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Quantitative and Qualitative Differences in the In Vivo Response of NKT Cells to Distinct α- and β-Anomeric Glycolipids


NKT cells represent a unique subset of immunoregulatory T cells that recognize glycolipid Ags presented by the MHC class I-like molecule CD1d. Because of their immunoregulatory properties, NKT cells are attractive targets for the development of immunotherapies. The prototypical NKT cell ligand α-galactosylceramide (α-GalCer), originally isolated from a marine sponge, has potent immunomodulatory activities in mice, demonstrating therapeutic efficacy against metastatic tumors, infections, and autoimmune diseases, but also has a number of adverse side effects. In vivo administration of α-GalCer to mice results in the rapid activation of NKT cells, which is characterized by cytokine secretion, surface receptor down-regulation, expansion, and secondary activation of a variety of innate and adaptive immune system cells. In this study, we have evaluated the in vivo immune response of mice to a set of structural analogues of α-GalCer. Our results show that, contrary to current thinking, β-anomeric GalCer can induce CD1d-dependent biological activities in mice, albeit at lower potency than α-anomeric GalCer. In addition, we show that the response of NKT cells to distinct GalCer differs not only quantitatively, but also qualitatively. These findings indicate that NKT cells can fine-tune their immune responses to distinct glycolipid Ags in vivo, a property that may be exploited for the development of effective and safe NKT cell-based immunotherapies.


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4 Abbreviations used in this paper: GalCer, galactosylceramide; GC, GalCer; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MFI, mean fluorescence intensity.
3 days after α-GCs18 administration, after which NKT cell numbers slowly return to normal levels around days 7–10 by homeostatic mechanisms. Thus, the response of NKT cells to glycolipids is characterized by rapid cytokine secretion, surface receptor down-modulation, expansion, and homeostatic contraction.

In vivo activation of NKT cells with α-GCs18 also leads to bystander activation of a variety of innate and adaptive immune system cells, including NK cells (43, 45, 46), B cells (45, 47), conventional T cells (45, 48, 49), and dendritic cells (50–53). These properties make α-GaLCer attractive for the development of immunotherapies and for inclusion in vaccine adjuvants. Indeed, α-GaLCer has been successful in treating certain metastatic tumors and infections, for prevention of autoimmunity, and in enhancing the efficacy of vaccines in mice (reviewed in Refs. 5, 8, 11, and 54–56). However, administration of α-GCs18 can also lead to adverse effects, including liver toxicity (57), abortions (30), and exacerbation of atherogenesis (58) in mice.

One way to develop effective and safe NKT-cell based immunotherapeutics would be to use structural analogues of the prototypical NKT cell ligand α-GCs18. For example, an analog, termed OCH (α-GC9; for structures of glycolipids, see Fig. 1), with a shortened sphingosine base was suggested to be superior to α-GCs18 in preventing experimental autoimmune encephalomyelitis (EAE) in mice. However, little is known regarding the in vivo immunomodulatory activities of altered lipid ligands such as OCH (59). Therefore, we have analyzed the in vivo immune response of mice to a panel of α-GaLCer analogues, in terms of their capacity to induce cytokine secretion, down-regulation of their TCR and NK1.1 markers, in vivo NKT cell expansion, NKT cell-mediated trans-activation of innate and adaptive immune system cells, and prevention of EAE. Our results show that, contrary to current thinking, β-anomeric GaLCer can activate NKT cells in vivo, albeit at reduced efficiency than α-anomeric GalCer. We also found that the response of NKT cells to distinct GalCer differs not only quantitatively but also qualitatively. These findings have important implications for understanding the biology of NKT cells and for developing effective NKT cell-based therapies.

Materials and Methods

Mice

Female C57BL/6 (B6) mice, 6–8 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). CD1d–/– mice (60) were from the tenth backcross to B6. Jα18–/– mice (61) backcrossed at least 10 times to B6 were obtained from Dr. M. Taniguchi (RIKEN Research Center of Allergy and Immunology, Yokohama, Japan). All mice were bred and housed in the animal facility at Vanderbilt University School of Medicine.

