Supporting Information

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SI Materials and Methods

Glycolipid Analogs of α-Galactosylceramide (α-GalCer), Mice, and Cell Lines. All the glycolipids were dissolved in 100% DMSO at a concentration of 1–2 mg/mL. For in vivo experiments, all compounds were diluted to 1 μg/mL in saline just before injection of 100 μL diluted glycolipid or 100 μL 0.1% DMSO into mice. Pathogen-free BALB/c and C57BL/6 female mice aged 6–10 wk were obtained from the National Laboratory Animal Center. CD1d-deficient BALB/c mice were obtained from the Jackson laboratory. Mouse lung and breast cancer cell stable clones, TC1-GFP-Luc and 4T1-GFP-Luc, were established from TC1 and 4T1, respectively, and selected for the sustained expression of GFP and luciferase proteins.

Mouse Cancer Models. To monitor the survival of mice with lung metastasis, C57BL/6 mice (6–8 wk, female, $n = 5$) were injected intravenously with 2×10^5 syngenic lung cancer cells (TC1) suspended in 0.1 mL of PBS. Mice were treated intravenously with indicated glycolipids at 0.1 μg/mouse right after tumor inoculation. Glycolipid was repeated at the same dosage once a week for a total of four doses. The survival was monitored for 35 d.

To assess therapeutic effects of glycolipids on melanoma, C57BL/6 mice (6–8 wk, female) were injected subcutaneously with 2×10^5 syngenic melanoma (B16) cells suspended in 0.1 mL of PBS. After 3 d, groups of C57BL/6 mice ($n = 5$) were treated intravenously with the indicated glycolipids at 0.1 μg/mouse and repeated once a week for 4 wk. The tumor volume was recorded every 3 d for 24 d by measuring with a caliper along the long axis (a), the short axis (b), and the height (c). Tumor volumes (mm3) were calculated by the formula: $a \times b \times c$.

To detect the tumor growth on real-time, C57BL/6 mice (6–8 wk, female) were injected subcutaneously with 2×10^5 syngenic lung cancer (TC1-GFP-Luc) cells suspended in 0.1 mL of PBS. BALB/C mice (6–8 wk, female) were inoculated with 2×10^5 syngenic breast cancer (4T1-GFP-Luc) subcutaneously on the right lower back. In both cancer models, groups of mice ($n = 6$) were treated intravenously with indicated glycolipids at 0.1 μg/mouse 3 d after tumor implantation and repeated once a week for 4 wk. The real-time tumor growth was monitored by Xenogen's IVIS® 200 Series and Living Image® Software (Xenogen).

Binding Stability of Mouse CD1d (mCD1d)–Glycolipid Complexes with Vα14 *i*NKT Cells. DN3A4-1.2 hybridoma cells were incubated with mCD1d–glycolipid complexes for 1 h at 4 °C, and then stained by anti-mouse IgG1–PE antibody (BD Biosciences PharMingen) for bound mCD1d at 4 °Cfor 30 min. The unbound complexes were washed away, the anti-mCD1d (clone 1B1; BD Biosciences PharMingen) blocking antibody (40 μg/mL) was added to prevent the rebinding of the dissociated mCD1d–glycolipid complexes, and the cells were incubated at 37 °C with rocking. At the indicated time point, an aliquot of the dimer-stained cells was washed and fixed with 4% paraformaldehyde and analyzed by flow cytometry. The decay plot was drawn as the time versus log of (the percentage of the bound dimer complex at the indicated time/the percentage of the bound dimer complex at time = 5). Using linear regression in Graphpad Prism software, the half-life of mCD1d–glycolipid complex binding to murine *i*NKT cells was determined by $t1/2 = -\log 2/\text{slope}$.

