

FIGURE 6. Implication of regulatory T cells after α -GalCer treatment. **A**, An increased number of Foxp3 regulatory T cells in MLNs from α -GalCer-treated mice was observed. cDNAs obtained from total MLNs of infected B6 and J α 281 $^{-/-}$ mice treated, or not, with α -GalCer were submitted to quantitative real-time PCR with specific primers and probed for Foxp3 and actin (five mice per group). After normalization to actin expression, results were expressed as an increase in Foxp3 expression in α -GalCer-infected B6 or J α 281 $^{-/-}$ mice compared with infected B6 or J α 281 $^{-/-}$ mice. This experiment was repeated twice with similar results. **B**, B6 mice were depleted of CD25 $^{+}$ cells with anti-CD25 mAb i.p. 3 days before α -GalCer treatment. Twenty-four hours later, all mice were infected. Similarly infected α -GalCer-treated B6 mice, anti-CD25-treated B6 mice, and B6 mice treated with isotype Abs were used as controls (five mice per group). Results are representative of two independent experiments.

significant increase in cytokine production with time (day 8). This late IFN- γ production indicates that other cells within the responding immune population (e.g., CD4 $^{+}$ T cells from the lamina propria) are specifically activated and probably are responsible for the death of 75% of the J α 281 $^{-/-}$ mice and the mild inflammation observed in the intestines of surviving mice.

NKT cells can be activated through different pathways. Activation through TCR ligation by CD1d-associated glycolipid is one possibility. Alternatively, IL-12 might activate NKT cells directly, in the absence of TCR engagement (38, 39), or might synergize its effect to that of TCR engagement (40). The activation pathway responsible for NKT cells activation after *T. gondii* infection remains unclear. It is indeed unknown whether TCR engagement by *Toxoplasma* Ag or through recognition of self Ag is required. Recently, Brigl et al. (40) have described a model in which NKT cells in the presence of IL-12 were activated after recognition of self Ags presented by CD1d. IL-12 was first made by DCs in response to microbial products, and this cytokine, in turn, activated NKT cells to up-regulate CD69 expression and IFN- γ production. One of the potential *Toxoplasma* Ag responsible directly or indirectly for NKT activation is the surface Ag-1 (SAG1) protein, the major surface protein of the parasite. The SAG1 molecule induces the dominant Ab response during infection (41) and a strong Th1 immune response characterized by high levels of IFN- γ production by CD4 T cell from the lamina propria and CD8 T lymphocytes (42, 43). SAG1 is a GPI-anchored protein and could be a potential ligand for CD1d molecule.

The hypothesis of the activation of NKT cells through TCR recognition of CD1d-presented Ag is attractive in our model. How-

ever, after oral infection with *T. gondii*, CD1 $^{-/-}$ (B6 background) mice developed an acute and lethal ileitis within 7 days despite the absence of NKT cells. This suggests that CD1d may act via several alternative pathways. Besides its activity on NKT cell activation, CD1d is important for the activation of IELs (33) that down-regulate the intestinal inflammation after *T. gondii* infection. Indeed, upon Ag activation these IEL secrete copious amounts of TGF- β that participate in the maintenance of gut homeostasis (28). The lack of CD1 expression leads to the absence of protective IELs, and the absence of regulatory mechanisms overcome the absence of inflammatory NKT cells. In addition, the CD1d molecule is expressed on both the apical and the basolateral membranes of intestinal epithelial cells (44), and its ligation induces IL-10 secretion by these cells (45). Thus, the regulation of CD1 expression and its recognition by the TCR could play important roles in the regulation of intestinal inflammatory processes.

In this model of pathogen-driven inflammatory disease, NKT cells are important for the initiation of the robust Th1 inflammatory immune response in the intestine after oral parasite infection. Alternatively, α -GalCer and related glycolipids can modulate NKT cell responses toward a Th2-like profile (11, 12, 46). Our observations demonstrate that α -GalCer treatment has an impact on the intestinal immune response by shifting the cytokine profile production by NKT cells toward a Th2 phenotype, resulting in orientation of the lamina propria CD4 response. A single dose of α -GalCer prevented the development of lethal ileitis after infection with *T. gondii*. This treatment resulted in a Th2 immune response characterized by the production of IL-4, IL-10, and IL-13 by intestinal NKT cells. The major cytokine implicated in this protection is IL-10, because the beneficial effect of α -GalCer treatment was completely abrogated in IL-10-deficient mice.

Our data are in full agreement with previous work reporting the high susceptibility of IL-10-deficient mice to the development of lethal ileitis after oral *T. gondii* infection (47). This susceptibility is associated with the defect of T cells to produce IL-10, because mice with an inactivation of the IL-10 gene restricted to T cells generated by Cre/loxP-mediated targeting of the IL-10 gene succumb to severe immunopathology upon infection with *T. gondii* (48).

IL-10 secreted by NKT cells also participated in the protective effect of α -GalCer treatment, because double-chimeric mice in which NKT cells alone were impaired in IL-10 secretion were more susceptible to the development of ileitis than controls after α -GalCer injection. However, other IL-10-producing cells are also implicated, because treatment with α -GalCer reduced the mortality of these double-chimeric mice. Regulatory CD25 $^{+}$ T cells are the likely candidates, because they are present in the intestine, and the anti-CD25 treatment blocked the protective effect of α -GalCer injection.

IL-10 produced by NKT cells has been shown to exert an important regulatory function in experimental models of different pathologies, such as diabetes (49) and allergic encephalomyelitis (50). The link between the shift in the cytokine profile produced by NKT cells toward a Th2 profile and the activation of regulatory CD4 $^{+}$ T cells is as yet unknown. IL-10-producing CD4 $^{+}$ NKT cells are involved in the generation of regulatory CD8 $^{+}$ T cells after Ag exposure in the anterior chamber of the eye (51). Several reports indicate that NKT cells may contribute to immunoregulation via DC maturation (52). DC maturation in the presence of IL-10 may equally induce T regulatory 1 or Th3 regulatory T cells (53). Secretion of IL-4 and IL-10 by intestinal NKT cells after α -GalCer treatment may act directly on local DCs during induction of the polarization of the immune response and promote a Th2 profile. There is evidence that DCs that mature in the presence of NKT cells produce greater amounts of IL-10

and lose the ability to secrete IL-12, a phenotype consistent with a tolerogenic function (17).

The participation of IL-4 in this process cannot be ruled out. The role of IL-4 seems to be complex in toxoplasmosis. Our data indicate that neutralization of IL-4 cannot render α -GalCer-treated mice as susceptible as wild-type, infected, untreated mice, indicating the participation of other cytokine, such as IL-10. In addition, these experiments might indicate, as suggested by Nickdel et al. (54), that IL-4-deficient mice are more resistant than wild-type mice to the development of ileitis. However, our data for IL-4 corroborate previous findings reporting that treatment with α -GalCer or OCH (a synthetic glycolipid that has shorter hydrophobic chain) improves mucosal Th1/Th2 cytokine balance by increasing IL-10 and IL-4 production and prevents experimental colitis in mice (55).

The important role played by NKT cells in the regulation of the intestinal immune response has also been previously suggested in a colitis model induced by chemical agents such as dextran sodium sulfate (56) or oxazolone (57). The pathogenic pathway leading to tissue injury in dextran sodium sulfate-induced colitis and, by extension, in Crohn's disease was attributed to production of Th1 cytokines such as IFN- γ and to the presence of NK1.1⁺ T cells (56). However, the pathogenic pathway leading to tissue injury in oxazolone colitis was also associated with NKT cells secreting IL-13 (57).

The presence of IL-10-secreting T regulatory lymphocytes has been associated with regulation of intestinal inflammation (33), and in our model these cells may be ultimately responsible for the protective effect seen after treatment with α -GalCer. These data illustrate the dual potential of NKT cells in orienting distinct (i.e., Th1 or Th2) immune responses depending on the stimuli used.

After activation with *T. gondii*, NKT cells are important mediators of the immune response via a robust IFN- γ -mediated effect that limits parasite replication and allows for parasite clearance. However, this early and influential response is not without drawbacks and can be detrimental to the host. This response, when uncontrolled, leads to the development of an acute inflammatory process and death within 7 days of infection in this experimental model of pathogen-driven ileitis. Our data highlight the crucial role of NKT cells derived from the gut in the modulation of intestinal homeostasis.

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Disclosures

The authors have no financial conflict of interest.

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Preferential T_H2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokines by bystander cells *in vivo*

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Abstract

The altered glycolipid ligand OCH is a selective inducer of T_H2 cytokines from NKT cells and a potent therapeutic reagent for T_H1-mediated autoimmune diseases. Although we have previously shown the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells, little is known about the extrinsic regulatory network for IFN- γ production. Here we demonstrate that OCH induces lower production of IFN- γ , not only by NKT cells but also by NK cells compared with α -galactosylceramide. OCH induced lower IL-12 production due to ineffective primary IFN- γ and CD40 ligand expression by NKT cells, and resulted in lower secondary IFN- γ induction. Co-injection of a sub-optimal dose of IFN- γ and stimulatory anti-CD40 mAb compensates for the lower induction of IL-12 by OCH administration. IL-12 converts OCH-induced cytokine expression from IL-4 predominance to IFN- γ predominance. Furthermore, CpG oligodeoxynucleotide augmented IL-12 production when co-administrated with OCH, resulting in increased IFN- γ production. Taken together, the lower IL-12 production and subsequent lack of secondary IFN- γ burst support the effective T_H2 polarization of T cells by OCH. In addition, highlighted in this study is the characteristic property of OCH that can induce the differential production of IFN- γ or IL-4 according to the availability of IL-12.