Reagents

α-GCs18 (KRN7000; (25,35,4R,1R,3S,4S,5R,6R,7R,8S,9S,10R,11S,12R,13R)-hexacosanyl-2-aminooctacosanoyl-1,3,4,octadecanetriol) (62), α-GC9 (OCH; (25,35,4R,1R,3S,4S,5R,6R,7R,8S,9S,10R,11S,12R,13R)-hexacosanyl-2-aminooctacosanoyl-1,3,4,octadecanetriol) (59), α-GC10 (25,35,4R,1R,3S,4S,5R,6R,7R,8S,9S,10R,11S,12R,13R)-hexacosanyl-2-aminooctacosanoyl-1,3,4,octadecanetriol) (62), and β-GCa26 (25,35,4R,1R,3S,4S,5R,6R,7R,8S,9S,10R,11S,12R,13R)-β-hexacosanyl-2-aminooctacosanoyl-1,3,4,octadecanetriol) (62) were synthesized at Kirin Brewery (Gunma, Japan), as described (62). Disialoganglioside GD3 was obtained from Matreya (Pleasant Gap, PA). Synthetic ceramide, phosphatidylethanolamine, β-GCa0 (psychosine; β-galactosyl-β1′-2-amino-1,3-octadecanediol), β-GCa8 (β-galactosyl-β1′-1′-N-octanoyl-2-amino-1,3-octadecanediol), and β-GCa12 (β-galactosyl-β1′-1′-N-dodecanoyl-2-amino-1,3-octadecanediol) were obtained from Avanti Polar Lipids (Alabaster, AL). All β-anomeric glycolipids were synthesized from β-anomeric precursors. Nuclear magnetic resonance spectroscopy analysis of β-GCa12 was unable to detect α-anomeric forms (data not shown), although this assay does not permit detection of α-anomeric forms at <1% of the β-anomeric form. All glycolipids were reconstituted in PBS containing 0.5% polysorbate-20 (Sigma-Aldrich, St. Louis, MO) and sonicated at 80°C when needed. Before each experiment, glycolipids were freshly diluted from stock solutions. Fluorescently labeled tetrameric CD1d molecules loaded with various glycolipids were prepared as described previously (37, 63). Anti-TCRβ conjugated with FITC or allophycocyanin, anti-NK1.1-PE or -PerCP-Cy5.5, anti-B220-PerCP, anti-CD3-PerCP, anti-IL-4-allophycocyanin, anti-IFN-γ-FITC, anti-CD69-FITC, anti-CD11c-allophycocyanin, anti-CD80-FITC, and anti-CD86-PE were purchased from BD Pharmingen (San Diego, CA). Synthetic myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide (MOGs35-55) was obtained from Bio-Synthesis (Lewisville, TX).

Flow cytometry

Single-cell suspensions of the spleen were prepared and stained with fluorescently labeled mAbs as described (45). In all experiments, dead cells were excluded from the analysis by electronic gating. For identification of NKT cells, B220+ cells were excluded from the analysis by electronic gating. The NKT cell population was identified as CD1dα-GCs18-tetramer+ and TCRβ+ cells. For analysis of intracellular cytokines of NK cells, CD3+ cells were excluded by electronic gating, and NK1.1+ cells were considered as NK cells. In some experiments, splenocytes were activated with ionomycin (1 μM) and PMA (20 ng/ml) for 6 h, followed by intracellular detection of cytokine expression by NK cells. For staining of dendritic cells, FcRs were first blocked by addition of anti-CD16/32 Abs (BD Pharmingen) and dendritic cells were identified on the basis of high expression of CD11c. For intracellular staining, 2 × 106 splenocytes were activated with glycolipids in the presence of Golgi Plug (BD Pharmingen) in U-bottom 96-well plates for 6 h. Intracellular cytokine staining was performed with Cytofix/Cytoperm reagents (BD Pharmingen) according to the manufacturer’s protocol. Data acquisition was performed on a FACSCalibur instrument (BD Biosciences, San Jose, CA), and data were analyzed by FlowJo software (Tree Star, Ashland, OR).

Measurement of in vitro TCR down-modulation

In vitro measurements of TCR down-modulation were performed as described (39). Briefly, 2 × 105 splenocytes were suspended in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, 2 mM glutamine, antibiotics, and 10 mM HEPES (complete medium), and plated in multiple wells of a U-bottom 96-well plate. The cells were incubated with glycolipid Ags to a final concentration of 100 ng/ml for the time periods indicated in the figures. Cells were then briefly centrifuged (1 min, 500 × g) to facilitate rapid NKT cell contact with APCs. The mean fluorescence intensity (MFI) of CD1d-tetramer staining on B220 TCRβ+ tetramer+ cells was measured at the time points indicated in the figures. The percentage of TCR expression on NKT cells was calculated by the following formula: 100 × (geometric MFI of tetramer staining among gated NKT cells in the experimental condition – geometric MFI of tetramer staining for background)/geometric MFI of tetramer staining among gated NKT cells in the control condition – geometric MFI of tetramer staining for the background).

Measurement of in vivo and in vitro responses to glycolipids

For in vivo measurements of cytokines, IgE, and cellular responses to glycolipids, mice were injected i.p. with 200 μl of different concentrations of glycolipids suspended in 0.025% polysorbate-20 in PBS (vehicle). After different time points, mice were bled, and cytokines and IgE levels were measured in the serum. Alternatively, for evaluation of distinct cell subsets, mice were sacrificed after different time points, spleens were harvested, and single-cell suspensions were stained with mAbs followed by flow cytometry. For measurements of in vitro responses to glycolipids, untreated or glycolipid-injected mice were sacrificed, and spleen cell suspensions were plated in U-bottom wells of 96-well plates at 2 × 106 cells per well in complete medium. Cells were incubated with increasing doses of glycolipids for 6 h. For proliferation assays, 1 × 106 μl of [3H]thymidine incorporation was measured in the serum. Alternatively, for evaluation of distinct cell subsets, cells were bled for 24 h after the addition of the well, and cells were incubated for an additional 12 h. Cells were then harvested with a cell harvester (Tomtec, Orange, CT), and 1×106 incorporation was monitored with a betaplate reader (Wallac, Gaithersburg, MD). For evaluation of cytokine secretion in vitro, supernatants were harvested after 60 h of culture with glycolipids and stored until measurement of cytokine levels by ELISA.