Isolation and Generation of Human Vα24 *i*NKT Cell Lines and Immature Monocyte-Derived Dendritic Cells. The naïve Vα24 *i*NKT cells were separated from peripheral blood cells using anti-Vα24-FITC antibody (Beckman Coulter), followed by anti-FITC microbeads (Miltenyi Biotec). The isolated NKT cells were incubated in media containing 50 U/mL IL-2 (R&D Systems) and replenished with fresh media every 3 d. Anti-CD14 mAbs coupled to magnetic beads (Miltenyi Biotec) were used to isolate CD14+ cells from leukopaks. Immature dendritic cells were generated from the CD14+ cells after 2-d incubation in the presence of 300 units/mL GM-CSF (R & D Systems) and 100 units/mL IL-4 (R&D Systems). Vα24 *i*NKT cell lines pulsed with C1, C34, C17, or DMSO only were generated as follows. After irradiation with 2,000 rad, the immature dendritic cells were cocultured with syngenic Vα24 *i*NKT cells in the presence of mock (DMSO only) or different glycolipids (C1, C34, and C17) at 100 ng/mL for 1 d. The cells were expanded in the presence of 50 U/mL IL-2 for 10–14 d after lipid removal. The same procedures were repeated once for further stimulation and expansion of *i*NKT cells. All *i*NKT cell lines (naïve, mock, C1, C34, and C17 pulsed) were shown to express Vα24 T cell antigen receptor (>95% purity).

Determination of Cytokines Secretion by Human *i*NKT Cells. The 1×10^5 naïve Vα24 *i*NKT cells were cocultured with 5×10^4 irradiated, immature CD14+ dendritic cells in the presence of glycolipids at 100 ng/mL in a 96-well plate. After 18 h, the supernatants were collected for the measurement of IFN-γ, IL-4, and IL-10 productions using Beadlyte® Human Cytokine kit and Luminex® 100™ reading system.

Fluorescence Activated Cell Sorter (FACS) Analysis of Vβ Usages in Mice and in Humans. Pooled B6 splenocytes ($n = 3$) were stimulated in vitro with indicated glycolipids at 100 ng/mL for 3 d and analyzed with Vβ8.1/8.2, Vβ7-specific antibodies (BD Biosciences PharMingen) by FACS within the gated NK1.1 + /CD3+ population. Human Vα24 *i*NKT cell lines expanded by C1, C34, C17, and mock were further purified by magnetic cell sorting (MACS) and stained with Vβ11 antibody (Beckman Coulter) for 30 min, and cells were washed and analyzed by flow cytometry.

Binding Avidity of CD1d-Loaded Glycolipids with Vα24 *i*NKT Cells. Human CD1d:Ig dimer (BD Biosciences PharMingen) was loaded with glycolipids or vehicle at a molar ratio of 1:10 at 37 °C overnight. C1-expanded Vα24 *i*NKT cell line was incubated with various concentrations of dimer-glycolipid complexes for 30 min at 4 °C, followed by staining with anti-mouse IgG1–PE mAb (A85-1) for another 30 min in the buffer containing azide (0.05%) to prevent internalization. After washing away the unbound dimer-glycolipid complexes, the cells were stained by anti-Vα24 and anti-Vβ11 antibodies for 30 min at 4 °C. After washing and fixation with 4% paraformaldehyde (PFA), the bound dimer-glycolipid complexes in the Vα24 + /Vβ11+ population were analyzed by flow cytometry. The binding curve and linear fit of the Scatchard transformation were plotted by Graphpad Prism software.