Introduction

NKT cells are a unique subset of CD1d-restricted T lymphocytes that express TCR and some NKR. NKT cells recognize glycolipid antigens such as α -galactosylceramide (α GC) by an invariant TCR α chain composed of V α 14-J α 18 segments in mice and V α 24-J α 18 segments in humans, associated with TCR β chains using a restricted set of V β genes (1, 2). NKT cells rapidly secrete large amounts of cytokines including IL-4 and IFN- γ upon antigen stimulation and are effective regulators of T_H1/T_H2 balance *in vivo* (3–5). We have previously demonstrated that *in vivo* administration to mice of altered glycolipid ligand, OCH, ameliorates experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and type I diabetes by enhancing IL-4-dependent T_H2 responses without inducing IFN- γ production and pathogenic T_H1 responses (6–8).

Recently, we have clarified the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells (9). IFN- γ production by NKT cells was more susceptible

to the sphingosine length of glycolipid ligand than that of IL-4, and the length of sphingosine chain determined the half-life of NKT cell stimulation by CD1d-associated glycolipids. IFN- γ production by NKT cells required longer T cell stimulation than did IL-4 production and the transcription of the IFN- γ gene required *de novo* protein synthesis by activated NKT cells. The NF- κ B family member transcription factor c-Rel was preferentially transcribed in α GC-stimulated, but not in OCH-stimulated, NKT cells and was identified as essential for IFN- γ production by activated NKT cells. Therefore, the differential duration of NKT cell stimulation, due to the binding stability of individual glycolipid antigens to CD1d molecules, determines whether signaling leads to effective c-Rel transcription and IFN- γ production by activated NKT cells.

Upon stimulation by α GC *in vivo*, NKT cells rapidly affect the functions of neighboring cell populations such as T cells, NK cells, B cells and dendritic cells (DCs) in a direct or indirect manner (10–13). The serial production of IFN- γ by NKT cells

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and NK cells has been demonstrated, suggesting that activated NKT cells may influence further IFN- γ production by other cells including NK cells (3, 10). A C-glycoside analog of α GC has been shown to induce a superior T_H1 -type response than α GC does by inducing higher IFN- γ production by NK cells. IL-12 was indispensable for the T_H1 -skewing effect of the glycolipid, indicating the importance of IL-12 in enhanced IFN- γ production *in vivo* (14). Furthermore, α GC-stimulated NKT cells can act as an adjuvant *in vivo* by inducing the full maturation of DCs, as manifested by augmented co-stimulatory molecules and enhanced mixed leukocyte reactions (11). Accordingly, α GC-stimulated NKT cells were shown to express CD40 ligand (CD40L, CD154), which can engage CD40 on antigen-presenting cells and stimulate them to produce IL-12 (15, 16). Furthermore, IFN- γ production and T_H1 -type responses were impaired in CD40-deficient mice (5). A growing body of evidence suggests that both extrinsic and intrinsic factors compose an intricate network for controlling IFN- γ production and T_H1 polarization after intensive stimulation of NKT cells by superagonistic glycolipid such as α GC.

Although the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells has been elucidated, little is known about the effect of OCH on bystander cells and the extrinsic regulatory network for IFN- γ production and T_H1 polarization. Considering the lower IFN- γ production by OCH compared with extensive IFN- γ production by α GC *in vivo*, OCH may affect the functions of neighboring cell populations in a different manner from that of α GC. In the current study, we demonstrate that OCH induces less effective production of IFN- γ and IL-12 by bystander cells possibly due to lower expression of CD40L by NKT cells. Co-administration of stimulatory anti-CD40 mAb in combination with IFN- γ enhanced the production of IL-12 induced by OCH *in vivo*, and IL-12 modulated OCH-induced cytokine expression by augmenting IFN- γ . Consistent with these results, co-administration of CpG oligodeoxynucleotide (ODN) with OCH preferentially induced IFN- γ production possibly through augmented IL-12 production. Considering that NKT cell responses to CD1d-presented self-antigens are modified by IL-12 to induce massive IFN- γ production during the course of microbial infection (17), OCH, at least partly, mimics the physiological behavior of the putative self-antigen for NKT cells in the context of cytokine milieu *in vivo*.

Methods

Reagents and antibodies

Murine IL-12, IFN- γ and Flt3-ligand (Flt3L) were purchased from Peprotech EC (London, UK). Anti-CD40 mAb (HM40-3) was purchased from BD Biosciences PharMingen (San Diego, CA, USA). Mouse anti-IFN- γ (R4-6A2) was purified from ascites of hybridoma obtained from American Type Culture Collection. Glycolipids were solubilized in dimethyl sulfoxide ($100 \mu\text{g ml}^{-1}$) and stored at -20°C until use. The following CpG ODN was synthesized: CpG ODN, 5'-GCATGACGTT-GAGCT-3'.

Mice

C57BL/6 (B6) mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). MHC class II-deficient

I-A $^b\beta$ -/- mice with the B6 background were purchased from Taconic (Germantown, NY, USA). All animals were kept under specific pathogen-free conditions and used at 7–12 weeks of age. Animal care and use were in accordance with institutional guidelines.

Induction of bone marrow-derived DCs

Bone marrow cells were isolated by flushing femurs of B6 mice and re-suspended in culture medium supplemented with murine Flt3L (100 ng ml^{-1}) as described in (18). Cells were harvested from the culture after 10 days and subjected to co-culture experiment with NKT cells.

Flow cytometry and intracellular cytokine staining

Spleen cells or liver mononuclear cells harvested after stimulation with glycolipids *in vivo* were cultured in complete media containing GolgiStop (BD PharMingen, San Jose, CA, USA). Then cells were incubated with Fc block (anti-mouse Fc γ IIIR/IIIR mAb clone 2.4G2) and were stained with biotinylated anti-NK1.1 mAb (PK136), washed with PBS and then stained with peridinin chlorophyll protein/cyanine 5.5-anti-CD3 mAb and streptavidin-allophycocyanin (APC). Then cells were washed twice with PBS and fixed in BD Cytotfix/Cytoperm solution for 20 min at 4°C . After fixation, cells were washed with BD Perm/Wash solution and re-suspended in the same solution containing either PE-anti-IFN- γ mAb (XMG1.2) or PE-conjugated isotype control Ig for 30 min at 4°C . Then samples were washed and the stained cells were analyzed using a FACS Calibur instrument (Becton Dickinson) with CELLQuest software (Becton Dickinson). Identification of iNKT cells by Dimer XI Recombinant Soluble Dimeric Mouse CD1d (BD PharMingen) was performed as described previously (19). For analysis of CD40L expression, spleen cells harvested after stimulation with glycolipids *in vivo* for indicated periods of time were cultured in complete media containing biotinylated anti-CD40L mAb (MR1) for 2 h. Cells were harvested, washed with PBS and stained with FITC-anti-CD3 mAb, PE-anti-NK1.1 mAb and streptavidin-APC for 20 min. CD40L expression was analyzed in CD3/NK1.1 double-positive cell.

Microarray

Microarray analysis was performed as described previously (9). In brief, I-A $^b\beta$ -/- mice pre-treated with anti-asialo GM $_1$ antibody were injected with α GC or OCH ($100 \mu\text{g kg}^{-1}$). Total RNA was isolated from liver NKT cells (purified as CD3+ NK1.1+ cells) and applied to microarray by using U74Av2 arrays (GeneChip System, Affymetrix, Santa Clara, CA, USA). From data image files, gene transcript levels were determined using algorithms in the GeneChip Analysis Suit software (Affymetrix).

Quantitative reverse transcription-PCR

Quantitative reverse transcription-PCR was conducted using a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals) as described previously (9). Primers used for the analysis of gene expression are as follows; CD40L (F) CGAGTCAACGCCCATTCATC, (R) GTAATCAAA-CACTCCGCC.

ELISA

The level of cytokine production in cell culture supernatants or in serum was evaluated by standard sandwich ELISA, employing purified and biotinylated mAb sets (11B11/BVD6-24G2 for IL-4, R4-6A2/XMG1.2 for IFN- γ and 9A5/C17.8 for IL-12) and standards (OptEIA set, BD PharMingen) as described previously (9). After adding a substrate, the reaction was evaluated using a Microplate reader (BioRad).

Statistics

For statistic analysis, non-parametric Mann-Whitney test was used to calculate significance levels for all measurements. Values of $P < 0.05$ was considered statistically significant.

Results

OCH induces lower IFN- γ expression than α GC in both NKT cells and NK cells *in vivo*

Although NKT cells are a major source of IL-4 after glycolipid administration *in vivo*, activated NKT cells are shown to affect the functions of bystander cells such as T cells, NK cells, B cells and DCs in a direct or indirect manner, resulting in

possible secondary augmentation of IFN- γ production by these cells. To evaluate the contribution of NKT cells and other cells for IFN- γ production after glycolipid administration, we performed kinetic analysis of cytokine production by splenic NKT cells, NK cells, T cells and other cells after *in vivo* administration of glycolipids. IFN- γ production was detected both in NKT cells and NK cells (Fig. 1A), and neither CD3+ T cells nor CD3-NK1.1- cells showed significant IFN- γ production 2 or 6 h after glycolipid administration. α GC induced a larger population of IFN- γ -producing NKT cells than OCH did which is consistent with the previous report (9). The kinetic analysis revealed that IFN- γ production by NKT cells was dominant in earlier time points (2 h) after glycolipid administration and IFN- γ production by NK cells was comparable or even higher at later time points (6 h) (Fig. 1B), suggesting that IFN- γ production by NKT cells preceded IFN- γ production by NK cells as reported previously (3, 10). As CD3+NK1.1+ cells do not always represent CD1d-restricted iNKT cells, we compared IFN- γ production by CD1d-dimerX-positive T cells after treatment with α GC or OCH. Again, α GC induced a larger population of IFN- γ -producing iNKT cells than OCH did (Fig. 1C). Interestingly, α GC induced a much larger population of IFN- γ -producing NK cells than

