α-Lactosylceramide as a Novel "Sugar-Capped" CD1d Ligand for Natural Killer T Cells: Biased Cytokine Profile and Therapeutic Activities

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The invariant natural killer T cells (iNKT) cells have emerged as an important regulator of immunity to infection, cancer, and autoimmune diseases. They can be activated by glycolipids that bind to CD1d. The most effective iNKT ligand reported to date is α-galactosylceramide (α-GalCer), which stimulates iNKT cells to secrete both Th-1 and Th-2 cytokines. Indiscriminate induction of both types of cytokines could limit the therapeutic potential of iNKT ligands, as Th-1 and Th-2 cytokines play different roles under physiological and pathological conditions. Therefore, a ligand with a biased cytokine-release profile would be highly desirable. Here, we report the synthesis and biological activity of α-lactosylceramide (α-LacCer). Our data demonstrate that α-LacCer can stimulate iNKT cells to proliferate and release cytokines, both in vitro and in vivo. Interestingly, while α-LacCer is approximately 1000-times less efficient than α-GalCer in inducing Th-1 cytokines, it is as potent as α-GalCer in the induction of Th-2 cytokines; therefore, α-LacCer is a novel compound that induces a biased cytokine release. Processing by β-glycosidase was critical for α-LacCer activity. Moreover, in vivo experiments suggest that α-LacCer is at least as potent as α-GalCer in the treatment of tumors and experimental autoimmune encephalomyelitis.

Introduction

Lipids and glycolipid antigens can be presented by MHC-I-like CD1 molecules to non-MHC-restricted T lymphocytes.[1] CD1d is the protein that presents glycolipids to a particular cell population called invariant natural killer T cells (iNKT cells). The iNKT cells can coexpress both the natural killer marker NK 1.1 and the semi-invariant T-cell receptor (TCR), which is formed by Vα14–Jα18 and Vβ8.2/Vβ7/Vβ2 chains in mice (Vα14i NKT cells) and by Vα24–Jα18 and Vβ11 chains in humans (Vα24i NKT cells).[2–4] The iNKT cells secrete the proinflammatory T helper 1 (Th-1) and immunoregulatory Th-2 cytokines, together with other chemokines, within 2 to 6 h of the stimulation. The secretion of cytokines allows iNKT cells to regulate many in vivo conditions, including malignancy, infection, and autoimmune diseases.[5,6]

α-Galactosylceramide (α-GalCer) is currently the most active glycolipid antigen known. Extracted from the marine sponge *Agelas mauritianus* by the Kirin Brewery Company in 1993, α-GalCer was first found to prolong the lifespan of B16 cells intraperitoneally inoculated mice.[7,8] Among the α-GalCer analogues, the most desirable model agonist for research and clinical usage is KRN-7000, which has an 18-carbon sphingosine and 26-carbon acyl chain attached to the sugar moiety.[9] α-GalCer can be readily loaded onto CD1d on the surface of antigen-presenting cells (APCs), and the CD1d–α-GalCer complex is recognized by the TCR on iNKT cells. This interaction transiently triggers a massive iNKT cell response, which is characterized by the production of cytokines, including both Th-1 and -2 cytokines. As a result, the functions of other cell types, such as natural killer (NK) cells, dendritic cells (DC), and some subsets of B- and/or T cells, are also affected.

α-GalCer has been demonstrated to have antitumor activity on a broad range of tumor cell lines in mouse transplant tumor models, including melanomas and lymphomas, and also colon, prostate, lung, breast, and renal cancers.[10–12] The activation of NK cells in response to the IFN-γ (interferon-γ) secreted by iNKT cells has been shown to be critical for its antitumor activity. Several clinical trials, both phase I and -II, have been conducted on the basis of positive results from animal tests. However, no clear clinical benefits have been observed, although α-GalCer treatment on humans is generally safe.[13] Mechanistic studies revealed that the treatment effects of α-GalCer were highly variable, and depended on the iNKT cell numbers in patients' blood. Furthermore, α-GalCer caused the iNKT cells to become unresponsive for a period of time, which even lasted for more than one month in a mouse model.[14] In addition to cancer treatment, α-GalCer has also shown efficacy in the

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treatment of autoimmune diseases in mouse models, including autoimmune diabetes[15,16] and experimental autoimmune encephalomyelitis (EAE).[17–19] Some promising effects of α-GalCer on organ transplants and atherosclerosis have also been shown in animal models.[20,21] However, observations show that α-GalCer plays different roles in these autoimmune diseases. For example, the activation of iNKT cells can suppress diseases such as type I diabetes and multiple sclerosis, but in the case of atherosclerosis the activation of iNKT cells will promote the disease.[2] The latest results have shown that the cosecretion of Th-1/Th-2 cytokines might cause the conflicts. IFN-γ from activated iNKT cells, for instance, can ameliorate allergic asthma, but interleukin-4 (IL-4) secreted at the same time will increase the severity.[22–24] Success in using α-GalCer-loaded immature or mature DCs for the treatment of cancer in clinical trials further demonstrated that the activation and expansion of iNKT cells represents the critical feature in cancer treatments, such as for lung cancer.[25,26] However, the clinical data obtained so far suggest that the compounds currently available can have severe limitations.

Recent studies have demonstrated that α-GalCer could induce anergy of iNKT cells, perhaps due to paralysis associated with the most potent agonist. In addition, since Th-1 and Th-2 cytokines are usually reciprocal inhibitors under physiological and pathological conditions, an agonist that stimulates both equally well will have limited clinical benefit. On the basis of these considerations, a less active agonist that can avoid anergy but stimulate lopsided Th-1/Th-2 cytokine release would be worth exploring. Here, the biological activities of a newly synthesized glycolipid antigen—α-lactosylceramide (α-LacCer)—have been assayed, and it has been found that this compound shows iNKT cell activation intensity similar to that of α-GalCer, but with a different cytokine release profile.

Results and Discussion

α-LacCer can stimulate cytokine release in in vitro assays

The structures of the key glycolipids mentioned here—α-GalCer, α-LacCer, and α-GlcCer—are shown in Figure 1.