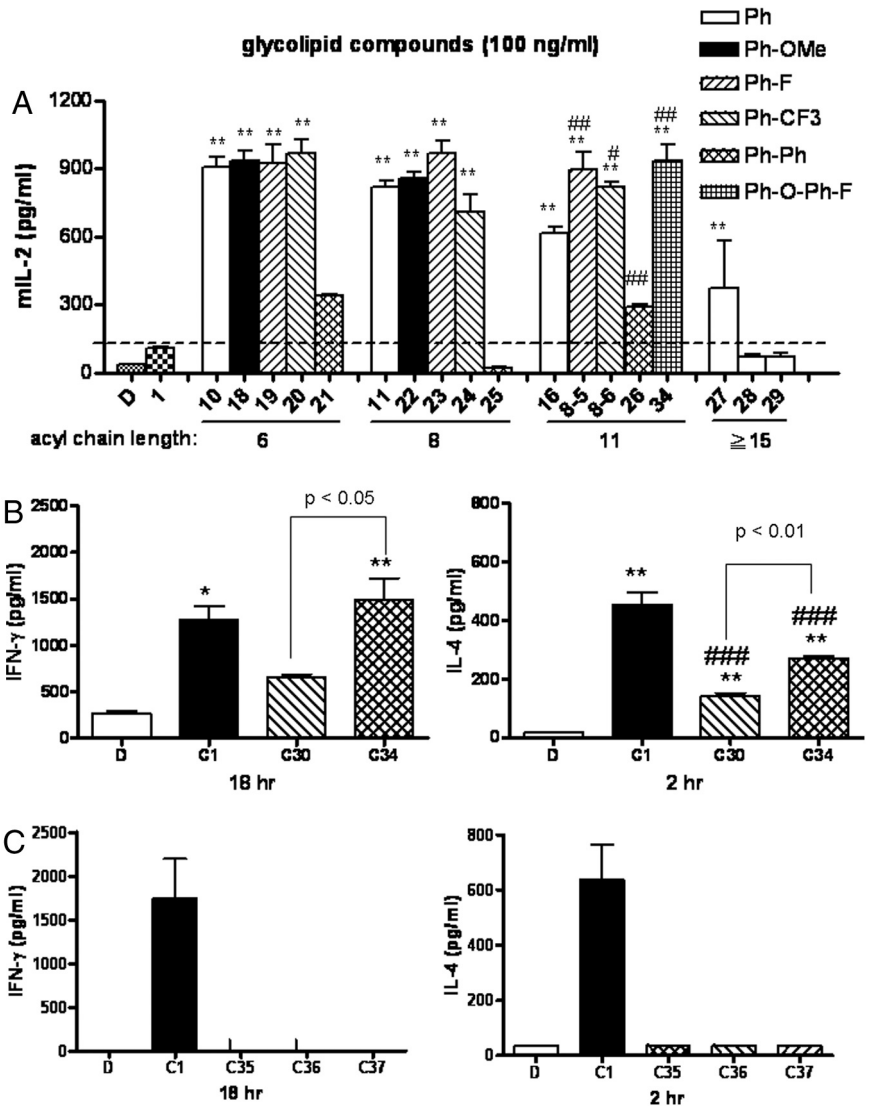


Fig. S1. The capacity of α-GalCer analogs to induce cytokines in murine Vα14 *i*NKT hybridoma or mouse serum. (*A*) Cytokine production by murine NKT hybridoma in vitro. 1×10^5 murine DN3A4-1.2 hybridoma cells were cultured in a mCD1d-coated 96-well plate and pulsed with the indicated glycolipid antigens at 100 ng/mL for 18 h. IL-2 secretion in the supernatant was measured by ELISA. Assays were performed in triplicates, and data were presented as mean ± SD. The symbol ** represents $p < 0.01$, compared to C1; #, $p < 0.05$; and ##, $p < 0.01$, compared to C16 (one-way ANOVA). (*B*–*C*) Cytokine induction in mice by glycolipids in vivo. Sera from BALB/C mice were collected at 2 and 18 h after intravenous injection with indicated glycolipids at 0.1 μg/mouse. The secretions of IFN-γ and IL-4 were measured from three mice after stimulation, and the values were presented as mean ± SD. The symbol * represents, $p < 0.05$; **, $p < 0.01$, compared with DMSO; and ###, $p < 0.001$, compared with C1 (one-way ANOVA). The comparison between C30 and C34 was shown on the figure with *p* values using a two-tailed Student t test.