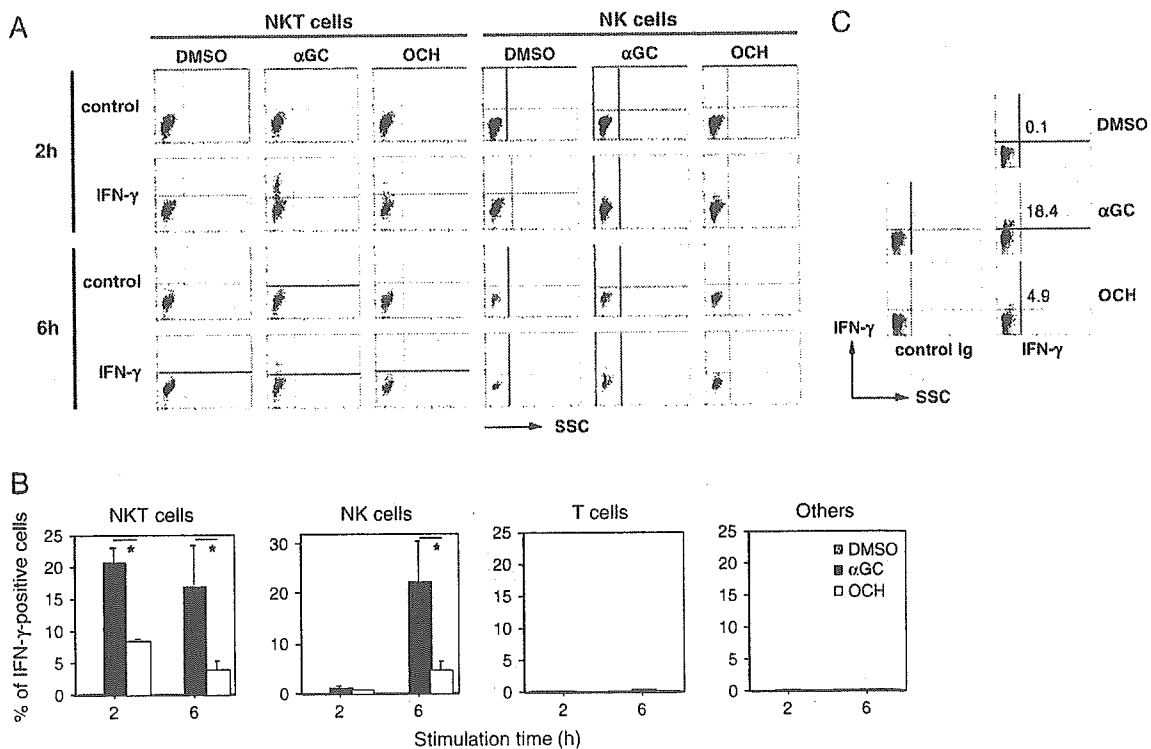


Fig. 1. Expression of IFN- γ by NKT cells and non-NKT cells after administration of glycolipid ligands. B6 mice were treated intra-peritoneally with 2 μ g per mouse of either α GC or OCH, and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular cytokine staining as described in Methods. (A) Data analyzed for CD3+NK1.1+ NKT cells, CD3-NK1.1+ NK cells, CD3+NK1.1-T cells and CD3-NK1.1- cells were shown for the presence of intracellular IFN- γ . Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). (B) Plotted values represent the percentage of IFN- γ -positive cells (mean \pm SD for triplicate samples) in the gated population after treatment with dimethyl sulfoxide (DMSO) (hatched bar), α GC (filled bar), or OCH (open bar). (C) Data analyzed for CD1d-DimerX-positive iNKT cells were shown for the presence of intracellular IFN- γ 2 h after glycolipid treatment. The experiments shown are representative of three independent experiments. * $P < 0.05$.

OCH, suggesting that OCH induces less IFN- γ production than α GC not only by direct effect on NKT cells but also by indirect effect on NK cells. To exclude the possibility of the contamination of activated non-CD1d-restricted T cells into NKT fractions or activated NKT cells into NK cells fraction due to the down-regulation of TCR, we conducted the following experiments. First, α GC-loaded DimerXI-stained cells were concentrated in the NK1.1+CD3+ population and <0.4% of cells were reactive to α GC-loaded DimerXI either in NK1.1+CD3- or NK1.1-CD3+ cell populations. Second, >95% of α GC-loaded DimerXI-reactive spleen cells were positive for both CD3 and NK1.1 after stimulation with glycolipids. Third, most of the intracellular IFN- γ -positive CD3- cells were DX5 positive 2 and 6 h after stimulation with glycolipids (data not shown). These results indicated that the contamination of IFN- γ -producing cells into the other fractions was minimum.

α GC-induced IFN- γ production by NK cells is partly dependent on IFN- γ produced by NKT cells

To determine the effect of IFN- γ on consequent IFN- γ production by NK cells, we treated mice with anti-IFN- γ mAb before administration of α GC, and then examined IFN- γ -producing cells using intracellular staining. As shown in Fig. 2, there was no significant difference in the frequency of IFN- γ -producing NKT cells after administration of α GC with or without anti-IFN- γ mAb. Meanwhile, co-administration of anti-IFN- γ mAb showed ~35% reduction in IFN- γ -producing NK cells after α GC treatment (Fig. 2, right panel). These results suggested that NKT cell-derived IFN- γ was involved in α GC-induced IFN- γ production by NK cells to some extent, but an IFN- γ -independent mechanism might be involved in indirect up-regulation of IFN- γ production by NK cells after α GC administration *in vivo*.

OCH administration does not induce effective IL-12 production

As DCs were demonstrated to be activated after *in vivo* administration of α GC (11, 20) to produce large amount of IL-12 (21) and IL-12 is one of the most potent inducers of IFN- γ (22), we performed kinetic cytokine analysis of serum levels of IL-12 (p70) together with IFN- γ and IL-4 after intraperitoneal injection of the glycolipids into B6 mice. As shown in Fig. 3, administration of α GC induced a rapid elevation of IL-12 and a delayed elevation of IFN- γ in B6 mice. In contrast, administration of OCH induced a rapid elevation of IL-4 comparable to that induced by α GC with significantly less amount of elevation of IFN- γ , resulted in T_H2 skewing as described previously. Although the level of IL-12 in serum was observed 6 h after α GC injection, OCH injection induced one-tenth amount of serum IL-12 level compared with α GC. In addition, freshly isolated liver NKT cells co-cultured with Flt3L-induced DCs produced significantly higher amount of IL-12 in the presence of α GC compared with OCH. Meanwhile, Flt3L-induced DCs loaded with either α GC or OCH exerted comparable amount of IL-4 production (Fig. 3B), demonstrating directly that DCs loaded with OCH produce less IL-12 upon co-culture with NKT cells than DCs loaded with α GC, and therefore suggest that the *in vivo* effects of OCH are not simply due to its preferential presentation by antigen-presenting cells that produce less IL-12. Taken together, these results indicated that OCH administration did not induce effective IL-12 production *in vivo*.

Lower expression of CD40L on OCH-stimulated NKT cells

Activated NKT cells stimulate DCs to produce IL-12 through the engagement of CD40 on DCs with CD40L inducibly expressed on NKT cells (15, 21). Furthermore, a C-glycoside analog of α GC induced a superior IFN- γ production by NK cells than α GC does in an IL-12-dependent manner (14),

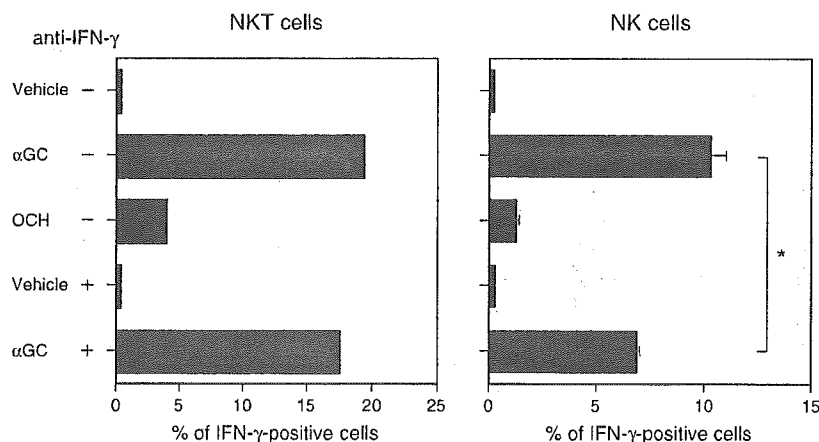


Fig. 2. α GC-induced IFN- γ production by NK cells is partly dependent on IFN- γ production by NKT cells. B6 mice were treated intra-peritoneally with 2 μ g per mouse of glycolipids with or without 500 μ g per mouse of anti-IFN- γ mAb. Four hours after treatment, spleen cells were harvested and subjected to intracellular cytokine staining. Plotted values represent the percentage of IFN- γ -positive cells (mean \pm SD for triplicate samples) in the gated population for CD3+NK1.1+ NKT cells (left) or CD3-NK1.1+ NK cells (right). Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). The experiments shown are representative of three independent experiments. * $P < 0.05$.