As the initial test for α-LacCer's activity in iNKT cells, the iNKT hybridoma assay was applied. The glycolipid-treated CD1d-expressing A/20 cells were used to stimulate the iNKT hybridoma cells, and the IL-2 released by stimulated hybridoma cells was quantified. Although not as potent as α-GalCer, α-LacCer still reached a significant activity level in the in vitro stimulation of iNKT hybridoma cells (Figure 1).

In the in vitro cytokine stimulation assay, a mouse spleen cell mixture containing both APC and iNKT cells, was treated with the glycolipids. IFN-γ and IL-4, which were taken as representative Th-1 and Th-2 cytokines, respectively, were quantified. α-GalCer and α-LacCer showed different cytokine-release profiles in this in vitro stimulation experiment (Figure 2). For IFN-γ, the Th-1-type cytokine, α-GalCer induced a significant iNKT cell cytokine release at a concentration of 0.01 ng mL^{-1}; α-LacCer was 1000- to 10000-times less efficient. In contrast, in the case of IL-4, which represents the Th-2 cytokine, α-GalCer
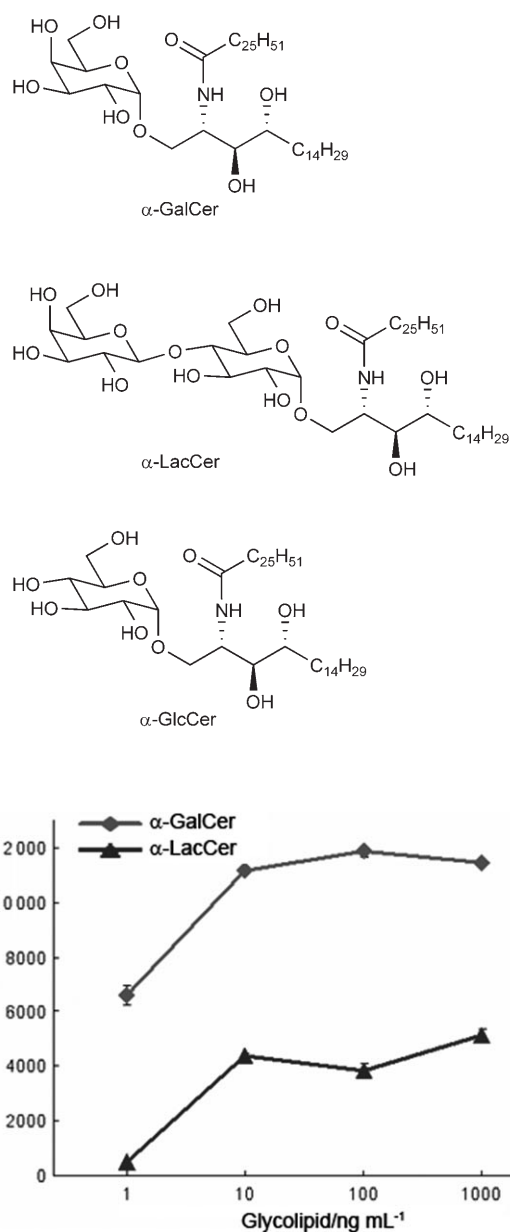

Figure 1. Structures of glycolipids and in vitro hybridoma stimulation by α-GalCer and α-LacCer.

and α-LacCer had similar stimulating capabilities. Therefore, α-LacCer is biased to induce more Th-2 cytokine than α-GalCer.

β-Galactosidase is critical to α-LacCer's activity

α-LacCer has a disaccharide "head", instead of a monosaccharide as in the case of α-GalCer. To find out whether the disaccharide is processed before binding to CD1d, α-LacCer's iNKT cell-stimulation activity was tested in the presence of a β-galactosidase inhibitor in culture. PETG (2-phenylethyl β-D-thiogalactoside) was used as the β-galactosidase inhibitor, and the α-galactosidase inhibitor, DGJ (deoxygalactonojirimycin hydrochloride), was used as a control. After being pretreated with β-galactosidase inhibitor (2 mM), the splenocytes were cultured with glycolipid antigens. Once the β-galactosidase inhibitor
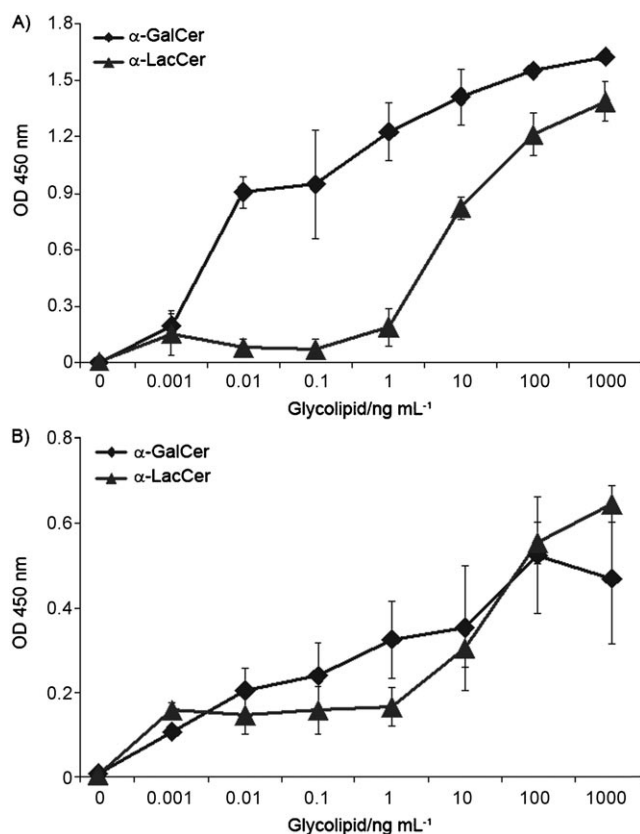
Figure 2. In vitro stimulation of A) Th-1 (IFN-γ) and B) Th-2 (IL-4) cytokines by glycolipids.

Figure 3. Effect of β-galactosidase inhibitor on α-LacCer's ability to stimulate cytokine release of both A) IFN-γ and B) IL-4.

was added, α-LacCer lost its ability to activate iNKT cells to produce either Th-1 or Th-2 cytokines (Figure 3).