NK cell cytotoxicity assay

NK cell cytotoxicity was evaluated against the NK cell target cell line YAC-1, in a standard 51Cr release assay. Data are presented as the percent specific lysis, which was calculated as follows: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release)).
Induction of EAE in mice and clinical evaluation of disease

Active EAE in B6 mice was induced as described previously (64). Briefly, 8- to 10-wk-old female B6 mice were immunized s.c. with 200 μg of MOG<sub>35-55</sub> peptide emulsified in CFA (BD Biosciences) on days 0 and 7. Mice also received 250 ng of pertussis toxin (Invitrogen Life Technologies, Carlsbad, CA) i.p. on days 0 and 2. Mice were treated with different concentrations of glycolipids or vehicle on days 0, 4, and 7 by i.p. injection. Clinical symptoms were monitored daily after the first immunization. The clinical score was graded as follows: 0, no disease; 1, tail limpness; 2, hindlimb weakness; 3, hindlimb paralysis; 4, forelimb weakness; 5, quadriplegia; and 6, death. Cumulative disease scores were calculated by adding daily disease scores from the day after immunization until the end of the experiment.

Evaluation of MOG<sub>35-35</sub>-specific T cell responses

For measurement of MOG<sub>35-35</sub>-specific T cell responses, mice were immunized for induction of EAE and treated with glycolipids as described above. Mice were sacrificed at 11 days, and the lymph nodes were harvested. A single-cell suspension of lymph node cells was prepared, and 2 x 10<sup>5</sup> cells were restimulated in vitro with 0 or 30 μg/ml MOG<sub>35-35</sub> peptide in complete medium for 60 h. The supernatant was harvested at the end of the culture, and IFN-γ and IL-4 levels were measured by ELISA.

ELISA

A standard sandwich ELISA was performed to measure mouse IFN-γ, IL-4, IL-12, and IgE levels. Capture and detection Abs for IFN-γ and IL-4 were obtained from R&D Systems (Minneapolis, MN); those for IL-12 and all cytokine standards were from BD Pharmingen. Capture and detection Abs for IgE as well as IgE standards were obtained from BD Pharmingen. For detection, streptavidin-HRP conjugate (Zymed Laboratories, San Francisco, CA) was used, and the color was developed using H<sub>2</sub>O<sub>2</sub>/3,3',5,5'-tetramethylbenzidine (DakoCytomation, Carpinteria, CA) as the substrate.

Results

In the present study, we have compared the in vitro and in vivo responses of NKT cells to distinct synthetic glycosylceramides. As a reference for comparison, we used the well-characterized α-α-anomeric GalCer KRN7000 (α-GCs18), which has an acyl chain of 26 hydrocarbons and a sphingosine base of 18 hydrocarbons (Fig. 1). We have studied four β-α-anomeric GalCer, β-GCa0 (also called psychosine), β-GCa8, β-GCa12, and β-GCa26 that differ from each other in the length of their acyl chains (Fig. 1), and two α-α-anomeric GalCer, α-GCs9 (also called OCH) and α-GCs10, which have a shortened sphingosine base, as compared with the prototypical α-GCs18 (Fig. 1).

Capacity of distinct glycosylceramides to induce in vitro proliferation and cytokine production by splenocytes

We first compared the in vitro proliferative and cytokine responses of total splenocytes to distinct glycosylceramides. Splenocytes from wild-type B6 mice, CD1d-deficient, or Ja18-deficient mice were treated in vitro with increasing doses of glycolipid Ags, ranging from 0 to 1000 ng/ml, and evaluated for proliferative and IFN-γ and IL-4 responses. Results (Fig. 2A) showed that α-GCs18 was most potent in inducing proliferation and cytokine production. Although it has been suggested that β-α-anomeric GalCer do not efficiently activate NKT cells (38), we found that β-GCa12 (Fig. 2A) and β-GCa26 (data not shown), but not β-GCa0, β-GCa8, ceramide, phosphatidylethanolamine, and disialoganglioside GD3 (data not shown), were able to induce proliferation and cytokine production when used at high doses (100–1000 ng/ml). To confirm that these activities of β-GCa12 are mediated by engagement of the invariant TCR expressed by NKT cells, we generated tetrameric CD1d molecules loaded with this reagent and stained splenocytes and liver mononuclear cells from wild-type, CD1d<sup>−/−</sup>, and Ja18<sup>−/−</sup> mice. Results (Fig. 2B) demonstrated significant staining in wild-type, but not CD1d<sup>−/−</sup> and Ja18<sup>−/−</sup> mice, whereas empty tetramers were unable to stain cells of mice from any genotype.

Although the responses to α-GCs9 and α-GCs10 in these assays were indistinguishable from each other, proliferative responses to these analogues were reproducibly weaker than to α-GCs18 (Fig. 2A). Interestingly, levels of IL-4 in the cultures treated with α-GCs9, α-GCs10, and α-GCs18 were very similar, whereas levels of IFN-γ were consistently lower in cultures treated with α-GCs9 and α-GCs10, compared with α-GCs18-treated cultures. These findings with α-GCs9 are consistent with prior studies (59, 65).