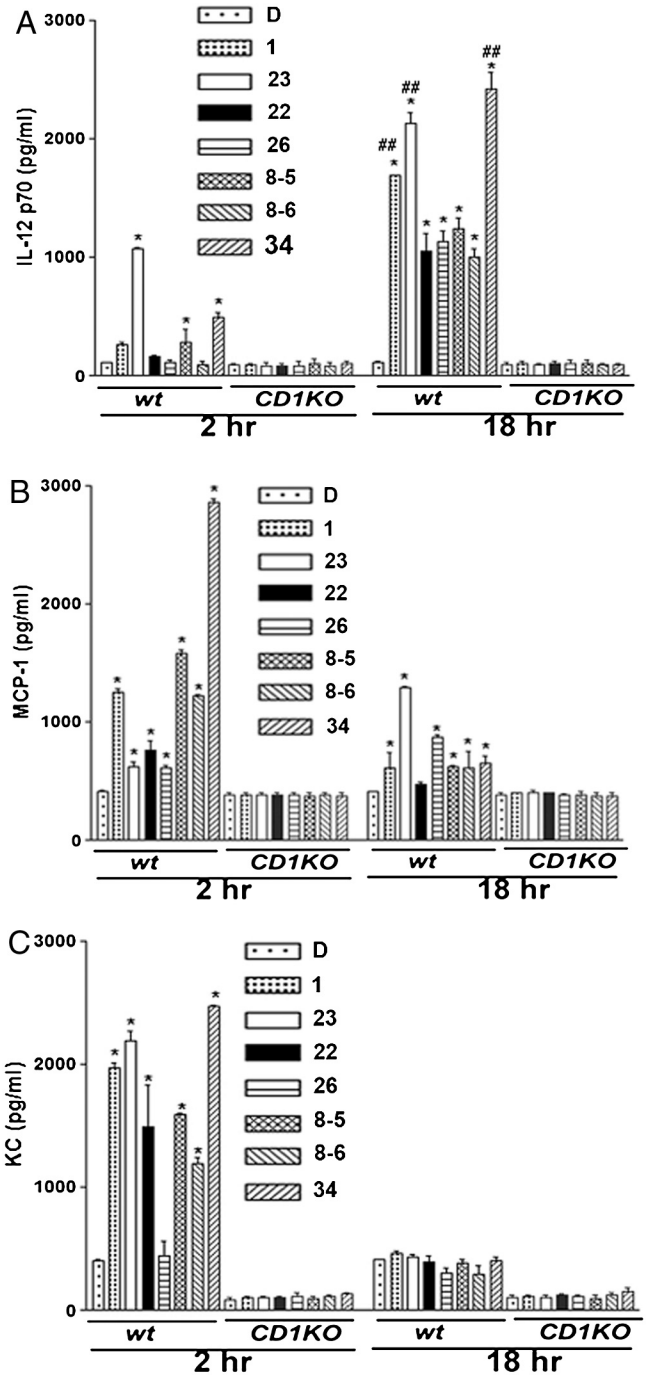


Fig. S2. Phenyl glycolipids induced secretions of IL-12p70, MCP-1, and KC were CD1d dependent in mice. Wild-type and CD1d knockout BALB/c mice were i.v. injected with indicated glycolipids (0.1 μg/mouse) or vehicle. Sera collected at 2 h and 18 h postinjection were analyzed for IL-12p70 (*A*) and chemokines such as MCP-1 (*B*), and KC (*C*). Assays were performed in triplicates, and data were presented as mean ± SD. The symbol * represents $p < 0.05$, compared with DMSO; and ##, $p < 0.01$, compared with 7DW8-5 (8-5) using a two-tailed Student t test.