This data demonstrate that the removal of the outside sugar is critical to α-LacCer activity. The processing of α-LacCer, which hydrolyzed α-LacCer to α-GlcCer, played a critical role in finishing the final modification of the glycolipid antigens. This extra modification process might cause a time delay in the glycolipid-antigen presentation and this could then affect the final cytokine profile of α-LacCer, which is different to that obtained when α-GlcCer is applied directly (Figure 4).

α-LacCer has to be hydrolyzed to α-GlcCer to become an active antigen. However, recent work has shown that the 4′-OH of α-GlcCer does not form hydrogen bonds with CD1d, so modification at this position should not affect the binding with CD1d.[27] Accordingly, besides the time delay, another possible reason for decreasing activity might be that part of the α-LacCer that has not been hydrolyzed to α-GlcCer competes with α-GlcCer to bind with the CD1d and cause subdued activity relative to α-GlcCer itself.

With reference to previous work on α-GlcCer[28] and according to our own results, α-LacCer and α-GlcCer showed distinguishable cytokine stimulation properties. For monosaccharide glycolipids, differences in cytokine stimulation activities mostly arise from differences in the stabilities of CD1d–glycolipid–TCR complexes.[28] For the activity difference between α-LacCer and α-GlcCer, however, the complex stability is not an issue. The extra processing procedure should be the main reason. This is
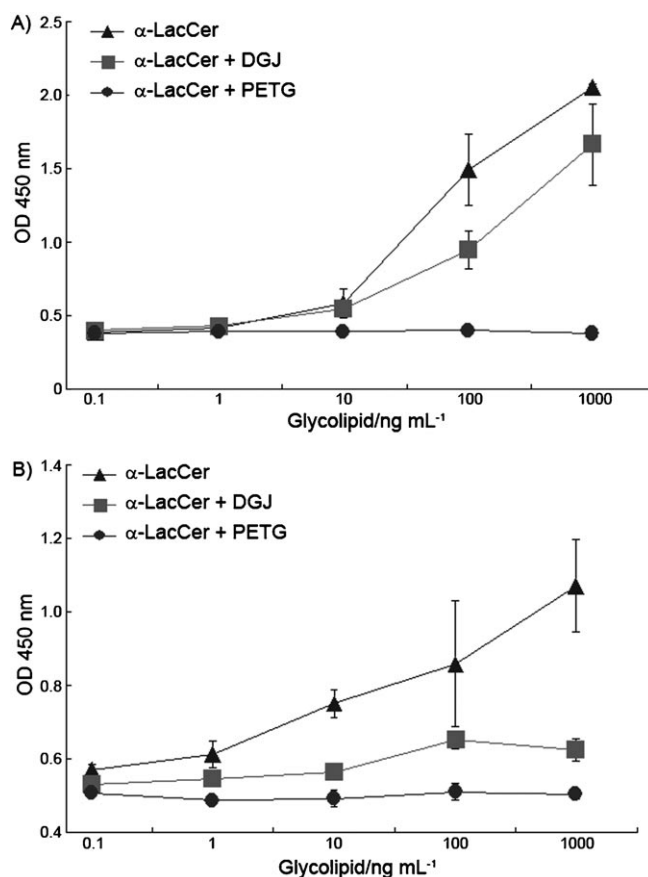
evidence that the stimulatory ability and overall immunological output of the glycolipid antigens are not only related to the binding between CD1d–glycolipid–TCR, but are also affected by the in vivo antigen-processing procedure.

T cells are activated in vivo by α-LacCer

Glycolipids were injected into mice to determine whether sugar capping changed the glycolipids' in vivo T-cell stimulation profiles. Different T-cell populations from glycolipid-treated mice spleens were analyzed by flow cytometry. For the iNKT cells (β-TCR+/CD1d-tetramer+), both α-GalCer and α-LacCer could stimulate and retain a significant iNKT cell population even ten days after compound injection (Figure 5 A). α-LacCer reached a stimulation strength close to that of α-GalCer. After being activated, both CD4+ and CD8+ T cells should acquire a memory cell phenotype, which is characterized by CD62Llow/CD44hi. The memory T cells in the mice spleen also showed the proliferation effects relative to the vehicle control after glycolipid injection (Figure 5 B and C). However, a statistical difference between α-GalCer and α-LacCer could not be recognized.
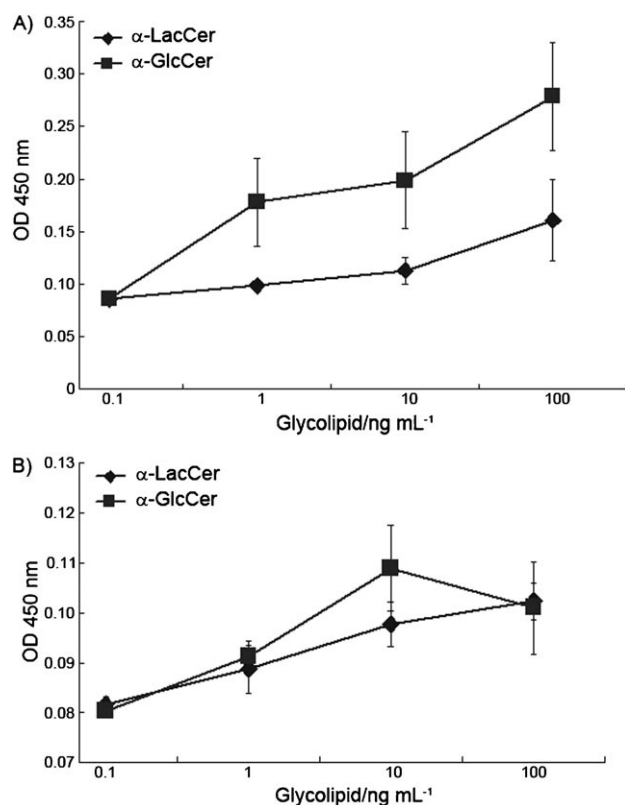
Figure 4. Stimulation of A) IFN-γ and B) IL-4 cytokine release by α-GlcCer and α-LacCer.

α-LacCer stimulates iNKT cell proliferation with similar kinetics both in the spleen and liver

It has been shown that when α-GalCer activates iNKT cells, the TCR, NK1.1, and other iNKT cell-surface markers will initially be suppressed but will then return to normal after 12 to 24 h. The iNKT cells rapidly proliferate and expand extensively, and reach their maximum cell number three days after injection.[1,29–31] The time course experiment showed that α-LacCer had exactly the same iNKT cell stimulation time pattern as α-GalCer, which also reached the apex 72 h after injection, both in the spleen and in the liver (Figure 6). However, while α-LacCer and α-GalCer have comparable optimal activities, α-LacCer is less effective than α-GalCer in terms of maximum expansion of the iNKT cell population in the spleen.