Importantly, none of the cultures from splenocytes of CD1d- and Ja18-deficient mice induced significant proliferation or cytokine secretion (Fig. 2A), indicating that responses observed in wild-type splenocytes required CD1d-restricted NKT cell activation.

Relative capacity of glycolipids to down-regulate expression of the invariant TCR expressed by NKT cells in vitro

Our previous results have shown that NKT cells down-regulate their TCR within a few hours of stimulation with α-GCs18 (39). The extent of TCR down-regulation provides a direct measure of the extent of T cell activation (65, 66). Therefore, we stimulated NKT cells with α-GCs18, β-GCa12, α-GCs9, and α-GCs10 in vitro for different time points and analyzed levels of TCR expression using tetrameric CD1d molecules loaded with α-GCs18 (CD1d-tetramers). In these experiments we used a glycolipid dose of 100 ng/ml for all analogues, as well as a higher dose for β-GCa12 (1000 ng/ml), because it is a less potent activator of NKT cell responses (see Fig. 2A). We found that all analogues tested were capable of down-regulating expression of the NKT cell receptor and that β-GCa12 was the least effective (Fig. 3). TCR down-regulation mediated by α-GCs18 was more rapid than for any of the other reagents tested. However, whereas TCR levels on NKT cells activated with α-GCs18 started to increase again at 24 h after activation, TCR levels of NKT cells stimulated with all other analogues continued to decrease until at least 24 h following the initial activation.

In vivo dynamics of the NKT cell population

We (39) and others (43, 63) have previously shown that in vivo administration of α-GCs18 induces a rapid decline in CD1d-tetramer<sup>+</sup> cells, which is largely due to transient down-regulation of the invariant TCR by these cells (39–41). NKT cells gradually reappear around 24 h after α-GCs18 administration, and then undergo a period of significant proliferation, expanding 5- to 10-fold in the spleen, peripheral blood, and bone marrow. Following this period of expansion, levels of NKT cells return to basal levels at 7–12 days after their initial activation. Therefore, we next compared the capacity of distinct glycolipids to induce the disappearance and expansion of NKT cells in vivo. We focused our analysis on NKT cells in the spleen, because NKT cell expansion is most profound in this organ (39, 40). Consistent with our in vitro experiments (Figs. 2A and 3), α-GCs18 is most potent in causing the disappearance of NKT cells from the spleen (Fig. 4, A and B). β-GCa0 and β-GCa8 were unable to induce any reduction in the prevalence of TCR<sup>+</sup>CD1d-tetramer<sup>+</sup> cells, even when used at a dose of 50 μg/mouse (Fig. 4C). Although β-GCa12 (Fig. 4, A and B) and β-GCa26 (data not shown) had no detectable effect when administered at a low dose (5 μg/mouse), these reagents were clearly capable of inducing a slight reduction in NKT cell TCR surface levels and a concomitant reduction in the overall numbers of TCR<sup>+</sup>CD1d-tetramer<sup>+</sup> cells when used at a high dose (50 μg/mouse). In the case of α-GCs9 and α-GCs10 (Fig. 4, A and B),
FIGURE 1. Structures of glycolipids used in this study. \( \alpha \)-GCs18 (also called KRN7000) is the prototypical \( \alpha \)-GalCer and contains a C26 acyl chain and a C18 sphingosine base. \( \beta \)-GCa0 (also called psychosine), \( \beta \)-GCa8, \( \beta \)-GCa12, and \( \beta \)-GCa26 are \( \beta \)-anomeric GalCer that differ from each other in the length of the acyl chain and in the structure of the sphingosine base. \( \alpha \)-GCs9 (also called OCH) and \( \alpha \)-GCs10 differ from each other and from \( \alpha \)-GCs18 in the length of the sphingosine base.
FIGURE 2. Capacity of distinct glycosylceramides to activate splenocytes. A. In vitro proliferative and cytokine responses of splenocytes to glycolipid stimulation. Total splenocytes (2 x 10^5/well) from wild-type B6 mice, CD1d-/- mice, and Jα18-/- mice were stimulated with graded doses of the indicated glycolipids. After 72 h, proliferation was assessed by [3H]thymidine incorporation. Supernatants were harvested at 60 h of culture to monitor IL-4 and IFN-γ responses. The proliferation results shown represent the mean ± SE of triplicate wells, and the cytokine results represent the mean ± SE of two mice. Representative data of three similar experiments are shown. B. The reactivity of β-GCa12 is dependent upon interaction with the invariant TCR expressed by NKT cells. Splenocytes or liver mononuclear cells from the indicated mice were stained with anti-TCR-β-FITC Abs and PE-labeled, tetrameric CD1d molecules loaded with the indicated glycolipids. Numbers indicate the percentage of cells within each quadrant. Data are representative of two separate experiments.
significant TCR down-modulation was observed, but this was less profound than that observed with α-GC18, resulting only in a slight apparent loss of NKT cell numbers early (8 h) following glycolipid administration.