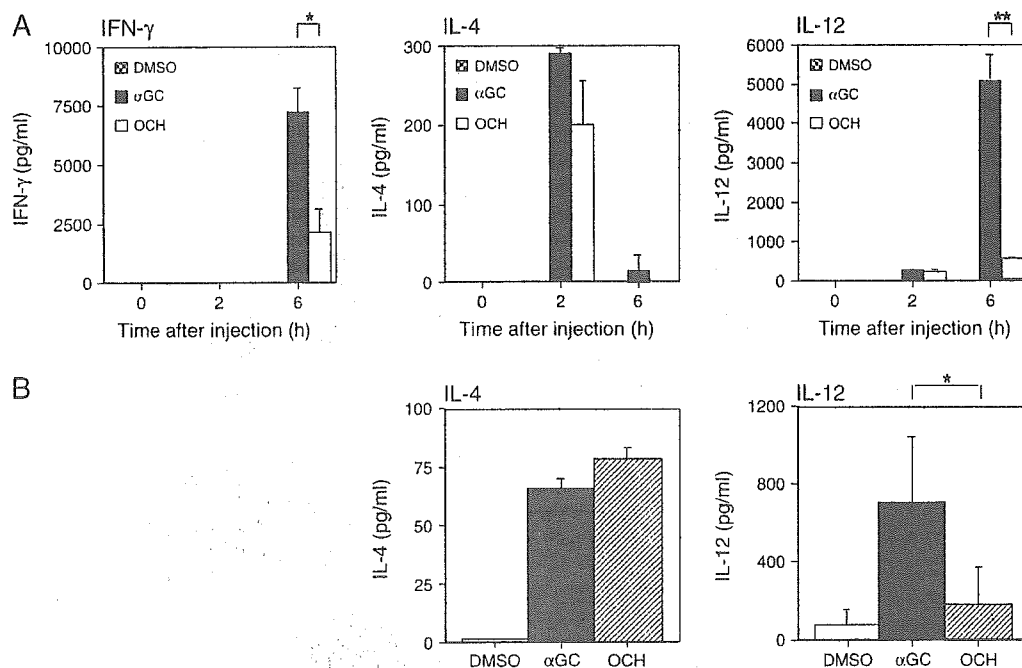


Fig. 3. OCH administration does not induce effective IL-12 production. (A) B6 mice were injected intra-peritoneally with vehicle alone, 2 µg per mouse of αGC or OCH and serum samples were collected at indicated times after injection. Serum levels of IFN-γ, IL-4 and IL-12 (mean ± SD) were determined by ELISA. This figure represents one of two experiments with similar results. * $P < 0.05$, ** $P < 0.01$. (B) Freshly isolated liver NKT cells were co-cultured with Flt3L-induced DCs in the presence of αGC or OCH for 72 h. Levels of IL-4 and IL-12 were determined by ELISA. Data are expressed as mean ± SD for triplicate wells and representative data of two similar experiments are shown. * $P < 0.05$.

which suggests that IFN-γ production by NK cells might be regulated by IL-12. To clarify the mechanisms of lack of IL-12 production upon stimulation with OCH, we compared the inducible expression of CD40L on NKT cells after *in vivo* administration of glycolipids. Microarray analysis revealed that CD40L transcripts were inducibly expressed in NKT cells 1.5 h after stimulation with αGC and disappeared 12 h after stimulation. In contrast, OCH treatment induced approximately one-third of CD40L transcription compared with the effect of αGC (Fig. 4A). Consistent with the data of microarray analysis, real-time PCR analysis confirmed the preferential up-regulation of CD40L transcript after αGC stimulation (Fig. 4B). To demonstrate the differential expression of CD40L between αGC-stimulated and OCH-stimulated NKT cells, surface expression of CD40L on NKT cells were compared by flow cytometry after *in vivo* treatment with the glycolipids. As shown in Fig. 4(C), αGC induced higher expression of CD40L than OCH did on the surface of NKT cells. If compared quantitatively by mean fluorescence intensity of CD40L-positive subsets after treatment with either glycolipid, OCH treatment induced less CD40L expression on NKT cells compared with the effect of αGC (Fig. 4C, right panel). These results indicated that CD40L expression on αGC-stimulated NKT cells was significantly higher than that on OCH-stimulated NKT cells.

Co-administration of IFN-γ and CD40 stimulation augments IL-12 production by OCH *in vivo*

Although the CD40 pathway plays an intrinsic role in physiological conditions in eliciting IL-12 production, effective

production of bioactive IL-12 by DCs requires another signal mediated by innate signals such as microbial stimuli (23) or by IFN-γ (24–26). Therefore, OCH-induced expression of CD40L and IFN-γ may not be effective to initiate IL-12 production from DCs *in vivo*. To test this hypothesis, we examined whether co-administration of stimulatory anti-CD40 mAb and/or IFN-γ confer OCH to induce higher IL-12 production. As shown in Fig. 5, administration of IFN-γ, stimulatory anti-CD40 mAb or combination of both reagents did not induce IL-12 expression *in vivo*. On the contrary, OCH-induced IL-12 production was partially augmented by co-administration of anti-CD40 mAb. Furthermore, concomitant administration of IFN-γ and stimulatory anti-CD40 mAb with OCH induced IL-12 production. These results suggest that the signals through CD40 and IFN-γ provided by OCH-stimulated NKT cells did not lead to efficient production of IL-12.

Co-administration of IL-12 augments IFN-γ production by OCH *in vivo*

A series of experiments so far indicated that OCH was less effective for induction of CD40L, IFN-γ and consequent IL-12 production than those induced by αGC. To examine directly the role of IL-12 production in less effective IFN-γ production by NKT cells and NK cells after OCH administration, we tested whether co-administration of IL-12 with OCH induces IFN-γ *in vitro* and *in vivo*. As shown in Fig. 6(A), IL-12 augmented IFN-γ production from spleen cells after *in vitro* treatment with

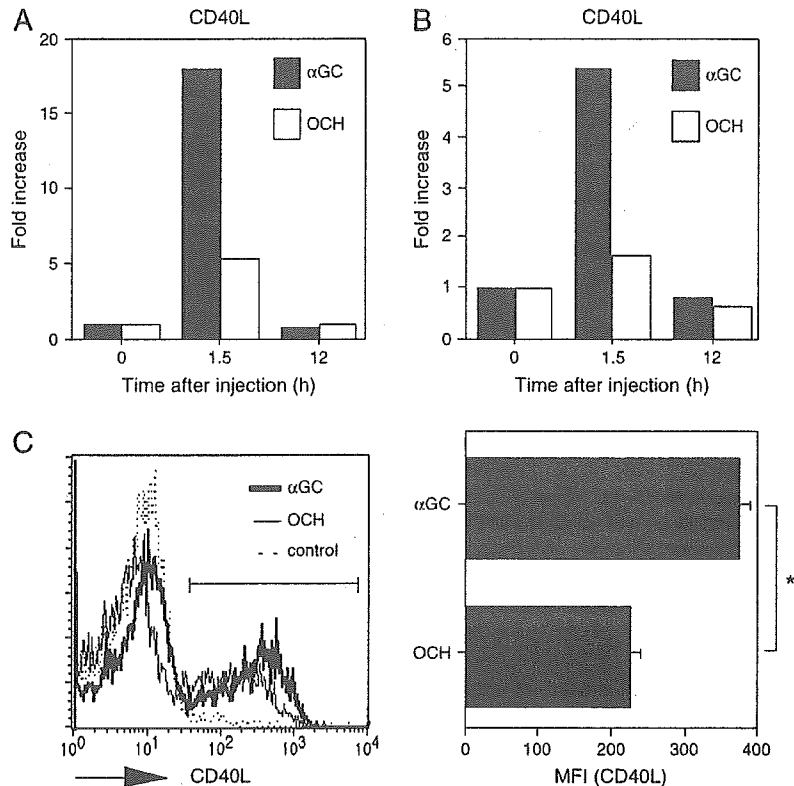


Fig. 4. Expression of CD40L on NKT cells stimulated with α GC or OCH. (A) Plotted values represent data of Affymetrix microarray analysis for indicated genes. The α GC- or OCH-stimulated liver NKT cells (purified as CD3+ NK1.1+ cells) as well as unstimulated NKT cells were analyzed at the indicated time points and the data represent to the relative values for glycolipid-treated samples when the value in NKT cells derived from untreated animals was defined as 1. (B) Real-time PCR analysis for the expression of CD40L mRNA. Data are presented as a fold induction of cytokine mRNAs after glycolipid treatment. The amount of mRNA in NKT cells derived from untreated animals was defined as 1. (C) Cell-surface expression of CD40L on α GC-stimulated (bold line) or OCH-stimulated (thin line) NKT cells. CD40L expression was analyzed in CD3/NK1.1 double-positive cell. Dotted line represents the histogram of control staining. B6 mice were injected intra-peritoneally with either α GC or OCH and liver mononuclear cells were isolated at the indicated time point. Cell-surface expression of CD40L was analyzed by flow cytometry (left) and plotted (right) as mean fluorescence intensity (MFI). Data are expressed as mean \pm SD for duplicate samples. This figure represents one of two experiments with similar results. * $P < 0.05$.

OCH in a dose-dependent manner. Higher doses of IL-12 induced IFN- γ production even without OCH and the effect of OCH is concealed in this condition. Interestingly, IL-12 treatment inhibits IL-4 production by OCH-stimulated spleen cells in a dose-dependent manner, suggesting the reciprocal regulation of cytokine production by IL-12. Next we examined the effect of co-administration of sub-optimal dose of IL-12 together with OCH. As shown in Fig. 6(B), co-administration of OCH and IL-12 induced significantly higher production of IFN- γ compared with either treatment alone, although sub-optimal dose of IL-12 alone failed to induce IFN- γ production. In contrast, co-administration of IL-12 did not enhance the IL-4 production 2 h after OCH administration *in vivo*. As both NKT cells and NK cells are important sources of IFN- γ after glycolipid stimulation, we evaluated the frequency of IFN- γ -producing NKT and NK cells after co-administration of OCH with IL-12. As shown in Fig. 6(C), IL-12 augmented the proportions of IFN- γ -producing cells in both cell populations, but not in conventional T cells, when co-administered with OCH. These results demonstrated that the properties of OCH

for less effective IFN- γ production by NKT cells and NK cells could be compensated by co-administration of IL-12.