α-LacCer has antitumor effects similar to those of α-GalCer in the mouse model

Transplantable MC38 tumor was used as a model to test the glycolipid antigens' antitumor activities. Most of the tumors reached 0.5–1.0 cm diameters around ten days after inoculation. At this point, the tumor-bearing mice were treated by intraperitoneal (i.p.) injection of drugs or vehicle. The tumors treated with the glycolipid antigens shrank in size one day after injection. This shrinkage was transient, however, as the tumors reassumed growth on day three. However, both glycolipid antigens did inhibit the tumor growth rate (Figure 7 A).

Figure 7 B shows a comparison of the best-fit regression lines of the transformed Gompertz growth curves for tumors treated with α-GalCer, α-LacCer, and only with DMSO. The slope (ζ) of the regression line is the specific growth rate of each tumor. It shows clearly that the growth rates for the tumors treated with α-GalCer and α-LacCer are very similar (ζ_α-GalCer = −0.0317; ζ_α-LacCer = −0.0241), and that both of them are much slower than those of the tumors treated with vehicle only (ζ_DMSO = −0.0527). The growth rate was thus reduced by 40 to 50 % when the tumor was treated with glycolipids. The injection of α-GalCer or α-LacCer can thus suppress the growth rates of transplanted tumors, relative to the untreated control.

α-LacCer also has anti-autoimmune disease effect in the mouse model

Previous studies have demonstrated that α-GalCer has preventive effects on EAE if administrated either before or shortly after induction of EAE. To determine potential therapeutic effects of the drugs, we treated mice at seven days after immunization with the MOG peptide, which is generally used to induce EAE in C57BL/6 mice. At this point, the inflammation to the central nervous system (CNS) has been initiated and most of the immunized mice started to show some symptoms 12 days after immunization. Mice treated with either drug showed reduction in their EAE scores, especially for the peak stage and the healing stage (Figure 8). The α-LacCer-treated group showed a better healing curve than the α-GalCer-treated group. Thus, in the therapeutic model, α-LacCer appears to be slightly more efficient than α-GalCer. Since EAE can be ameliorated by an enhanced Th-2 response, the increased efficiency in inducing Th-2 cytokine can explain the superior activity of the α-LacCer.

Conclusions

Taken together, we have demonstrated that α-LacCer, a novel chemical, is capable of stimulating iNKT cells. α-LacCer showed a biased cytokine profile in relation to that of α-GalCer, and showed a slightly enhanced therapeutic effect in tumor rejection and inhibition of autoimmune encephalomyelitis. α-LacCer could therefore have better potential in clinical treatments.

Experimental Section

Mice and animal care: All mice were purchased from The Jackson Laboratory (Maine, USA) and housed in the Ohio State University and OncoImmune animal facilities. All experiments were carried out according to the approved protocols.

Cell lines and cell cultures: A20/CD1 and DN3A4-1.2 cells were kindly provided by Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, CA, USA). Both cell types were cultured in RPMI1640 with fetal bovine serum (10 %), L-glutamine (2.05 mM), and penicillin (1 %) at 37 °C in a CO_2 (5 %) atmosphere. The MC38 cells were cultured and maintained in DMEM with FBS (10 %), L-
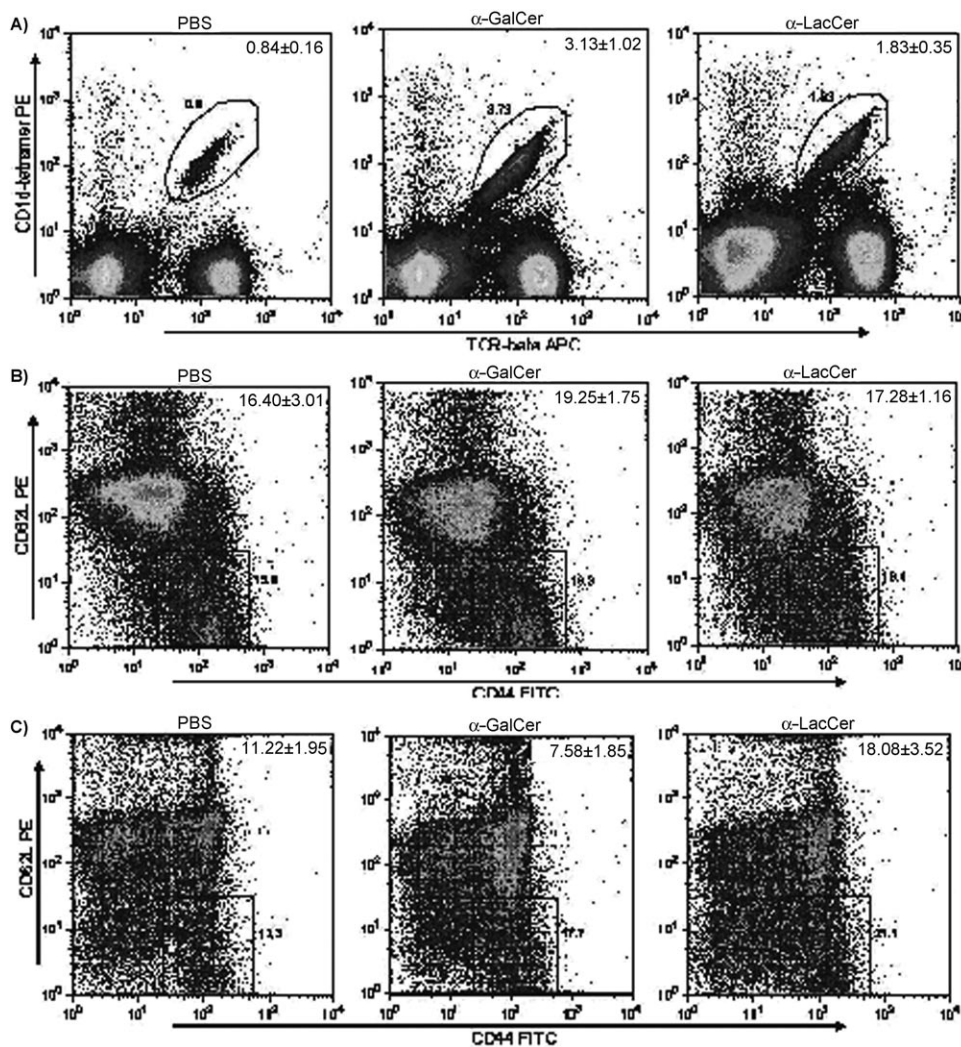
Figure 5. In vivo T-cell stimulation by glycolipids in A) iNKT cells (β-TCR$^+$/CD1d-tetramer$^+$), and B) CD4$^+$ memory T cells (CD62L^{low}/CD44^{hi}), C) CD8$^+$ memory T cells (CD62L^{low}/CD44^{hi}).