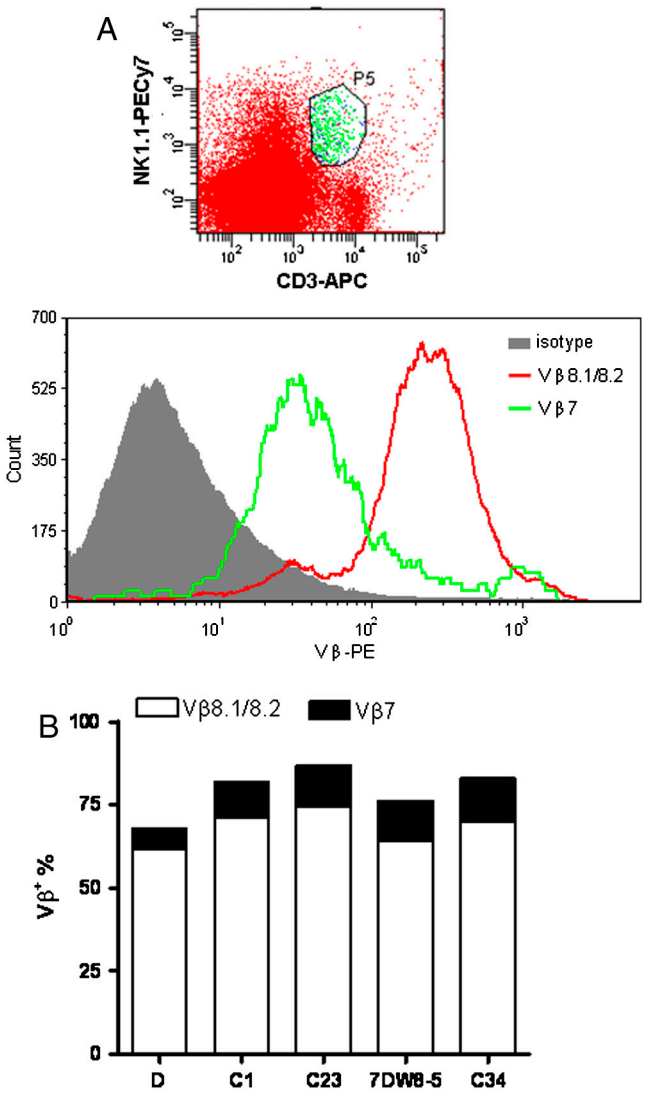
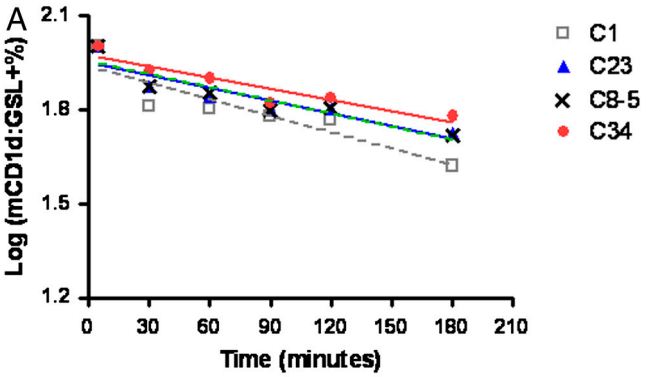


Fig. S3. TCR β-chain usage of Vα14 *i*NKT Cells upon phenyl glycolipids stimulation. The pooled splenocytes from 3–4 B6 mice were in vitro stimulated with DMSO (*D*) or indicated glycolipids at 100 ng/mL for 3 d. (*A*) NK1.1 + /CD3 + NKT cells were gated to determine the expression levels of different beta chains using Vβ-specific antibodies for FACS (fluorescence activated cell sorter) analysis. (*B*) The percentage of Vβ8.1/8.2- or Vβ7-positive NKT cells was presented as the average from 3–4 mice. This was one of the representative results from two independent experiments.

A



B

mCD1d-glycolipid complexes toward V α 14i NKT cells	t1/2 (min) mean±SD
C1	179.4±9.4
C23	215.3±10.1*
7DW8-5	215.8±1.6*
C34	247.2±10.0**

1. Data were combined from two independent experiments.
2. Tukey's Multiple Comparison Test of 1 way Anova was used for statistical analysis (comparing to C1: *, p<0.05; **, p<0.01).

Fig. S4. Binding stability of murine CD1d–glycolipid complexes with murine *i*NKT cells. (*A–B*) The mCD1d–glycolipid complexes were incubated with Vβ8.2 + Vα14*i*NKT hybridoma cells. After washing away the dissociated complexes at the indicated time points, the remaining bound complexes were analyzed by FACS. (*A*) Plot was shown as time (min) versus log of (the percentage of the bound dimer complex at the time indicated/the percentage of the bound dimer complex at time = 5). (*B*) Using linear regression in Graphpad Prism software, the half-life of dimer-glycolipid complexes with *i*NKT cells was determined as $t1/2 = -\log 2/\text{slope}$. The values were derived from the average of two independent experiments and calculated with one-way ANOVA statistical analysis. comparing to C1: *, $p < 0.05$; and **, $p < 0.01$.