Consistent with our prior studies, we found that α-GC18 induces profound in vivo NKT cell expansion, reaching levels that are increased 5- to 10-fold around 3 days after injection. Surprisingly, however, none of the other reagents tested induced significant NKT cell expansion, even when used at a dose of 50 μg/mouse (Fig. 4, A, B, and D). As α-GC18 induces NKT cell expansion even at doses as low as 0.5 μg/mouse (data not shown), these findings suggest that NKT cells can generate qualitatively distinct responses to structurally different glycosylceramides in vivo.

In addition to down-regulation of TCR expression, activated NKT cells down-regulate expression of the NK1.1 marker (39, 40, 67). In vivo, NK1.1 down-regulation becomes detectable around 24 h following α-GC18 administration and is maximal around 3 days, after which NK1.1 levels slowly return to normal, remaining significantly reduced for at least 10 days (39, 40). Thus, we evaluated different GalCer for this parameter of NKT cell activation (Fig. 4, E and F). We found, once again, that α-GC18 was most potent in inducing NK1.1 down-regulation, whereas little NK1.1 down-regulation was observed for β-GCa12, even at a dose of 50 μg/mouse. α-GC9 and α-GC10 efficiently induced NK1.1 down-regulation on NKT cells, but their effects were less sustained than α-GC18 (Fig. 4, E and F).

Serum cytokine and IgE levels

Glycolipid-activated NKT cells are capable of modulating Th cell responses by inducing cytokine production, in particular IL-4, IFN-γ, and IL-12 (9, 54, 55). Therefore, we measured the levels of these cytokines in the serum of mice at 2, 6, or 24 h after glycolipid treatment. Results for α-GC18 were consistent with prior studies (4, 45, 68), indicating an early burst of IL-4 (peaks at 2 h), followed by IL-12 (peaks at 6 h), and IFN-γ (peaks at 24 h) (Fig. 5A). Although a low dose of β-GCa12 did not result in any appreciable cytokine production, at a high dose, this reagent clearly induced IFN-γ and IL-12 production (most evident at the 6-h time point), but no detectable IL-4. In sharp contrast, α-GC9 and α-GC10 induced an early burst of IL-4 production that was similar to that induced by α-GC18. Consistent with prior studies (59), levels of IFN-γ induced by α-GC9 and α-GC10 were slightly lower at 6 h and substantially lower at 24 h, compared with α-GC18. Curi-}

osuly, however, and in contrast with the previous study (59), IFN-γ levels induced by α-GC9 and α-GC10 at early time points (2 h) were higher than those induced by α-GC18. The serum profiles of IL-12, produced in response to α-GC9 and α-GC10, were very similar to that of α-GC18.

Prior studies have indicated that a single injection of α-GC18 to B6 mice induces a profound increase in serum IgE levels (45, 68). Therefore, we compared the capacity of distinct glycolipids to induce this Ig isotype. All glycolipids tested induced a significant increase in serum IgE levels, but α-GC18 was most potent (Fig. 5B).

Cytokine production by NKT cells

Glycolipid-activated NKT cells trans-activate a variety of cell types (55). Consequently, cytokine levels in splenocyte cultures or the serum of mice in response to glycolipid treatment reflect the overall pool of cytokines produced by NKT cells themselves, together with those produced by a variety of cell types activated secondarily to NKT cells. Therefore, we evaluated IL-4 and IFN-γ secretion by NKT cells from glycolipid-treated mice using an intracellular staining assay. We found that cytokine profiles produced by NKT cells from naive mice in response to in vitro treatment with distinct glycolipids were nearly indistinguishable, but that β-GCa12 was the least effective (0-h time points in Fig. 6). In vivo administration of glycolipids invariably appeared to result in a rapid reduction of IL-4 production, but sustained IFN-γ production (8-h, 24-h, 3-day, and 7-day time points in Fig. 6). As in previous experiments, α-GC18 was the most effective inducer of cytokines, β-GCa12, although capable of inducing cytokines when used at a high dose, was the least effective, and responses mediated by α-GC9 and α-GC10 were indistinguishable from each other.

Activation status of B cells, NK cells, conventional T cells, and dendritic cells

In vivo activation of NKT cells results in the trans-activation of a variety of cell types, including NK cells (43, 45, 46), B cells (45, 47), conventional T cells (45, 48, 49), and dendritic cells (50–53). Therefore, we compared the activation status of NKT cells, NK cells, B cells, and conventional T cells using the activation marker CD69, and the activation status of dendritic cells by the expression levels of CD80 and CD86 after 24 h of in vivo treatment with glycolipids. We found significant up-regulation of CD69, CD80, and CD86 expression on all cell types evaluated, and by each of the glycolipids tested (Fig. 7). Akin to our earlier experiments,
FIGURE 4. In vivo dynamics of the NKT cell population in response to distinct glycolipids. Mice were injected with the indicated glycolipids (5 μg per mouse or as indicated; i.p.) or vehicle. Spleen cells were prepared at the indicated time points, stained with anti-TCRβ-FITC, CD1d/α-Gc18-tetramer-PE, anti-B220-PerCP, and anti-NK1.1-allophycocyanin, and analyzed by flow cytometry. A, Dot plots of TCRβ vs tetramer. B, Graphical representation of the data from A. C, Dot plots for TCRβ vs tetramer at 8 h after injection of the indicated glycolipids. D, Flow cytometry plots for TCRβ vs tetramer at 3 days after injection of the indicated glycolipids. Data shown represent the mean ± SE of two mice per time point. Numbers in A, C, and D indicate the average percentage of TCRβ+ tetramer”B220” cells for two mice. E, Dot plots of tetramer vs NK1.1. Numbers indicate the average percentage of tetramer”NK1.1”B220” cells for two mice. F, Percentage of NK1.1+ cells among tetramer” cells shown in E. Data shown represent the mean ± SE of two mice per time point. Data for A, B, E, and F are representative of four separate experiments with two to three mice in each group; data for C and D are from one experiment with two mice per group.
\(\alpha\text{-GCs18}\) was most potent and \(\beta\text{-GCa12}\) was the least potent stimulator. However, whereas a dose of 5 \(\mu\text{g}\) of \(\beta\text{-GCa12}\) per mouse induced no detectable TCR and NK1.1 down-regulation on NKT cells in vivo, this dose was able to increase CD69 expression on NKT and NK cells and to a lesser extent on B cells and conventional T cells, as well as increase CD80 and CD86 expression on CD11c\text{hi}\) dendritic cells.