7×Ph), 6.48 (d, $J=3.6$ Hz, 1H; H-1), 5.08 (d, $J=10.7$ Hz, 1H; PhCH$_2$), 5.04 (d, $J=11.5$ Hz, 1H; PhCH$_2$), 4.87 (d, $J=11.1$ Hz, 1H; PhCH$_2$), 4.81–4.73 (m, 6H; PhCH$_2$), 4.61 (d, $J=11.5$ Hz, 1H; PhCH$_2$), 4.57 (d, $J=$ 12.1 Hz, 1H; PhCH$_2$), 4.43 (d, $J=$ 11.4 Hz, 1H; PhCH$_2$), 4.40 (d, $J=$ 7.0 Hz, 1H; H-1′), 4.36 (d, $J=$ 12.0 Hz, 1H; PhCH$_2$), 4.31 (d, $J=$ 11.9 Hz, 1H; PhCH$_2$), 4.13 (t, $J=$ 9.6 Hz, 1H; H-1′), 4.01–3.96 (m, 3H), 3.93 (dd, $J=11.1$, 2.9 Hz, 1H), 3.81 (dd, $J=9.7$, 7.8 Hz, 1H), 3.74 (dd, $J=9.6$, 3.6 Hz, 1H), 3.62 (t, $J=$ 8.6 Hz, 1H), 3.54 (dd, $J=11.1$, 1.5 Hz, 1H), 3.45–3.39 ppm (m, 3H); ^{13}C NMR (125 MHz, CDCl$_3$): $\delta=161.22$, 139.19, 139.13, 138.80, 138.60, 138.32, 138.19, 138.05, 128.63, 128.43, 128.35, 128.24, 128.22, 128.05, 127.98, 127.92, 127.87, 127.81, 127.75, 127.74, 127.63, 127.60, 127.50, 127.45, 127.39, 127.13, 102.86, 94.58, 91.46, 82.51, 79.86, 79.69, 78.40, 78.04, 75.81, 75.48, 75.25, 74.75, 73.96, 73.69, 73.47, 73.20, 73.14, 73.10, 72.59, 68.15, 67.51 ppm; HRMS calcd for C$_{63}$H$_{64}$Cl$_3$NO$_{11}$Na: 1138.3443 [M+Na]$^+$; found: 1138.3459.

2,3,4,6-Tetra-O-benzyl-α-ᴅ-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-ᴅ-glucopyranosyl-(1→1)-(2S,3S,4R)-2-hexacosanoylamino-3,4-di-O-benzoyl-octadecan-1,3,4-triol (4): A suspension of acceptor **3**$^{[32]}$ (48 mg, 0.053 mmol), donor **2** (120 mg, 0.11 mmol), and 4 Å molecular sieves (0.3 g) in di-

glutamine (2.05 mᴍ), and penicillin (1%) at 37 °C in a CO$_2$ (5%) atmosphere.

Synthesis of α-lactosylceramide: The synthesis followed the protocol of preparation of α-GalCer (Scheme 1).$^{[32]}$

2,3,4,6-Tetra-O-benzyl-α-ᴅ-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-ᴅ-glucopyranosyl-(1→1)-trichloroacetimide (2): N-Bromosuccinimide (374 mg, 2.1 mmol) was added at 0 °C to a solution of phenyl-heptabenzyl-1-thio-ᴅ-lactose **1**$^{[33]}$ (1.03 g, 1.0 mmol) in acetone (9 mL) and water (1 mL), and the mixture was stirred for 30 min. The reaction was quenched by addition of saturated aqueous sodium bicarbonate. The organic solvent was removed in vacuo, and the remaining aqueous solution was extracted with ethyl acetate. After being dried over anhydrous sodium sulfate, the extract was concentrated, and then the residue was dissolved in dry dichloromethane (10 mL). Trichloroacetonitrile (1 mL, 10.0 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (70 μL, 0.5 mmol) were added. The reaction mixture was stirred for 2 h. The solvent was evaporated, and the residue was purified by chromatography to give the product (0.92 g, 82% yield). ^{1}H NMR (500 MHz, CDCl$_3$): $\delta=8.62$ (s, 1H; CCl$_3$C=NH), 7.39–7.15 (m, 35H;