Modification of cytokine profiles by pathogen-associated molecular patterns after OCH treatment *in vivo*

As sub-optimal dose of IL-12 was able to rescue defective IFN- γ production by administration of OCH alone, availability of IL-12 might be a crucial determinant for OCH-induced production of IFN- γ . As DCs and phagocytes produce IL-12 in response to pathogens during infection, pathogen-associated molecular patterns (PAMPs) are possible important determinants for cytokine profiles after OCH stimulation *in vivo*. We applied CpG ODN (27), which skews the host's immune milieu in favor of T_H1 responses by enhancing the production of pro-inflammatory cytokines including IL-12 (28), for analyzing cytokine profile of OCH. As shown in Fig. 7(A), CpG ODN alone induced no cytokine production within 6 h after injection. Concomitant injection of CpG ODN with OCH induced strong IFN- γ production (7.5-fold induction with 10 μ g per mouse of CpG ODN plus OCH and 14-fold induction with 100 μ g per

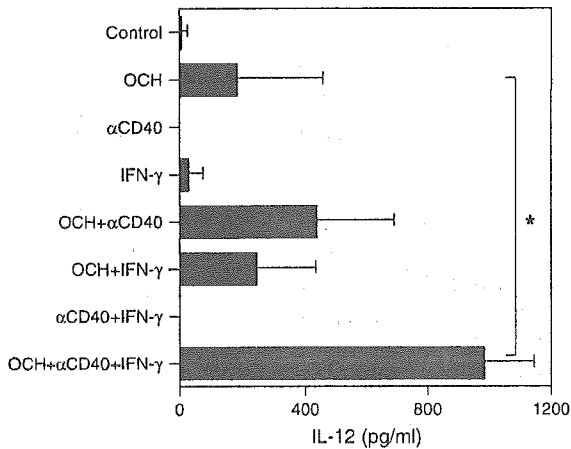


Fig. 5. Co-administration of IFN- γ and stimulatory anti-CD40 mAb augments IL-12 production after OCH administration *in vivo*. B6 mice were treated intra-peritoneally with 2 μ g per mouse of glycolipids in combination with murine IFN- γ (1 μ g per mouse) and/or stimulatory anti-CD40 mAb (100 μ g per mouse) and serum samples were collected 2 h after treatment. The level of IL-12 production was determined by ELISA. These data represent one of two experiments with similar results. * $P < 0.05$.

mouse of CpG ODN plus OCH) and induced moderate IL-4 production (2.6-fold induction with 10 μ g per mouse of CpG ODN plus OCH and 2.1-fold induction with 100 μ g per mouse of CpG ODN plus OCH). Accordingly, co-administration of OCH and 10 μ g per mouse of CpG ODN exhibited strong induction of IL-12 production (Fig. 7B, left panel), suggesting the synergic effect of OCH and CpG ODN for preferential up-regulation of IL-12. These results suggested that the PAMPs could be a considerable determinant for the cytokine profile following *in vivo* administration of OCH through regulating the availability of pre-inflammatory cytokines such as IL-12.

Discussion

In this study, we clarified the effect of OCH on bystander cell activation including the sequential IFN- γ production by NK cells and the functional conditioning of DCs. *In vivo* administration of OCH induced much lower IFN- γ production from both NKT and NK cells compared with that induced by α GC administration. NKT cell-derived IFN- γ was partially involved in inducing IFN- γ production by NK cells after α GC administration, implying that an IFN- γ -independent mechanism is also important for indirect up-regulation of IFN- γ production by NK cells after α GC administration *in vivo*. OCH administration induced lower CD40L expression by NKT cells compared with α GC administration, resulting in the lower production of IL-12 by DCs. Co-injection of stimulatory CD40 mAb and IFN- γ with OCH augmented the OCH-induced IL-12 production. Likewise, co-injection of IL-12 with OCH enhanced the production of IFN- γ by OCH administration alone. Furthermore, administration of OCH and CpG ODN into mice selectively induced IFN- γ production *in vivo*.

Consistent with the previous reports (9, 29), we here demonstrated that OCH administration induced less amount

of IFN- γ than that of α GC in iNKT cells. Supporting these observation is another report in which truncation of the phytosphingosine lipid chain of α GC increases the relative amounts of IL-4 release by human NKT cells (30).

The functional relevance between NKT cells and NK cells was demonstrated in which NK-sensitive tumor incidence was higher and the time of tumor development was earlier in NKT cell-deficient mice compared with B6 mice (31). Considering that NKT cell-deficient mice still possess NK cells (32), NKT cells might serve as a modulator of NK cell function in tumor immunity, though the molecular mechanisms of how NKT cells modulate NK cells has not been clarified yet. Recently, β -anomeric galactosylceramide has been reported to have a capacity to reduce numbers of NKT cells without inducing typical NK cell-mediated responses (29, 33). We demonstrated in this study that OCH-induced IFN- γ production by NK cells was lower compared with that induced by α GC. This is at least partly due to the lower induction of IFN- γ by OCH-stimulated NKT cells and the lower induction of IL-12 by DCs, leading to weak activation of NK cells. There is a report showing that OCH and α GC can induce comparable amount of IFN- γ by NK cells 8 and 24 h after stimulation (29), even though serum levels of IFN- γ induced by OCH treatment were significantly lower than that by α GC treatment 6 or 24 h after stimulation. Since the major producer of IFN- γ *in vivo* after treatment with glycolipids at the later time points were demonstrated to be NK cells (3, 10), it is not clear whether cells other than NKT cells or NK cells could be the IFN- γ producer after α GC stimulation in their experimental condition. Although the basis for the discrepancy is not clear, it may be related to the difference in the synthetic methods of those glycolipids. Nevertheless, we reproducibly confirmed the *in vivo* ameliorating effects of OCH in various autoimmune mouse models including EAE, CIA and inflammatory bowel disease (7, 8, 34) through the differential induction of various cytokines.

The CD40 pathway plays an intrinsic role in physiological conditions by eliciting IL-12 production by DCs (35, 36). However, cross-linking of CD40 alone has been shown to be incapable of inducing IL-12 production by DCs. Schulz *et al.* (23) has demonstrated that effective production of bioactive IL-12 by DCs through T cell activation should be initiated by innate signals such as microbial stimuli. Activated T cell-mediated IL-12 production by DCs through CD40 signaling requires another signal, for example, IFN- γ (24–26), which is also shown to be required for uncommitted immature DCs to develop the capacity to produce high levels of IL-12 upon subsequent contact with naive T cells (25). Consistent with the observation, IFN- γ enhances gene transcription encoding both the p40 and p35 components of IL-12, resulting in a particularly marked production of the heterodimeric IL-12 (37, 38). Intriguingly, α GC-induced expression of IL-12R on NKT cells requires the production of IFN- γ by NKT cells and the production of IL-12 by DCs (21). In addition, IL-12 itself has been shown to act directly on DCs to promote IL-12 production (39). α GC provides dual signals to DCs by up-regulating CD40L on NKT cells and by inducing IFN- γ production by NKT cells, resulting in a large amount of IL-12 production by DCs. Our reconstitution experiment clearly showed that signals through CD40 and IFN- γ provided by OCH lead to small