**NKT cell-mediated trans-activation of NK cells**

Because the capacity of ligand-activated NKT cells to trans-activate NK cells plays a critical role in the in vivo response of NKT cells, we evaluated glycolipids for their effects on this aspect of the immune response in greater detail. First, we (39) and others (43) have previously shown that \(\alpha\text{-GCs18}\) treatment of mice results in a transient reduction in the prevalence of NK1.1\text{hi} TCR\text{β} cells, representing mostly NK cells (as well as some NKT cells that have down-regulated their surface TCRs), by a mechanism that remains undefined. All glycolipids tested in this assay were able to induce a short-term reduction in the prevalence of NK cells, with similar kinetics (Fig. 8, A and B). Second, we tested NK cells for IFN-\(\gamma\) secretion by intracellular staining at different time points after in vivo treatment with glycolipids (Fig. 8, C–E). Consistent with their effects on the disappearance of NK cells, all glycolipids tested were able to induce IFN-\(\gamma\) production by these cells. Third, we evaluated the capacity of splenocytes to lyse YAC-1 target cells, at 24 h after glycolipid injection. All glycolipids tested were effective at inducing the lysis of YAC-1 target cells, albeit at distinct efficiency (Fig. 8F).

Collectively, these findings indicate that all glycolipids tested are capable of inducing NK cell activation in terms of CD69 induction (Fig. 7A), transient NK cell depletion (Fig. 8, A and B), IFN-\(\gamma\) synthesis (Fig. 8, C–E), and cytotoxic effector function (Fig. 8F).

**Impact of distinct glycosylceramides on the progression of EAE**

Finally, we compared the capacity of glycolipids to modulate EAE disease, induced in B6 mice by immunization with the MOG\text{35–55} peptide. Based on our prior experience with this model (64), we selected the dose of 2 \(\mu\text{g}\)/injection/mouse of each of the glycolipids as well as a dose of 20 \(\mu\text{g}\)/injection/mouse in the case of the \(\beta\text{-anomeric GalCer}\) administered to mice on days 0, 4, and 7 after EAE induction. Results (Fig. 9A and Table I) showed that \(\alpha\text{-GCs18}\), \(\alpha\text{-GCs9}\), and \(\alpha\text{-GCs10}\) have similar potency in preventing EAE. However, in most experiments, \(\alpha\text{-GCs18}\) was more potent than any of the other glycolipids in preventing EAE (Fig. 9A and Table I). At the low dose, \(\beta\text{-GCa12}\) and \(\beta\text{-GCa26}\) slightly ameliorated EAE disease, whereas, consistent with our prior studies (64), at a higher dose, these reagents exacerbated disease, with typically an earlier onset of disease and increased mortality (e.g., in the experiment shown in Fig. 9A, 4 of 6 mice treated with 20 \(\mu\text{g}\) of \(\beta\text{-GCa12}\)/injection/mouse died, compared with 1 of 10 mice in the vehicle group).

To evaluate effects of glycosylceramides on MOG\text{35–55} peptide-specific immune responses, we measured cytokine responses of lymph node cells from mice induced with EAE and treated with glycolipids to in vitro stimulation with MOG\text{35–55}. Consistent with prior studies (64), \(\alpha\text{-GCs18}\) suppressed MOG\text{35–55}-induced IFN-\(\gamma\) production and enhanced MOG\text{35–55}-induced IL-4 production, compared with vehicle-treated mice (Fig. 9B). Treatment with \(\alpha\text{-GCs9}\) and \(\alpha\text{-GCs10}\) had similar effects on IFN-\(\gamma\), but little effect on IL-4 production. Finally, \(\beta\text{-GCa12}\) had no detectable influence.
FIGURE 6. NKT cell cytokine production following glycolipid administration. Mice were injected with the indicated glycolipids (5 μg per mouse or as indicated; i.p.) or vehicle. Spleen cells were prepared at the indicated time points, and 2 × 10⁶ cells were restimulated in vitro with glycolipids at a concentration of 100 ng/ml and cultured in the presence of Golgi Plug for 6 h to induce the intracellular accumulation of cytokines. Cells were harvested and surface stained with tetramer-PE and anti-B220-PerCP, followed by intracellular staining with anti-IL-4–allophycocyanin and anti-IFN-γ–FITC. Data are shown for NKT cells (B220<sup>+</sup> tetramer<sup>+</sup>). Numbers indicate the percentage of cells within each quadrant. Data are representative of three separate experiments.

