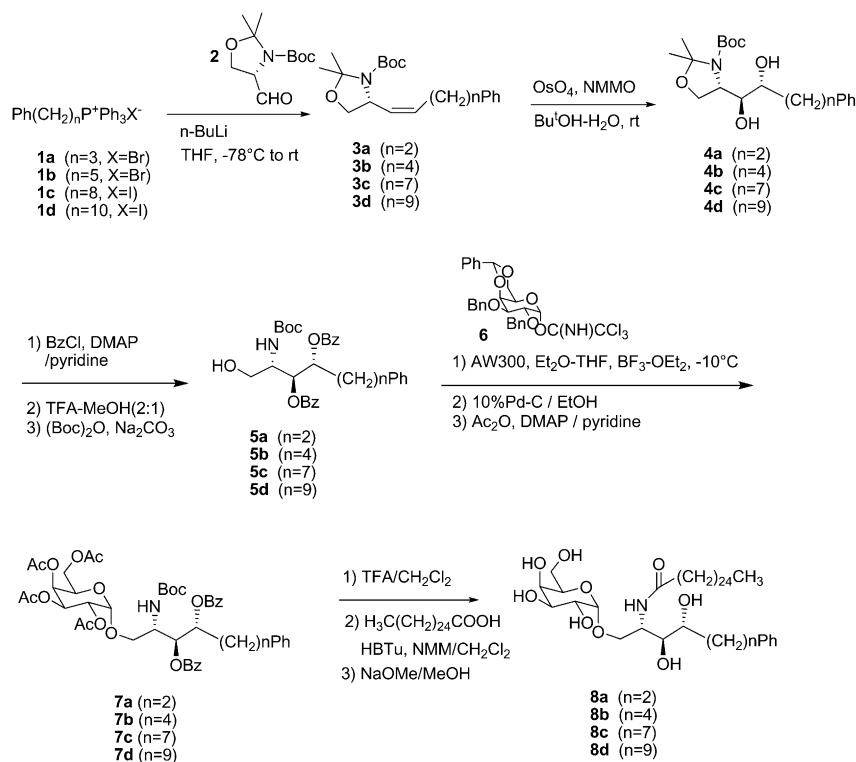
Fig. S1. The synthesis of a focused library of 25 α-GalCer analogues. Series B compounds were synthesized in a similar manner as the one we have already reported (1). Phytosphingosine chain analogues were synthesized according to the scheme described. Garner aldehyde 2 was coupled with Wittig reagents 1a–1d to give corresponding olefins 3a–3d. Compounds 3a–3d were treated with osmium tetroxide to give diols 4a–4d and an undesired isomer. The hydroxyl groups of the diol were protected with benzoyl groups and the isopropylidene group removed by TFA. After reprotection of the amine with Boc anhydride, the phytosphingosine analogues were glycosylated to yield mainly the α-linked product. After a series of protecting group manipulations, hexacosanoic acid was coupled to the free amine in the presence of HBTu. Deprotection of galactose protecting groups using 20% Pd(OH)$_2$ and deprotection of benzoyl groups using NaOMe afforded the target compounds. The synthesis of series A compound has been previously described (2).