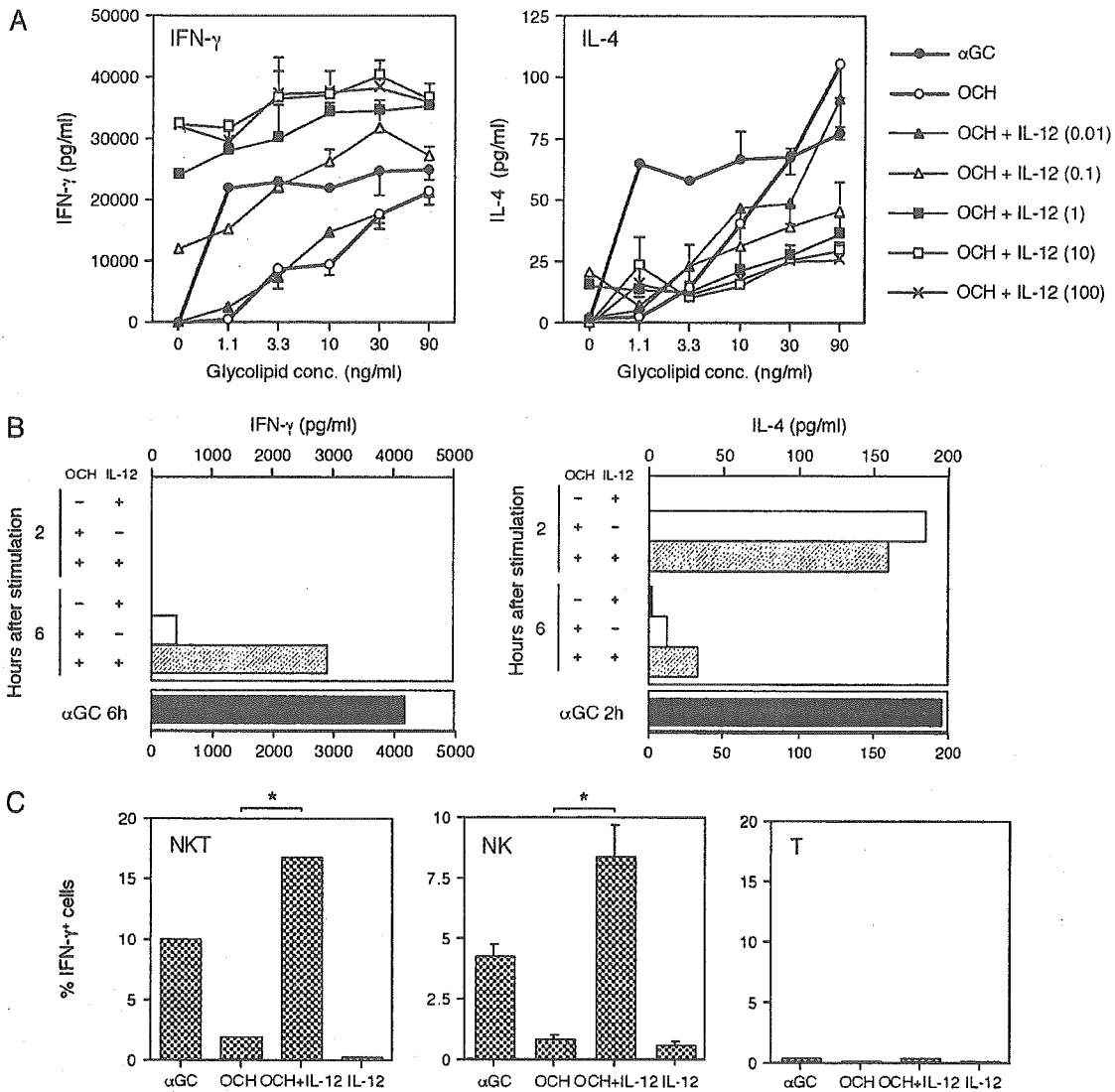


Fig. 6. Co-administration of IL-12 augments IFN- γ production by OCH. (A) Effects of IL-12 on cytokine production of splenocytes stimulated with glycolipids *in vitro*. Splenocytes were stimulated with various concentration of α GC or OCH in the presence or absence of IL-12 (with concentrations from 0.01 to 100 ng ml⁻¹) for 72 h and the levels of IFN- γ (left) or IL-4 (right) in the supernatants were measured by ELISA. Data are expressed as mean \pm SD for triplicate wells. This figure represents one of two experiments with similar results. (B) Effects of IL-12 on cytokine production after glycolipid administration *in vivo*. B6 mice were treated with 10 ng per mouse of IL-12, 2 μ g per mouse of OCH or OCH plus IL-12 and serum samples were collected at indicated times after injection. Serum levels of IFN- γ (left) and IL-4 (right) were determined by ELISA. This figure represents one of three experiments with similar results. (C) B6 mice were treated with 100 ng per mouse of IL-12 alone or in combination with 2 μ g per mouse of OCH and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular cytokine staining as described in Methods. NKT cells, NK cells and T cells were analyzed for the presence of intracellular IFN- γ as described in Fig. 1. Similar results were obtained by analyzing liver mononuclear cells after glycolipid administration (data not shown). Data are expressed as mean \pm SD for triplicate wells and represent one of two experiments with similar results. * $P < 0.05$.

amount of IL-12 production from DCs that is unable to trigger the IFN- γ burst by NKT cells and NK cells.

Treatment of mice with OCH together with sub-optimal doses of IL-12 resulted in significantly augmented IFN- γ production *in vivo*, indicating that the impaired IL-12 production by OCH is likely to be one of the major causes for less effective IFN- γ production *in vivo*. Similar observations were

reported previously, in which treatment of mice with sub-optimal doses of α GC together with sub-optimal doses of IL-12 resulted in strongly enhanced natural killing activity and IFN- γ production (21). These results indicate an important role for DC-derived IL-12 for glycolipid-induced activation of NKT cells and suggest that NKT cells may be able to condition DCs for subsequent immune responses. To further clarify the

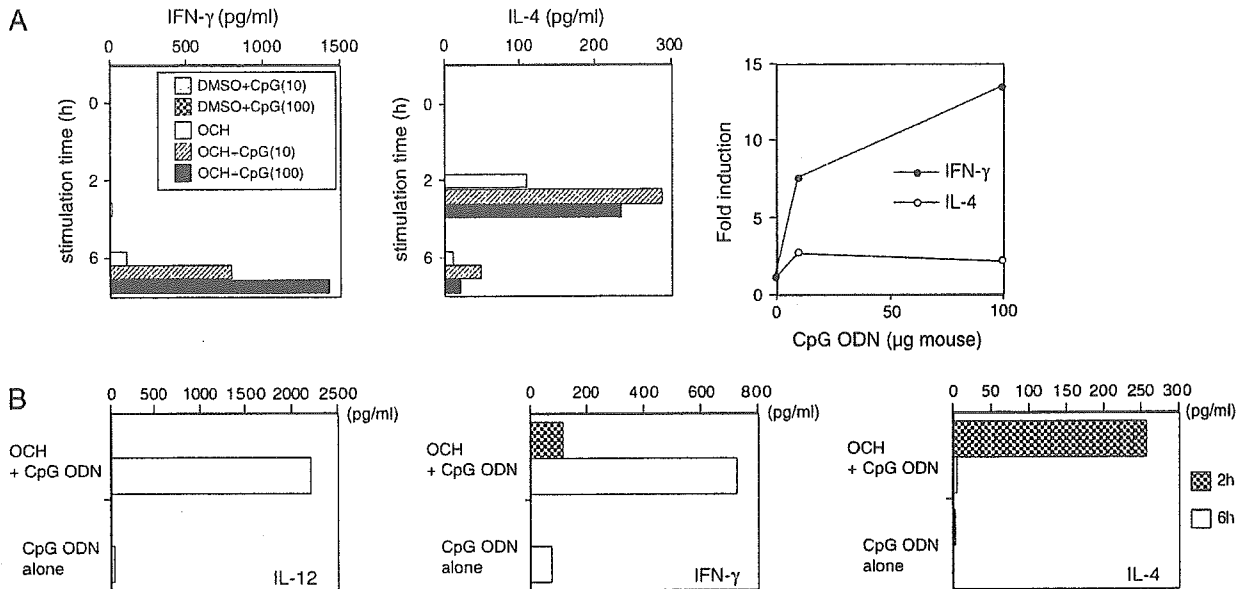


Fig. 7. Co-administration of CpG ODN augments IFN- γ production by OCH stimulation *in vivo*. (A) B6 mice were injected with 10 μ g per mouse or 100 μ g per mouse of CpG ODN alone or in combination with 2 μ g per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels of IFN- γ (left) or IL-4 (center) were determined by ELISA. The ratio of cytokine production was plotted in the right panel as fold induction for IFN- γ (at 6 h after injection) and IL-4 (at 2 h after injection). This figure represents one of two experiments with similar results. (B) B6 mice were injected with 10 μ g per mouse of CpG ODN alone or in combination with 2 μ g per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels of IL-12 (left), IFN- γ (center) or IL-4 (right) were determined by ELISA. This figure represents one of three experiments with similar results.

cooperative roles of IL-12 for effective IFN- γ production by glycolipid-stimulated NKT cells, CpG ODN (27) was co-administered with OCH, in which IFN- γ production was preferentially augmented in response to IL-12 expression. CpG ODN induces innate immune responses similar to bacterial DNA, and is one of the PAMPs expressed by a diverse group of microorganisms. Taken together, a variety of glycolipid antigens elicit differential effects, not only on NKT cells but also on bystander cells such as NK cells and DCs, which may modulate subsequent immune responses. Recently, Brigl *et al.* demonstrated that a bacterial infection can induce a predominantly T_H1 cytokine responses from self-antigen-primed NKT cells. In this instance, microbial products were recognized not by NKT cells directly, but by DCs, resulting in IL-12 secretion and subsequent potent IFN- γ production (17). Following the exposure of immune cells to exogenous antigens or infection, IL-12 is produced by DCs in response to CD40 signals or microbial products, and co-stimulates the responses of NKT cells to self-antigens, resulting in a significant augmentation of IFN- γ production but no detectable IL-4 production (40). It is noteworthy to point out that the behavior of OCH in response to IL-12 is analogous to that of the putative self-antigen for NKT cells (Fig. 6). Therefore, NKT cells also respond to OCH in a diverse manner according to the availability of IL-12, which can be induced by a wide variety of pathogens, and thus OCH may be a useful tool to evaluate the physiological responses of NKT cells to various innate immune conditions.

Regarding the predominant effect of OCH on T_H2 polarization by NKT cells, several molecules have been identified that positively regulate T_H2 polarization, such as thymus-specific lymphopoietin (TSLP), OX40 ligand (OX40L) or prostaglandin (PG) E_2 . In the microarray analysis of glycolipid-stimulated NKT cells and DCs, no inducible transcription of TSLP and OX40L in NKT cells was observed 1.5 or 12 h after OCH treatment. Furthermore, synthetic pathway for PGs seems quiescent because the expression of PG H synthetase (or cyclooxygenase 2), a key enzyme initiating PG synthesis, was not induced in either NKT cells or DCs after treatment with OCH. Considering that all of these molecules are regulated transcriptionally upon stimulation, the involvement of these molecules for OCH-mediated T_H2 polarization seems minimum. Taken together, the results demonstrated in this study suggest that OCH induces T_H2 predominance by a default pathway.

In summary, we have demonstrated here that OCH-mediated dominant T_H2 polarization is accomplished not only by the preferential IL-4 induction by NKT cells but also by the evasion of the secondary IFN- γ burst. This effect of OCH is due to the ineffective induction of IFN- γ and CD40L by NKT cells and the subsequent reduction of IL-12 secretion. These results demonstrate the cellular mechanisms involved in altered glycolipid ligand (OCH)-induced T_H2 polarization and immune regulation *in vivo*. Therefore, proper assessment of the effects of the innate immune system on the host's response should be taken into consideration when modulating NKT responses *in vivo* by glycolipids, such as OCH.