Discussion

We have performed a detailed comparison of the immunomodulatory properties of a panel of GalCer analogues, in terms of their effects on NKT cells themselves, distinct immune responses that are activated downstream of NKT cells, and disease modulation. Our findings indicate that β-GalCer with long acyl chains (C ≥ 12), when used at high concentrations, can effectively activate cytokine secretion by NKT cells and induce the trans-activation of a variety of cell types. In addition, among all GalCer tested, only α-GCs18 (KRN7000) was able to induce significant NKT cell expansion in vivo, although other GalCer were able to effectively trans-activate a number of cell types in response to NKT cell stimulation. None of these activities were seen in the absence of CD1d or Jα18 expression, indicating that they require CD1d-restricted Ag presentation to invariant NKT cells. Finally, in our hands, α-GCs18 was as potent, or more potent, than analogues with a shortened sphingosine base, α-GCs9 (OCH; C = 9) and α-GCs10 (C = 10), for modulating EAE disease. Collectively, our findings indicate that NKT cells can fine-tune their responses to distinct glycolipids.

Our results with β-GCa12 and β-GCa26 indicate that these reagents, when used at high concentrations, effectively induce cytokine secretion in splenocyte cultures in vitro, and in the serum in vivo. The cytokine profiles induced by these reagents were similar to those induced by the prototypical α-GalCer, α-GCs18 (KRN7000). However, β-GalCer induced little IL-4 secretion in the serum. Furthermore, intracellular staining of NKT cells demonstrated significant IFN-γ and IL-4 production in response to β-GCa12, although responses were substantially reduced compared with the α-GalCer tested. Additionally, β-GCa12 effectively activated a variety of other cell types, including B cells, conventional T cells, dendritic cells, and NK cells, secondary to NKT cell stimulation. The capacity of β-GCa12 to induce the activation of NK cells was confirmed by a variety of means, including the induction of CD69 expression by NK cells, the transient disappearance of these cells in vivo around 8–48 h after treatment, production of intracellular IFN-γ, and in vitro cytotoxicity against YAC-1 target cells. The capacity of β-GCa12 and β-GCa26 to induce trans-activation of NK cells, in the face of relatively poor IL-4 secretion by NKT cells, may provide a possible explanation for the tendency of these reagents, when used at high doses, to exacerbate rather than suppress EAE disease (64).

In agreement with the findings reported here for β-anomeric GalCer, a recent report (69) has shown that β-GalCer can effectively activate NK cells. However, in apparent contrast with our findings, these investigators were unable to find evidence for significant NK cell stimulation mediated by β-GalCer (69). However, it is interesting to note that most of the experiments by Ortaldo et al. (69) and those reported here, were performed with β-GCa12 purchased from the same commercial source (i.e., Avanti Polar Lipids). In addition, we have confirmed most of our findings, in particular the NK cell activation data, with a separate reagent, β-GCa26, which was derived from a different source (i.e., Kirin Brewery). At present, the reasons for these apparent differences in
results are unclear. Possible explanations include details in experimental setup and sensitivity of assays used for NK cell activation.

One potential concern of our and other studies performed with α-anomeric GalCer is their potential low-level contamination with β-anomeric forms. Although nuclear magnetic resonance studies were unable to detect any β-anomeric forms among these α-anomeric Gal-Cers (data not shown), the relatively low sensitivity of this assay cannot exclude low levels (<1%) of contamination. Although we cannot formally exclude contamination with β-anomeric GalCers that we have studied: 1) these reagents were very potent in inducing the activation of CD80 and CD86 expression by dendritic cells, 2) they are relatively ineffective in inducing serum IL-4 secretion in vivo, 3) they are relatively ineffective in inducing IL-4 production by NK cells, while inducing significant IFN-γ, as measured by intracellular staining, and 4) when used at high doses, they exacerbate EAE. Taken together, these findings provide evidence for qualitative differences in the responses of mice to β- vs α-anomeric GalCers, arguing against contamination as the reason for our observed activities of β-GCa12 and β-GCa26.

It has been generally assumed that NKT cells recognize α-anomeric but not β-anomeric glycosphingolipids (38, 70). The latter, but not the former, are found in mammals, particularly in the nervous system (71, 72). Our findings that β-GalCer with long acyl chains, can be recognized by NKT cells and elicit the effector functions of these cells, raises the interesting possibility that endogenous β-anomeric glycosphingolipids may function as (one of)