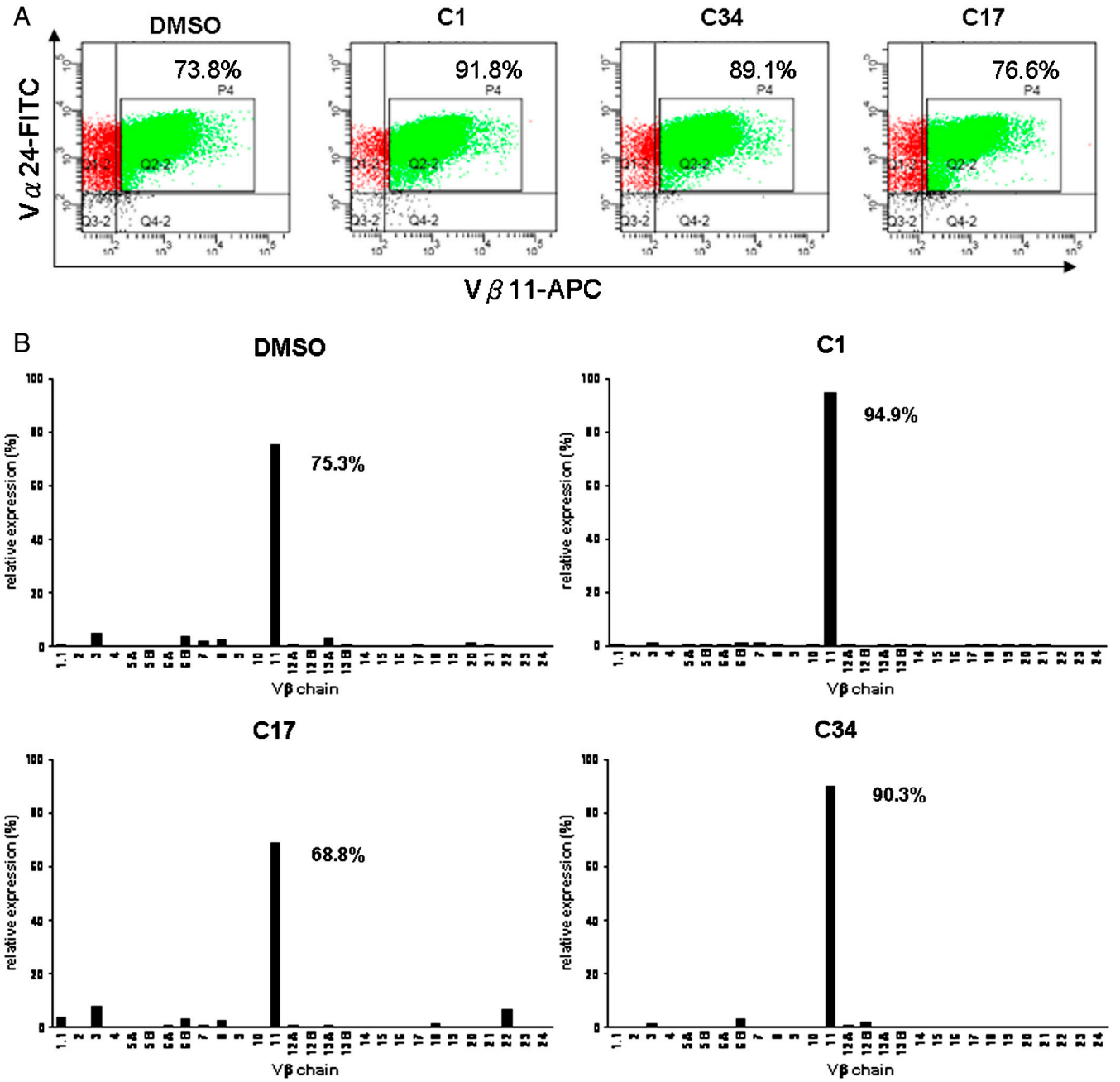


Fig. S5. The TCR β-chain usage of human Vα24 *i*NKT cells expanded by different glycolipids. Vα24 *i*NKT cells were isolated from human peripheral blood mononuclear cell (PBMC), twice pulsed with indicated glycolipids and expanded for 12–14 d. The expanded *i*NKT cells were further purified by MACS (magnetic cell sorting) with purity >95%. (*A*) The percentage of Vβ11+ in Vα24 *i*NKT cells was analyzed by flow cytometry. (*B*) The cDNA was prepared from the glycolipid-expanded Vα24 *i*NKT cells and quantitative real-time PCR was performed to detect the usage of TCR β chain of Vα24 *i*NKT cells. Data was representative of donor 4.

Table S1. The spectratyping results of mock-treated Vα24 *i*NKT cells

	Mock-treated				
Vβ(%)	donor 1	donor 2	donor 3	donor 4	donor 5
β1.1	0.77	0.10	0.11	0.69	2.81
β2	7.07	ND	ND	0.00	ND
β3	63.46	0.25	0.11	4.94	2.90
β4	0.09	0.01	0.08	0.35	0.61
β5A	0.66	0.38	0.51	0.28	2.46
β5B	0.18	0.08	0.32	0.38	0.26
β6A	0.73	0.52	8.68	0.54	7.52
β6B	1.50	5.19	2.04	3.68	29.68
β7	1.44	0.19	0.76	1.77	2.65
β8	0.92	0.25	3.52	2.60	0.95
β9	0.18	0.11	0.02	0.19	0.38
β10	0.71	ND	0.01	0.11	0.06
β11	11.34	90.70	77.19	75.29	19.57
β12A	0.04	0.02	1.16	0.87	3.39
β12B	0.07	ND	0.09	0.21	0.93
β13A	0.52	0.76	0.32	3.23	4.92
β13B	0.45	0.00	2.50	0.70	7.96
β14	1.47	0.07	0.63	0.27	0.41
β15	0.17	0.07	0.05	0.08	2.52