1. Fujio M, et al. (2006) Structure-based discovery of glycolipids for CD1d-mediated NKT cell activation: tuning the adjuvant versus immunosuppression activity. *J Am Chem Soc* 128: 9022–9023.
2. Liang PH, et al. (2008) Quantitative microarray analysis of intact glycolipid-CD1d interaction and correlation with cell-based cytokine production. *J Am Chem Soc* 130:12348–12354.
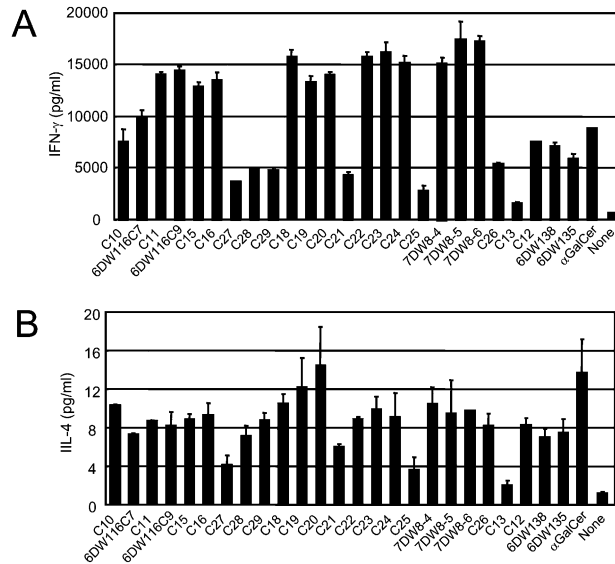
Fig. S2. Level of IFN-γ and IL-4 production by human *i*NKT cells in response to glycolipids presented by HeLa-hCD1d. Twenty thousand cells of a human *i*NKT cell line were cocultured with 2×10^4 human CD1d-transfected HeLa cells (HeLa-hCD1d) in the presence of 10 ng/mL of each glycolipid in a 96-well culture plate. After 24-h incubation, supernatants were collected and the concentrations of IFN-γ (*A*) and IL-4 (*B*) were determined by ELISA. Results represent one of three similar experiments using *i*NKT cell lines derived from different donors.
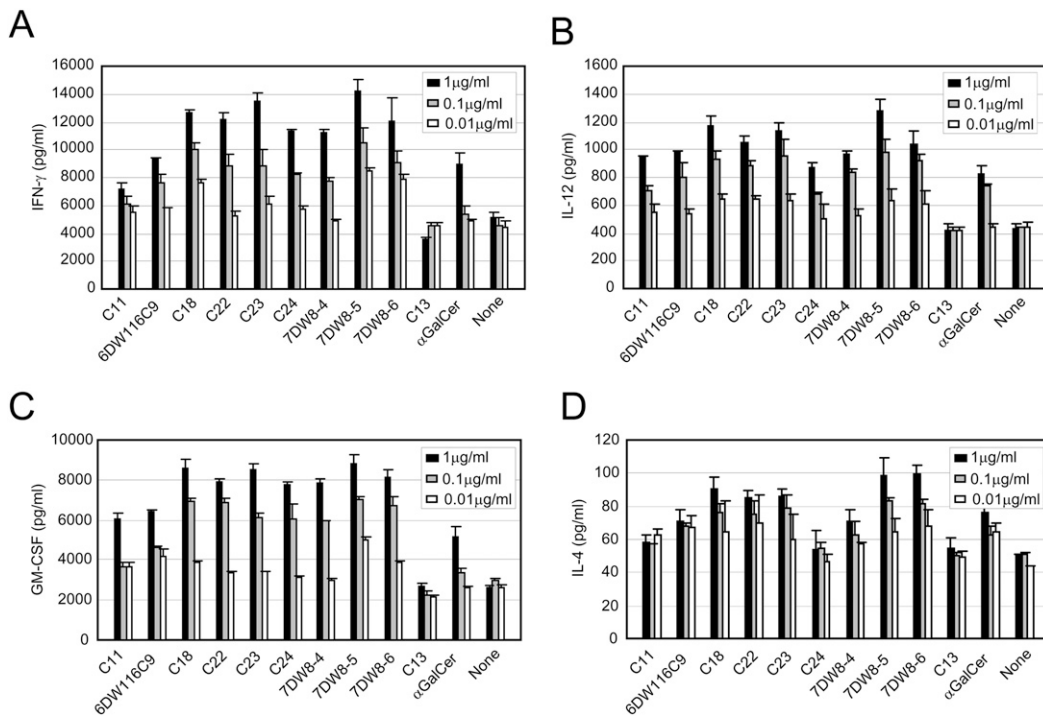
Fig. S3. Level of various cytokines produced by human *i*NKT cells in response to glycolipids presented by autologous DCs. Autologous immature DCs were generated from CD14+ PBMCs after 5 d incubation with GM-CSF and IL-4. Then, 2×10^4 cells of a human *i*NKT cell line were cocultured with 2×10^4 immature DCs in the presence of 0.1 μg/mL of glycolipids in a 96-well culture plate. After 24 h of incubation, supernatants were collected, and the concentrations of IFN-γ, IL-4, GM-CSF, and IL-12 were determined by ELISA. Error bars represent SD among triplicate wells. Results represent one of three similar experiments using *i*NKT cell lines derived from different donors.