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Abbreviations

APC	allophycocerythrin
CD40L	CD40 ligand
CIA	collagen-induced arthritis
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
Flt3L	Flt3-ligand
α GC	α -Galactosylceramide
iNKT	invariant NKT
NF- κ B	nuclear factor- κ B
ODN	oligodeoxynucleotide
OX40L	OX40 ligand
PAMP	pathogen-associated molecular pattern
PG	prostaglandin
TSLP	thymus-specific lymphopoietin

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Synthetic α -Mannosyl Ceramide as a Potent Stimulant for an NKT Cell Repertoire Bearing the Invariant $V\alpha 19$ - $J\alpha 26$ TCR α Chain

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Summary

A NKT cell repertoire is characterized by the expression of the $V\alpha 19$ - $J\alpha 26$ invariant TCR α chain ($V\alpha 19$ NKT cell). This repertoire, as well as a well-established $V\alpha 14$ - $J\alpha 281$ invariant TCR α^+ NKT cell subset ($V\alpha 14$ NKT cell), has been suggested to have important roles in the regulation of the immune system and, thus, is a major therapeutic target. Here, we attempted to find specific antigens for $V\alpha 19$ NKT cells. $V\alpha 19$ as well as $V\alpha 14$ NKT cells exhibited reactivity to α -galactosyl ceramide (α -GalCer). Thus, a series of monoglycosyl ceramides with an axially oriented glycosidic linkage between the sugar and ceramide moiety were synthesized and their antigenicity to $V\alpha 19$ NKT cells was determined by measuring their immune responses in culture with glycolipids. Comprehensive examinations revealed substantial antigenic activity for $V\alpha 19$ NKT cells by α -mannosyl ceramide.

Introduction

Natural killer T (NKT) cells are defined as lymphocytes bearing both the common NK marker NK1.1, a member of the NKR-P1 gene family, and the TCR-CD3 complex [1, 2]. The major component of NKT cells bearing the invariant $V\alpha 14$ - $J\alpha 18$ TCR α chain ($V\alpha 14$ NKT cells) [3, 4] is positively selected by the nonpolymorphic MHC class-I-like CD1d molecule in association with $\beta 2$ -microglobulin ($\beta 2m$) [5–9]. $V\alpha 14$ NKT cells are shown to be reactive to certain glycosphingolipids (GSLs) such as α -galactosyl ceramide (α -GalCer) in the context of CD1d [10]. An important feature of NKT cells is their ability to secrete both proinflammatory (Th1) cytokines such as IFN- γ and antiinflammatory (Th2) cytokines such as IL-4 and IL-10 upon stimulation through the invariant TCR [11–14], suggesting a pivotal role in immunoregulatory functions including tumor immunity [15, 16].

Recently, we demonstrated the presence of a novel NKT cell repertoire (designated the $V\alpha 19$ NKT cell) that was characterized by the expression of the $V\alpha 19$ - $J\alpha 26$ ($V\alpha 19$ -AJ33) invariant TCR α chain [17]. This invariant TCR was previously found in the peripheral blood of human, bovine, and mouse deficient in transporter associated with antigen-processing (Tap)-1 by PCR-

based techniques [18]. Positive selection of the invariant $V\alpha 19$ - $J\alpha 26$ TCR-bearing cells by the nonclassical MHC class I molecule MR1 has also been shown [19]. Similar to $V\alpha 14$ NKT cells, the cells of this repertoire also produce large amounts of immunoregulatory cytokines in response to the engagement of the invariant TCR and are considered to have important roles in the regulation of the immune system (M. Shimamura et al., submitted). In addition, they are suggested to participate in the control of IgA production in intestine [19]. Thus, the search for specific antigens for $V\alpha 19$ NKT cells is of extreme importance in developing new therapies for immunoregulatory disorders utilizing the function of the repertoire.

GSLs are one of the principal components in mammalian cell membranes [20, 21]. Most glycosphingolipids share a common β -linked glucosyl or galactosyl ceramide as a skeletal structure. A discovery of α -GalCer from marine sponges [22], which is stimulatory to the mammalian immune system by specifically activating $V\alpha 14$ NKT cells, prompted us to investigate unnatural GSLs as agents for stimulating $V\alpha 19$ NKT cells.

In the current study, we focused on GSLs related to α -GalCer as potential stimulants for $V\alpha 19$ NKT cells because $V\alpha 19$ as well as $V\alpha 14$ NKT cells are suggested to recognize antigens presented by nonclassical MHC class I molecules, presumably MR1. Therefore, an efficient method of producing α -GalCer-related GSLs was addressed. We synthesized GSLs by coupling an activated monosaccharide with a sphingosine precursor whose amino functionality was masked with an azide group. The present method enabled us to efficiently obtain a series of naturally occurring and related GSLs. As a result of comprehensive examinations, immunological activities toward $V\alpha 19$ NKT cells were found in α -mannosyl ceramide (ManCer).

Results and Discussion

Synthesis of GLSs

Several approaches have been reported for the synthesis of azidosphingosine, which is particularly useful as a glycosylation acceptor [23–28]. However, these methods are relatively time consuming and technically difficult. Here, a means of producing azidosphingosine with fewer steps and easier operations was established (Figure 1). Herold reported an efficient method of synthesizing sphingosine [29] starting with Garner's aldehyde 1 [30] as a precursor. Addition of 1-pentadecynyllithium to 1 afforded amino alcohol 2 in 72% yield as a mixture of diastereomers. The ratio of diastereomers was determined by ¹H NMR spectroscopy (51% yield for the anti product). Although these isomers could be separated at this point by repeating the chromatographic purification, it was easier to separate them after protection of the hydroxyl group with benzyl ether in the case of large-scale preparation. After the 3-OH group had been protected by the benzylation, a desired *anti* isomer of 2 was exposed to HCl-MeOH to give 3.

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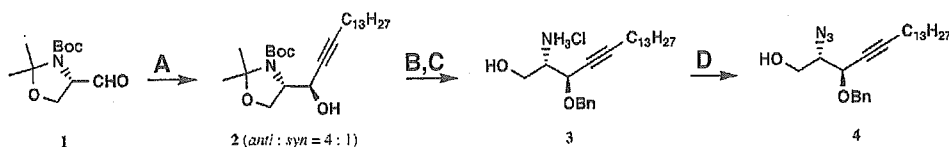


Figure 1. Synthesis of Azidosphingosine 4

Reagents and conditions. (A) 1-pentadecyne, *n*-butyllithium, hexamethylphosphoramide (HMPA), THF, -78°C , 72%. (B) NaH, BnBr, DMF, 0°C to rt. (C) 5% HCl/MeOH, 0°C to rt. (D) TfN_3 , NaOMe, DMAP, MeOH, room temperature, 81% yield from 2.

The amino alcohol 3 was then treated with TfN_3 (0.4 M in CH_2Cl_2) [31, 32], NaOMe, and DMAP in MeOH at room temperature to afford compound 4 in 81% yield from *anti*-2. As a result, the sphingosine building block 4 was prepared in 4 steps starting from Garner's aldehyde 1 in an overall yield of 40%.

Next, the appropriate glycosyl donors to be coupled with 4 were considered. We decided to use 2-*O*-benzylated glycosyl donors for coupling a monosaccharides with a 2-OH group and 2-azido-sugars for coupling glucosamine or galactosamine to preferentially obtain α -glycosides while taking into account the anomeric effect. To select these protective groups was advantageous to the simple deprotection of the final products. The thioether was chosen as a selective protecting group for the anomeric center.

The glycosylation of neutral sugars (glucose, galactose, mannose, xylose, fucose, talose, and altrose) with 4 was carried out by using DMTST (Dimethyl(methylthio) sulfonium trifluoromethane sulfonate) (Table 1). The α and β glycosides were separated by chromatography

with a silica-gel column eluted with *n*-hexane-ethyl acetate.

In the case of glucosyl and xylosyl donor, 6-*O*-acetyl-2, 3, 4-tri-*O*-benzyl thioglucoside 5 and 3-*O*-acetyl-2,4-*O*-dibenzyl thioxyloside 8 were selected because inseparable glycosylation products were obtained when a perbenzylated donor was used. The azide group in 12–18 was converted to a stearoyl amide group by hydrogenation with Lindlar's catalyst in the presence of stearic anhydride followed by hydrogenation with Perlmann's catalyst to afford the desired neutral-sugar-based GLSs (19–25) in good yield (Table 1). Although the altrosylceramide 25 obtained at this step was a mixture of anomers, the components were easily separable after peracetylation.

Glucuronic acid containing glycolipid was prepared from 12 α (Figure 2). After transformation of the azide in 12 α to a stearoyl amide group, Jones oxidation of the alcohol in 26 afforded carboxylic acid, which afforded 27 upon hydrogenation with Pearlman's catalyst in 69% yield from 12 α .

Table 1. Synthesis of Neutral Sugar-Based α -Glycosyl Ceramides

entry	substrate		products		isolated yield (%)		α -GLCs		yield (%)			
	R	R'	R	R'	α	β	R					
1	Glc	5	CH_2OAc	OBn	12 ^a	CH_2OH	OBn	77	19	19	CH_2OH	84
2	Gal	6	CH_2OBn	OBn	13	CH_2OBn	OBn	51	36	20	CH_2OH	84
3	Man	7	CH_2OBn	OBn	14	CH_2OBn	OBn	73	14	21	CH_2OH	62
4	Xyl	8	H	OAc	15 ^a	H	OH	35	36	22	H	55
5	L-Fuc	9	CH_3	OBn	16	CH_3	OBn	52	17	23	CH_3	99
6	Tal	10	CH_2OBn	OBn	17	CH_2OBn	OBn	68	11	24	CH_2OH	70
7	Alt	11	CH_2OBn	OBn	18	CH_2OBn	OBn	76 ($\alpha : \beta = 1 : 2$) ^{b, c}		25	CH_2OH	70 ^c

Reagents and conditions. (A) DMTST, MS 4 Å, CH_2Cl_2 , 0°C to room temperature. (B) H_2 , Pd-CaCO₃, stearic anhydride, AcOEt, room temperature. (C) H_2 , Pd(OH)₂, MeOH/AcOEt (2/1), room temperature.

^aAfter deacetylation with NaOMe/MeOH.

^bAt this step, α and β anomers could not be separated. These anomers were separated after peracetylation of altrosylceramide. The ratio of anomers was determined by ¹H NMR of peracetylated altrosylceramide.