β16	1.81	0.00	0.01	0.08	0.07
β17	0.26	0.16	0.30	0.85	2.56
β18	0.08	0.11	0.27	0.27	1.32
β19	0.08	0.00	0.09	0.20	0.31
β20	0.21	0.22	0.39	1.39	2.50
β21	0.72	0.23	0.09	0.84	0.85
β22	2.01	0.57	0.74	0.17	1.09
β23	2.94	0.00	0.00	0.02	0.06
β24	0.11	ND	0.01	0.00	1.28

The experiment was performed as described in Fig. S5*B*. ND, nondetected (CT value <35).

Table S2. The spectratyping results of C1-treated Vα24 *i*NKT cells

	C1-treated				
Vβ(%)	donor 1	donor 2	donor 3	donor 4	donor 5
β1.1	0.37	0.06	0.13	0.28	0.05
β2	0.00	ND	ND	ND	ND
β3	1.77	0.22	0.06	0.76	0.29
β4	0.11	0.01	ND	0.01	0.04
β5A	0.50	0.28	0.06	0.07	0.17
β5B	0.22	0.03	0.18	0.22	0.03
β6A	0.45	0.15	0.12	0.33	0.39
β6B	0.86	0.82	0.55	0.68	0.60
β7	0.35	0.12	0.82	0.65	0.17
β8	0.32	0.09	0.20	0.31	0.26
β9	0.09	0.02	0.01	0.02	0.01
β10	0.39	0.03	0.07	0.33	0.09
β11	90.94	97.31	93.88	94.95	96.26
β12A	0.02	0.00	0.03	0.21	0.15
β12B	0.08	0.00	0.06	0.04	0.07
β13A	0.41	0.08	2.43	0.21	0.21
β13B	0.41	0.00	0.17	0.11	0.12
β14	0.46	0.03	0.16	0.24	0.01
β15	0.12	0.18	0.03	0.03	0.01
β16	0.11	0.02	0.04	0.01	0.06
β17	0.17	0.06	0.05	0.06	0.54
β18	0.06	0.16	ND	0.08	0.06
β19	0.27	0.03	0.77	0.13	0.17
β20	0.12	0.07	ND	0.14	0.10
β21	0.96	0.17	0.04	0.09	0.08
β22	0.35	0.05	0.04	0.03	0.04
β23	0.07	0.00	0.04	0.00	0.00
β24	0.02	0.01	0.02	0.00	0.01

The experiment was performed as described in Fig. S5*B*. ND, nondetected (CT value <35).