A

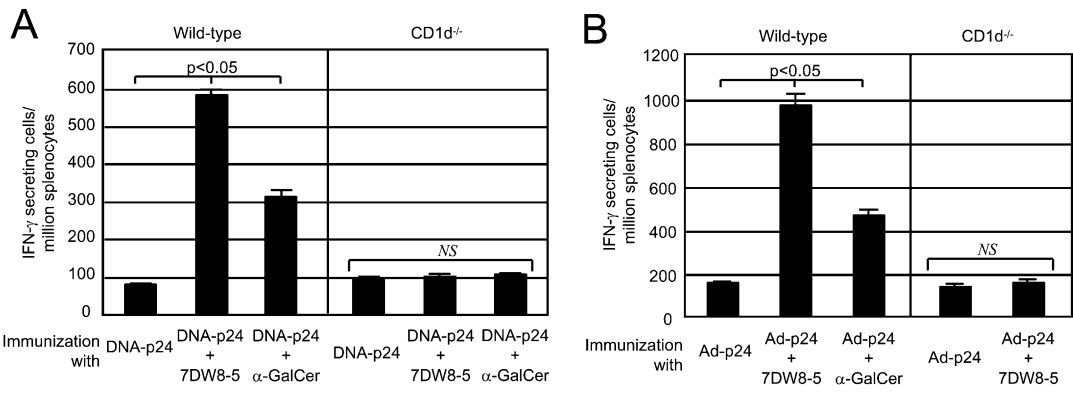| Glycolipid	# of spots in duplicate		Mean ± SD
C13	127	145	136 ± 9
C18	410	428	419 ± 9
C22	415	391	403 ± 12
C23	452	480	466 ± 14
7DW8-5	478	540	509 ± 31
7DW8-6	420	474	447 ± 27
α-GalCer	320	308	314 ± 6
None	102	104	103 ± 1

B

Glycolipid	# of spots in duplicate		Mean ± SD
C13	79	83	81 ± 2
C18	159	153	156 ± 3
C22	190	172	181 ± 9
C23	164	188	176 ± 12
7DW8-5	253	257	255 ± 2
7DW8-6	199	173	186 ± 13
α-GalCer	136	138	137 ± 1
None	78	76	77 ± 1

Fig. S4. Relative number of human PBMCs secreting IFN-γ in response to glycolipids presented by autologous DCs. Fifty thousand CD14⁻ PBMCs from two different donors (*A* and *B*) were cocultured with 5×10^4 autologous immature DCs in the presence of 100 ng/mL of each glycolipid in an ELISPOT plate, and 24 h later, the relative number of IFN-γ–secreting cells was determined by an ELISPOT assay. In both experiments, error bars represent the SD of duplicated wells.

A   CD86

Glycolipid	CD86 (MFI)
C13	599
C18	2795
C22	2685
C23	2971
7DW8-5	3416
7DW8-6	2665
α-GalCer	1830
DMSO	427

B   HLA-DR

Glycolipid	HLA-DR (MFI)
C13	670
C18	3825
C22	3907
C23	4031
7DW8-5	4746
7DW8-6	3877
α-GalCer	1814
DMSO	581

Fig. S5. The degree of DC maturation/activation induced by glycolipids. Human immature DCs (300,000) were first incubated with 0.1 μg/mL of each glycolipid for 1 h. Then, the DCs were cocultured with 6×10^5 human iNKT cells for 20 h, and the degree of DC maturation/activation was determined by FACS analysis. CD11c marker was used to gate on DCs, and the expression of DC maturation markers that include CD86 (*A*) and HLA-DR (*B*) were determined. DAKO Summit software was used to calculate mean fluorescence intensity. Results represent one of two similar experiments.

Fig. S6. Adjuvant effects of 7DW8-5 in CD1d-deficient mice. Groups of CD1d-deficient BALB/c mice, as well as WT BALB/c mice, were immunized intramuscularly with DNA-p24 (*A*) or Ad-p24 (*B*) together with 1 μg of each glycolipid. DNA-p24 immunized mice received additional boost with DNA-p24, as in Fig. 5 in the main text. Two weeks after the last immunization, splenocytes were collected and the relative number of IFN-γ–secreting CD8^{+} T cells was determined by an ELISPOT assay. In all experiments, data are expressed as mean ± SD of five mice, and results represent one of two similar experiments.