ethyl ether and tetrahydrofuran (5:1, 2 mL) was stirred at room temperature for 0.5 h. After the system had been cooled to −23 °C, trimethylsilyl trifluoromethanesulfonate (5 μL, 0.027 mmol) was added. The resulting mixture was stirred for an additional 2 h. Triethylamine was added to quench the reaction, and the mixture was filtered through a Celite pad. The filtrate was concentrated and purified by chromatography with hexane and ethyl acetate (4:1) to afford a white solid (63 mg, 64% yield). The newly formed glycosidic linkage between the saccharide and ceramide was characterized by ^{1}H NMR ($\delta=4.79$ ppm, $J_{H1',H2'}=3.7$ Hz) and ^{13}C–^{1}H coupling NMR ($\delta=98.5$ ppm, $J_{C1',H1'}=170.3$ Hz) as having the α configuration.$^{[34]}$ ^{1}H NMR (500 MHz, CDCl$_3$): $\delta=8.08$ (dd, $J=8.4$, 1.3 Hz, 2H; PhCO), 7.97 (dd, $J=8.5$, 1.3 Hz, 2H; PhCO), 7.61 (tt, $J=7.5$, 1.0 Hz, 1H; PhCO), 7.53 (tt, $J=7.5$, 1.2 Hz, 1H; PhCO), 7.49 (t, $J=7.9$ Hz, 2H; PhCO), 7.38 (t, $J=8.0$ Hz, 2H; PhCO), 7.37–7.13 (m, 35H; 7× PhCH$_2$), 6.76 (d, $J=9.3$ Hz, 1H; CONH), 5.77 (dd, $J=9.0$, 2.9 Hz, 1H; H-3), 5.43 (dt, $J=9.0$, 3.3 Hz, 1H; H-4), 5.04 (d, $J=10.7$ Hz, 1H; PhCH$_2$), 4.99 (d, $J=11.4$ Hz, 1H; PhCH$_2$), 4.83 (d, $J=11.3$ Hz, 1H; PhCH$_2$), 4.79 (d, $J=3.7$ Hz, 1H; H-1′), 4.78 (d, $J=11.0$ Hz, 1H; PhCH$_2$), 4.73 (d, $J=11.9$ Hz, 1H; PhCH$_2$), 4.72 (d, $J=11.4$ Hz, 1H; PhCH$_2$), 4.71 (d, $J=11.9$ Hz, 1H; PhCH$_2$), 4.67 (d, $J=11.0$ Hz, 1H; PhCH$_2$), 4.65 (m, 1H; H-2), 4.64 (d, $J=11.5$ Hz, 1H; PhCH$_2$), 4.57 (d,
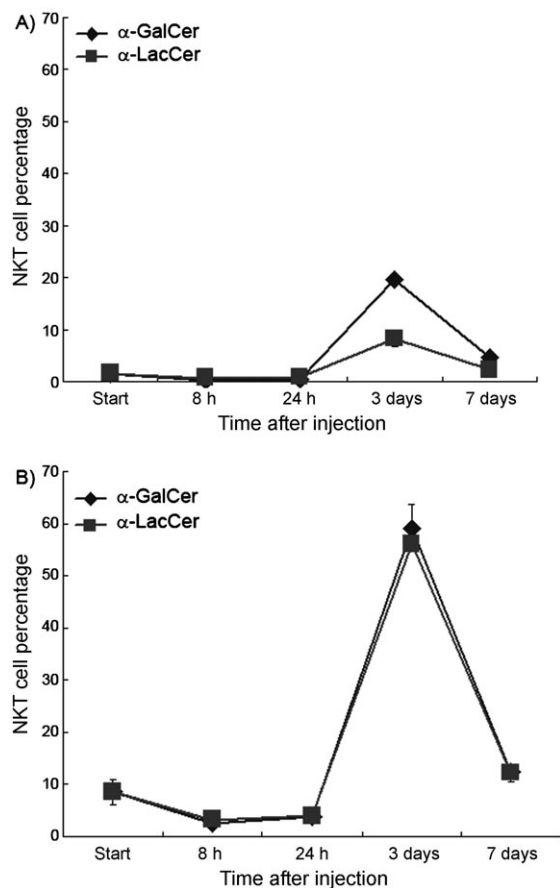
Figure 6. The iNKT cell percentage in the presence of glycolipids over time A) in the spleen, and B) in the liver.
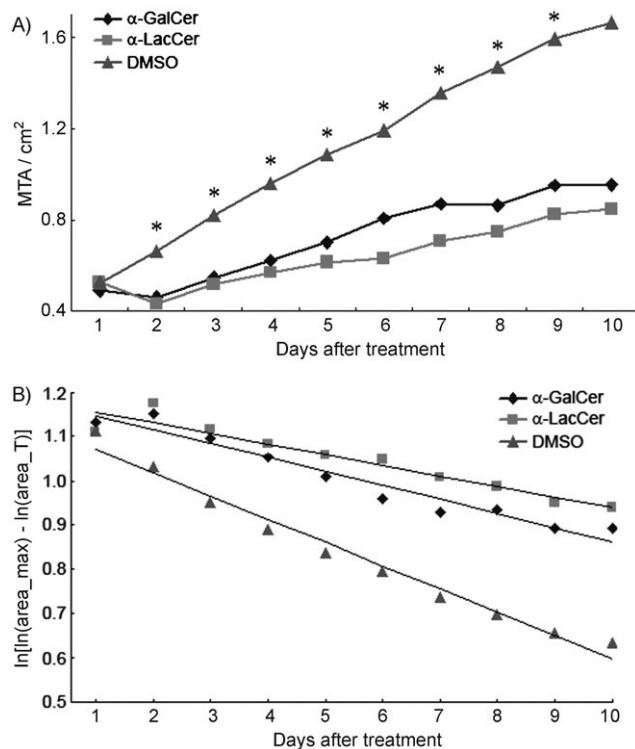