Table S3. The spectratyping results of C34-treated Vα24 *i*NKT cells

	C34-treated				
Vβ(%)	donor 1	donor 2	donor 3	donor 4	donor 5
β1.1	0.69	0.05	0.23	0.31	0.06
β2	1.93	ND	ND	ND	ND
β3	3.16	0.09	0.24	1.31	0.30
β4	0.32	0.00	0.01	ND	0.20
β5A	1.80	0.10	0.06	0.19	0.55
β5B	0.27	0.18	0.27	0.01	0.41
β6A	2.13	0.23	0.06	0.13	0.65
β6B	2.15	2.37	0.83	3.29	3.77
β7	1.82	0.17	0.12	0.09	0.77
β8	0.71	0.23	0.08	0.36	0.78
β9	0.14	0.02	0.01	0.02	0.02
β10	0.15	0.00	0.07	0.07	0.12
β11	72.27	95.54	92.03	90.33	83.41
β12A	0.57	0.02	0.04	1.03	0.46
β12B	0.49	0.01	0.09	1.96	0.19
β13A	3.62	0.08	3.32	0.30	0.56
β13B	0.85	0.11	0.19	0.02	0.91
β14	1.52	0.24	0.03	0.09	0.77
β15	0.13	0.02	0.02	0.02	0.03
β16	0.19	0.00	0.01	0.02	0.47
β17	0.48	0.15	0.06	0.07	0.92
β18	0.36	0.06	ND	0.03	0.09
β19	0.07	0.02	2.03	0.07	0.12
β20	0.72	0.05	0.01	0.16	0.49
β21	1.18	0.17	0.05	0.06	0.10
β22	2.11	0.07	0.05	0.05	0.18
β23	0.10	0.00	0.01	0.00	3.67
β24	0.02	0.00	0.08	0.00	0.00

The experiment was performed as described in Fig. S5*B*. ND, nondetected (CT value <35).

Table S4. The spectratyping results of C17-treated Vα24 *i*NKT cells

	C17-treated			
Vβ(%)	donor 2	donor 3	donor 4	donor 5
β1.1	0.05	3.34	3.79	0.07
β2	ND	ND	ND	0.00
β3	0.42	0.98	7.98	2.50
β4	0.16	0.06	0.07	0.00
β5A	0.31	2.95	0.42	0.13
β5B	0.18	0.56	0.26	0.08
β6A	0.34	6.98	0.85	0.02
β6B	1.96	1.27	3.11	1.85
β7	1.01	0.52	0.62	0.03
β8	0.85	4.62	2.43	3.09
β9	0.21	0.74	0.07	0.00
β10	0.05	ND	0.14	0.00
β11	92.05	58.13	68.76	67.35
β12A	0.02	4.73	1.15	0.00
β12B	0.04	0.92	0.24	0.00
β13A	0.54	4.20	0.59	22.02
β13B	0.59	0.24	0.14	2.33
β14	0.09	0.33	0.36	0.00
β15	0.02	3.08	0.08	0.00
β16	0.02	0.07	0.06	0.00
β17	0.14	2.77	0.37	0.05
β18	0.31	0.17	1.35	0.45
β19	0.01	0.55	0.14	0.00
β20	0.17	1.85	0.25	0.00
β21	0.27	0.78	0.16	0.04
β22	0.18	0.14	6.55	0.00
β23	0.01	0.01	0.05	0.00
β24	0.01	0.01	0.00	0.00

The experiment was performed as described in Fig. S5*B*. ND, nondetected (CT value <35). The number of Vα24 *i*NKT cells in donor 1 was insufficient for C17 stimulation.