**FIGURE 7.** Activation status of different cell types in glycolipid-injected mice. Mice were treated with vehicle or the indicated glycolipid analogues (5 µg per mouse or as indicated; i.p.). After 24 h, mice were sacrificed and single-cell suspensions of the spleen were prepared. A, Expression of the activation marker CD69 by NKT cells, NK cells, B cells, and conventional T cells. Spleen cells were stained with anti-CD69-FITC or its isotype control (filled), and with different combinations of tetramer-PE, anti-B220-PerCP, anti-TCRβ-allophycocyanin, and anti-NK1.1-PerCP-Cy5.5. CD69 expression was measured on NKT cells (B220+ TCRβ+ tetramer+), NK cells (B220+ TCRβ+ NK1.1+), B cells (B220+ TCRβ+) and conventional T cells (TCRβ+ tetramer+ B220+) in mice injected with glycolipid (thick line) or vehicle (thin line). B, Expression of CD80 and CD86 on dendritic cells. Spleen cells were stained with combinations of anti-CD11c, anti-CD80, anti-CD86, or their respective isotype control Abs (filled). Data are shown for CD11c+ cells after treatment with the glycolipid Ags (thick lines) or vehicle (thin lines). Data are representative of three separate experiments.
FIGURE 8. NKT cell-mediated trans-activation of NK cells by glycolipid Ags. Mice were injected (i.p.) with vehicle or the indicated glycolipids (5 μg per mouse or as indicated). Spleen cells were prepared at the indicated time points. A and B, Prevalence of NK cells. Cells were stained with anti-TCRβ-FITC, anti-NK1.1-PE, and anti-B220-PerCP, and analyzed by flow cytometry. Numbers in A indicate the mean percentage of NK cells (TCRβ⁺ NK1.1high) for two to three mice. Data from A are summarized in B as the mean ± SE percentage of NK cells in glycolipid-injected mice compared with uninjected mice. Data shown represents one of four similar experiments. C-E, Cytokine production by NK cells after glycolipid administration. Mice were injected with glycolipids, and at different time points, spleen cells were cultured in medium (C and E) or PMA (20 ng/ml) plus ionomycin (1 μM) (D) in the presence of Golgi Plug for 6 h to allow intracellular cytokine accumulation. Cells were harvested and surface-stained with anti-CD3-PerCP and anti-NK 1.1-PE, followed by intracellular staining with anti-IFN-γ-FITC. Data for the 24-h time point are shown in C and D, and data for different time points after glycolipid injection are shown in E. The percentages of IFN-γ-positive cells were determined after electronic gating on the CD3⁺ NK1.1high population. F, In vitro NK cell cytotoxicity. Splenocytes from mice injected with the glycolipid Ags were prepared after 24 h of administration and used as the cytotoxic effector cells against 51Cr-labeled YAC-1 cells in different E:T ratios. The data shown are the mean ± SE of two mice from representative of three separate experiments.
the physiological ligands for NKT cells. In this context, we have shown previously that cells deficient in β-D-glucosylceramide synthase fail to stimulate NKT cell hybridomas (37). Thus, our findings raise the possibility that β-D-anomeric glycolipid downstream of this enzyme could function as a natural ligand for NKT cells. However, it is unlikely that the physiological concentrations of β-D-anomeric endogenous glycolipids reach levels that are sufficient to directly activate NKT cells. Nevertheless, it has been recently proposed that NKT cells use a strategy for their activation involving weak responses to self Ags that are amplified by proinflammatory cytokines such as those produced during microbial infection (73). Our findings suggest that β-anomeric glycolipids may provide such a source of weakly reactive self Ags. Future studies will be designed to test this hypothesis.

A key observation in our studies is that only α-GC18 is capable of inducing significant in vivo expansion of NKT cells. Because α-GC18 induces NKT cell expansion over a wide range of in vivo doses (data not shown), this is not simply due to differences in the overall strength of TCR signaling, but rather quantitatively different responses of NKT cells to distinct glycolipid Ags. For example, despite the lack of NKT cell expansion mediated by α-GC9 and α-GC10, these reagents and α-GC18 promoted similar levels of IL-4 secretion by NKT cells and in the serum. Nevertheless, α-GC9 and α-GC10 appeared to be less effective than α-GC18 in inducing IFN-γ production by NKT cells. Furthermore, we did not find a substantial difference in the capacity of these reagents to induce the trans-activation of different innate and adaptive immune cells, including NK cells, and in their capacity to prevent EAE.

GalCer hold significant promise for the development of NKT cell-based immunotherapies. α-GalCer have been successfully used in treating certain metastatic tumors and infections, and for preventing autoimmunity (reviewed in Refs. 5, 8, 11, and 54–56). However, administration of α-GalCer can also lead to adverse effects, including liver toxicity (57), abortions (30), and exacerbation of atherogenesis (58) in mice. Although administration of α-Gal-Cer to humans appears to have no adverse consequences, at least in the short-term and at the doses used (74, 75), these reagents have significant potential for generating unwanted side effects. Therefore, the availability of glycolipid analogues that selectively induce certain effector functions of NKT cells holds great promise for the development of effective and safe NKT cell-based immunotherapies. Although it has been previously suggested that α-Gal-Cer with a shortened sphingosine base may have superior efficacy for prevention of autoimmune disease (59, 76), we were unable to reproduce those findings, at least at the doses that were tested. Nevertheless, our findings indicate that NKT cells can fine-tune their in vivo responses to distinct glycolipids, suggesting that it may be possible to generate NKT cell ligands that selectively promote certain NKT cell-mediated immune effector functions and have superior immunomodulatory activities.