Figure 7. A) Growth curves of glycolipid-treated transplanted tumors. Statistical comparisons (Dunnet multiple comparison) were made between the DMSO group and the glycolipid-treated groups. Asterisks indicate that the tumor size of the DMSO group is significantly larger than the two glycolipid treated groups; $*p < 0.05$; MTA: mean tumor area. B) The specific growth rate curves of differently treated tumor groups.
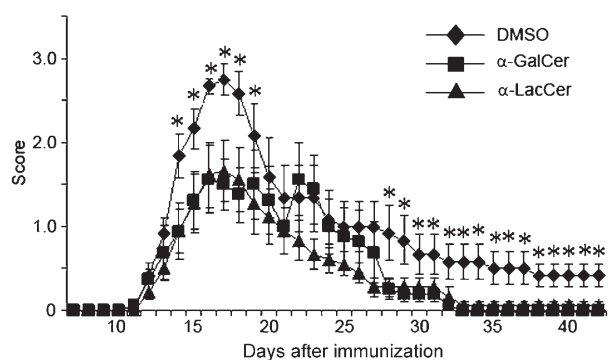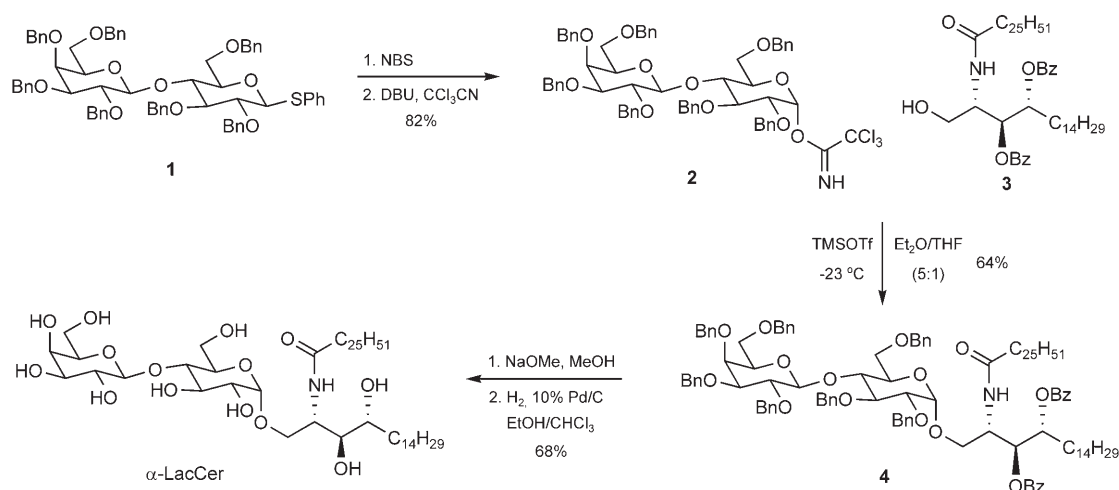
$J = 11.4$ Hz, 1 H; PhCH$_2$), 4.53 (d, $J = 12.1$ Hz, 1 H; PhCH$_2$), 4.38 (d, $J = 11.9$ Hz, 1 H; PhCH$_2$), 4.34 (d, $J = 7.7$ Hz, 1 H; H-1''), 4.32 (d, $J = 12.1$ Hz, 1 H; PhCH$_2$), 4.25 (d, $J = 11.9$ Hz, 1 H; PhCH$_2$), 3.92–3.89 (m, 2 H), 3.85 (dd, $J = 10.9$, 3.5 Hz, 1 H), 3.80–3.71 (m, 5 H), 3.56–3.53 (m, 2 H), 3.49 (dd, $J = 9.6$, 3.7 Hz, 1 H; H-2'), 3.39–3.32 (m, 2 H), 2.19 (t, $J = 7.5$ Hz, 2 H; RCH$_2$CONH), 1.94 (m, 2 H; H-5), 1.64 (m, 2 H), 1.43–1.22 (m, 68 H), 0.93 (t, $J = 6.8$ Hz, 3 H; CH$_3$), 0.92 ppm (t, $J = 6.9$ Hz, 3 H; CH$_3$); ^{13}C NMR (125 MHz, CDCl$_3$): $\delta = 173.1$, 166.2, 165.3, 139.4, 139.1, 138.8, 138.6, 138.3, 138.1, 138.0, 133.3, 132.9, 130.1, 129.9, 129.8, 129.77, 128.6, 128.4, 128.34, 128.31, 128.27, 128.2, 128.16, 128.1, 128.0, 127.9, 127.8, 127.7, 127.66, 127.6, 127.54, 127.5, 127.4, 127.39, 127.3, 127.0, 103.0, 98.5, 82.6, 80.0, 79.8, 79.3, 76.6, 75.3, 75.2, 74.7, 74.0, 73.7, 73.4, 73.3, 73.2, 73.1, 72.6, 72.5, 70.9, 68.2, 68.0, 67.5, 48.6, 36.7, 32.0, 29.8, 29.7, 29.68, 29.66, 29.61, 29.6, 29.5, 29.4, 29.38, 28.6, 25.73, 25.71, 25.7, 22.7, 14.1 ppm; HRMS calcd for C$_{119}$H$_{159}$NO$_{16}$Na: 1882.1590 [$M+$Na]$^+$; found: 1882.1578.

α-D-Galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1)-(2S,3S,4R)-2-hexacosanoylamino-octadecan-1,3,4-triol (α-LacCer):
A solution of protected lactosylceramide **4** (60 mg, 0.032 mmol) in dry methanol (2 mL) was treated with NaOMe (5 mg) for 30 min. The reaction mixture was neutralized with Amberlyst resin and filtered. The filtrate was concentrated, and the residue was dissolved in a mixture of CHCl$_3$/EtOH (4:1, 2 mL) followed by addition of Pd/C (10%, 4 mg). The resulting suspension was stirred under H$_2$ (1 atm) for 4 h. The catalyst was filtered off, and the filtrate was concentrated. The residue was purified by chromatography (CHCl$_3$/MeOH 4:1) to give the product (22 mg, 68% yield). ^{1}H NMR

Figure 8. Effects of glycolipids on EAE development in the mouse model. Asterisks indicate that the disease score of the DMSO-treated group is significantly higher than those of the glycolipid-treated groups (Dunnet multiple comparison); $*p < 0.1$.

(500 MHz, [D$_5$]pyridine): $\delta = 8.44$ (d, $J = 8.4$ Hz, CONH), 6.45 (br, 9 H; 9×OH), 5.46 (d, $J = 3.6$ Hz, 1 H; H-1'), 5.17 (m, 1 H; H-2), 5.02 (d, $J = 7.8$ Hz, 1 H; H-1''), 4.55 (dd, $J = 10.9$ Hz, 1 H), 4.49–4.39 (m, 5 H), 4.38–4.25 (m, 5 H), 4.17 (t, $J = 9.0$ Hz, 1 H), 4.11 (dd, $J = 6.1$, 3.3 Hz, 1 H), 4.09 (t, $J = 6.7$ Hz, 1 H), 4.05 (dd, $J = 9.4$, 3.7 Hz, 1 H), 2.41 (t, $J = 7.5$ Hz, 2 H; RCH$_2$CO), 2.23 (m, 1 H), 1.87 (m, 2 H), 1.78 (m, 2 H), 1.64 (M, 1 H), 1.43–1.17 (m, 68 H), 0.85 ppm (t, $J = 6.4$ Hz, 6 H; 2 × CH$_3$); ^{13}C NMR (125 MHz, [D$_5$]pyridine): $\delta = 173.0$, 105.4, 100.2, 82.2, 77.0, 76.3, 75.0, 73.2, 72.8, 72.2, 72.1, 69.8, 68.0, 61.9, 61.8, 51.1, 36.5,

Scheme 1. Synthesis of α-LacCer by the trichloroacetimidate method.

34.1, 31.9, 30.2, 29.9, 29.8, 29.77, 29.7, 29.68, 29.6, 29.59, 29.5, 29.4, 29.37, 29.2, 26.1, 22.7, 14.0 ppm; HRMS calcd for $C_{56}H_{109}NO_{14}Na$: 1042.7746 $[M+Na]^+$; found: 1042.7759.

In vitro hybridoma stimulation assay: Glycolipids were dissolved in DMSO at 1000× the indicated concentration and were then added to A20/CD1 cells (1×10^6 cells, 1 mL) to reach the indicated concentration. After being incubated for 16 h, the A20/CD1 cells were washed with medium. Then, pretreated A20/CD1 cells (0.1×10^6 cells) were mixed with DN3A4-1.2 hybridoma cells in medium (0.5×10^5 cells, 200 μL). The mixture was incubated for another 16 h, and the IL-2 in the supernatant was measured by ELISA. The ELISA was carried out by using the previously published procedure.[35] The supernatant was first incubated in a purified anti-IL-2-coated 96-well plate, and then the biotin-conjugated anti-IL-2 (both from EBioscience) second antibody was used. A HRP–streptavidin conjugate was applied to detect the second antibody with 3.3′,5.5′-tetramethylbenzidine (TMB) substrate. The plates were read at 450 nm.

In vitro cytokine stimulation assay: To perform the in vitro stimulation, single-cell suspensions from mice spleens were cultured with the glycolipid antigens, either α-GalCer or α-LacCer, at different concentrations for 72 h. The supernatants from the cultures were taken for ELISA to measure the released IFN-γ and IL-4. To perform the cytokine ELISA, supernatants were first cultured overnight in anti-IFN-γ or anti-IL-4 capture antibody-coated (BD Biosciences) wells. Biotin labeled rat-anti-mouse IFN-γ or IL-4 antibodies (BD Biosciences) were then used as second antibodies, followed by incubation with the streptavidan–HRP (BD Biosciences). The OPD kit (Sigma–Aldrich) was used to develop the color, and HCl (2 M) was added to each well to stop the reaction. The plates were read on a plate reader at a wavelength of 492 nm.

In vivo T-cell stimulation: Six-week old C57BL/6 mice were used. With three mice taken as a group, α-GalCer or α-LacCer in vehicle solvent (1:25 DMSO in pH 7.0 PBS solution, 8 μg, 200 μL) was intravenously (i.v.) injected through the mouse tail vein. Vehicle solvent without any compound was used as the control. Ten days after injection, the mice were sacrificed and the splenocytes were stained by fluorescence conjugated mAb against CD4, NK1.1, β-TCR (BD Biosciences), and PBS57-loaded CD1d-tetramer (NIH Tetramer Facility). All the stained samples were analyzed by flow cytometry.

Time course of iNKT cell in vivo stimulation in mice spleen and liver: Two compounds, α-GalCer and α-LacCer, were i.v. injected into C57BL/6 mice in vehicle solvent (1:25 DMSO in pH 7.0 PBS solution, 8 μg, 200 μL). At different time points (starting point, 8 h, 24 h, 3 days, and 7 days) mice were sacrificed, and iNKT cells in both the spleen and liver were stained and analyzed as in the previous experiment.

Antitumor growth activity of glycolipid antigens: The mouse transplant tumor was used as in vivo model to test the antitumor activity of glycolipid antigens. To induce tumor growth, MC38 cells (1×10^6 cells) were injected into the rear flanks of 6-week old male C57BL/6 mice. When the tumor had grown to 0.5–1.0 cm diameter, α-GalCer or α-LacCer in vehicle solvent (2 μg, 50 μL) was directly injected into the tumor. After the first injection, the mice were injected every other day, five times in total. For each treatment group, six mice with tumors were injected and another group of mice received only vehicle solvent as a negative control. The tumor size was measured every day until the tumor diameter was over 2 cm or other early removal criteria were reached.

The results were analyzed by the quantitation method. Since depicting tumor size over time results in nonlinear growth curves that are complicated to describe and compare, the Gompertz equation, $S(T) = S(0) \exp[(1-\exp(-\zeta T))C/\zeta]^{[36]}$, was utilized for further analysis of the growth rate of the tumors. The Gompertz equation can be transformed to $\ln[\ln(\text{area\_max}) - \ln(\text{area\_}T)] = \ln(C) - \zeta T$ to depict the growth rate of the tumor as a straight line when the size $\ln[\ln(\text{area\_max}) - \ln(\text{area\_}T)]$ is plotted against time (T). In this equation, area_max is a theoretical maximal area that the tumor can reach, and area_T is the tumor area T days after transplantation. The Gompertz constant, ζ, is termed the specific growth rate of the tumor and k is the constant unrelated to this rate.

EAE model: Myelin-oligodendrocyte-glycoprotein (MOG, peptide p35–55; Sigma–Genosys) was used to induce EAE. Eight-week-old female C57BL/6 mice were used. Equal volumes of MOG in complete Freund's adjuvant (CFA, Difco Laboratories; 4 mg mL^{-1}) and *Mycobacterium tuberculosis* H37Ra (Difco Laboratories) in CFA (8 mg mL^{-1}) were thoroughly mixed. The mixture (100 μL) was injected at three sites on the back of each mouse around the bottom of the tail (subcutaneously). Immediately after immunization, *Pertussis toxin* (PT, List Biological Laboratories, Inc.) in PBS

(200 µg, 200 µL) was intravenously injected into each mouse. Another injection followed 48 h later.

EAE developed after eight to nine days. The mice were observed every day and scored on a scale of 0–5 with gradations of 0.5 for intermediate scores. The criteria for scoring are listed as follows: 0) no clinical signs, 1) loss of tail tone, 2) wobbly gait, 3) hind-limb paralysis, 4) moribund, and 5) death. The treatments, α-GalCer or α-LacCer in vehicle solvent (1:25 DMSO in pH 7.0 PBS solution, 8 µg, 200 µL), were given on the same day as immunization. Vehicle solvent without the glycolipid antigen was used as the negative control. For each treatment, ten mice were used as a group.

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