^cYield for a mixture of anomers.

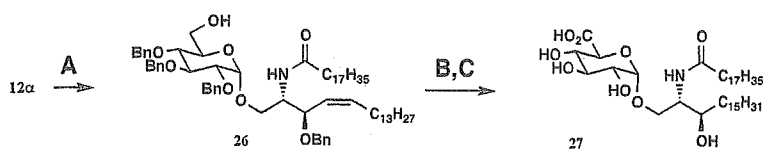


Figure 2. Synthesis of α -Glycosylceramide Containing Glucuronic Acid

Reagents and conditions. (A) H_2 , Pd-CaCO₃, stearic anhydride, AcOEt, rt. (B) Jones reagent, acetone, 60°C. (C) H_2 , Pd(OH)₂, MeOH:AcOEt=2:1, rt, 69% yield from 12 α .

For the sialylation of 4 (Figure 3), thiophenyl sialoside 28a showed no reactivity to 4 with DMTST or NIS-TfOH as a promoter probably because of the bulkiness of the anomeric position being a quarternally carbon center and the weak nucleophilicity of the thiophenyl group. Hence, a less bulky and more nucleophilic ethyl thiosialoside was selected as a donor. Using NIS-TfOH but not DMTST for coupling, this donor gave the sialoside 29b in a dramatically improved yield in a shorter reaction time. The glycosylation reaction was completed within 2 hr, judging from a TLC analysis (n-hexane-ethyl acetate), and the combined yield of α and β anomers was 84% ($\alpha/\beta = 2.3/1$). The desired β -sialoside 29b- β was converted to sialylceramide 32 in 3 steps with 82% yield (amide formation using hydrogenation with Lindlar's catalyst in the presence of stearic anhydride followed by hydrolysis of acetyl and methyl ester with lithium hydroxide and hydrogenation with Pearlman's catalyst).

Selective manipulation of 2-amino group in the sugar residue and the 2-amide group in the sphingosine moiety is required to synthesize glycosaminy and galactosaminy ceramide via the glycosylation of 4. Because of the difficulty in controlling the reactivity of these two functional groups, we chose ceramide 33 as a glycosyl acceptor in this particular case. Glycosyl imidates were chosen as more reactive glycosyl donors in order to compensate for the weak nucleophilicity of the acceptor. As a result (Figure 4), a glycoside 35 was obtained in 48% yield as a mixture of anomers upon the reaction of 33 and 34 with TESOTf as a promoter. Each isomer was separated by silica-gel column chromatography. The coupling of 36 and 33 afforded 37 in 29% yield with BF₃ · Et₂O as a promoter. In this case, α -anomer was formed as the sole product. 35 α and 37 were hydrogenated with Pearlman's catalyst in the presence of 0.1

N aqueous HCl to afford 38 and 39 in a quantitative yield, respectively.

Immunological Activities of the Synthesized GSLs

Alpha-GalCer, a specific activator for $V\alpha 14$ NKT cells, was tested for its potential to activate $V\alpha 19$ NKT cells in a continuous search for specific antigens for this novel NKT cell repertoire. Total MNCs were prepared from livers of invariant $V\alpha 19$ -J $\alpha 26$ transgenic (Tg)⁺ TCR $\alpha^{-/-}$ as well as C57BL/6 and $\beta 2m^{-/-}$ mice for the determination of the biological activity of GSLs. They were used as responders without further fractionation because (1) only $V\alpha 19$ Tg⁺ cells are generated as TCR⁺ cells in $V\alpha 19$ Tg⁺ TCR $\alpha^{-/-}$ mice, (2) the liver is the organ with the largest proportion of NKT cells among MNCs ($V\alpha 19$ Tg⁺ NK1.1⁺ cells are about 30%), and (3) liver MNCs include populations of dendritic cells, macrophages, and B cells, which have the potential to function as antigen-presenting cells (APCs). The liver MNCs were cultured in the presence of α -GalCer, and the immune responses were monitored by measuring cytokine secretion in the culture fluid and cell proliferation (Figure 5A). Similar to C57BL/6 cells, $V\alpha 19$ Tg⁺ cells seemed to have some responsiveness to α -GalCer. In contrast, both C57BL/6 and $V\alpha 19$ Tg⁺ cells showed no detectable responsiveness to β -GalCer. $V\alpha 19$ NKT cells are the sole component of the NKT cell population in $V\alpha 19$ Tg⁺ TCR $\alpha^{-/-}$ mice, whereas $V\alpha 14$ NKT cells are the major subset in C57BL/6 mice. Therefore, these findings strongly suggest that $V\alpha 19$ NKT cells as well as $V\alpha 14$ NKT cells [10] recognize α -glycosylated ceramides.

To test this hypothesis, we synthesized a series of α -glycosyl ceramides with a naturally occurring monosaccharide residue and examined their antigenic activity (Figure 5A). $V\alpha 19$ Tg⁺TCR $\alpha^{-/-}$ but not C57BL/6 cells

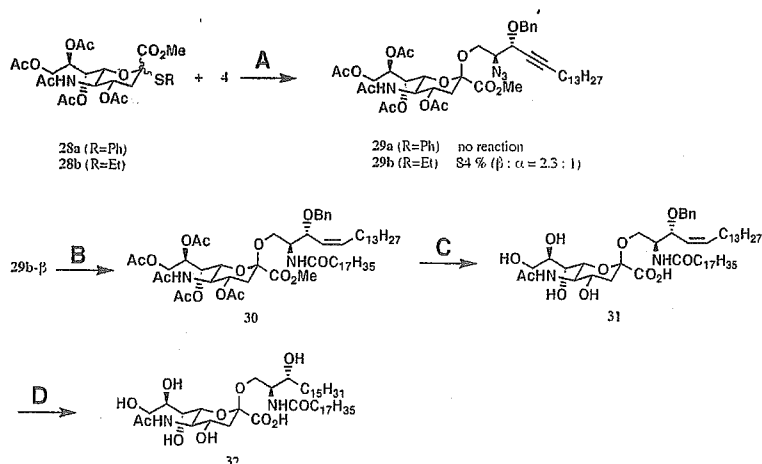


Figure 3. Synthesis of β -Sialyl Ceramide

Reagents and conditions. (A) NIS, TfOH, MS 4 Å, CH₂Cl₂:(CH₂Cl)₂=2:1, -40°C to -30°C. (B) H_2 , Pd-CaCO₃, stearic anhydride, AcOEt, rt. (C) LiOH, MeOH:H₂O=4:1, 0°C to rt. (D) H_2 , Pd(OH)₂, MeOH, rt, 82% yield from 29b- β .

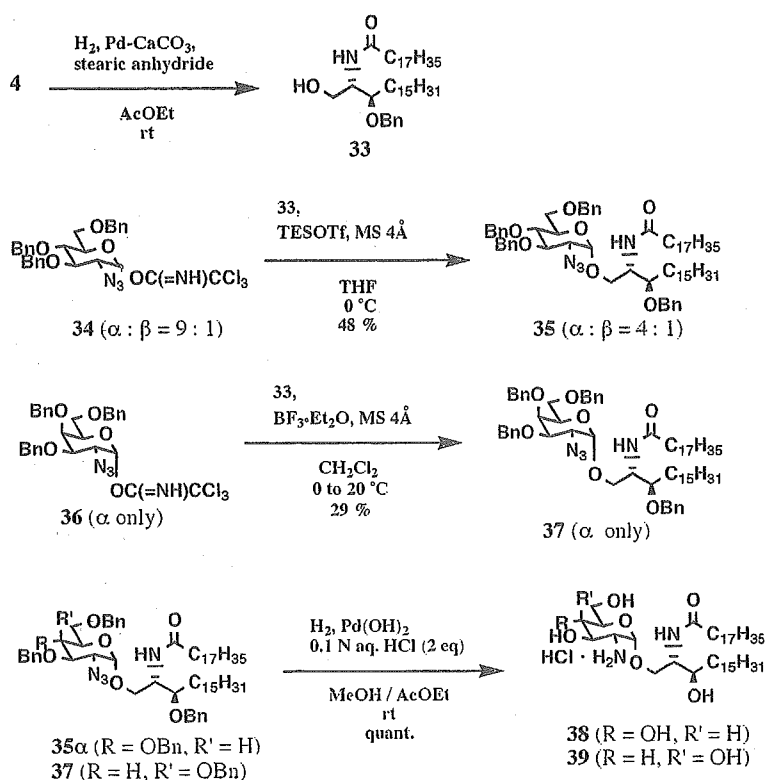


Figure 4. Synthesis of α -Glucosaminy and α -Galactosaminy Ceramides

For the synthesis of glucosaminy and galactosaminy ceramides, 2-azide-glycosyl donors (34, 36) were used. They were coupled with 3-O-benzyl ceramide. deprotection of the benzyl groups and conversion of the azide to the amino group in the adducts (35, 37) were performed in a successive procedure to afford the final products (38, 39).

were most efficiently induced to proliferate and produce IL-4 and IFN- γ by α -ManCer rather than α -GalCer. In contrast, C57BL/6 cells were responsive to α -GalCer and, to a certain extent, to α -glucosyl and glucuronyl ceramide (α -GlcCer and α -GlcUACer). Thus, these results suggest that V α 19 NKT cells recognize α -ManCer as a specific antigen in contrast to V α 14 NKT cells reactive to α -GalCer [10], α -GlcCer [10], and α -GlcUACer [33, 34]. Few NKT cells are generated in β 2m $^{-/-}$ mice because the expression of β 2m associated with non-classical MHC class I molecules is required for the positive selection of these NKT cell repertoires. Less reactivity by β 2m $^{-/-}$ cells to α -ManCer further supports that the immune responses to α -ManCer were conferred to V α 19 NKT cells in V α 19 Tg $^+$ TCR $\alpha^{-/-}$ mice.

The biological activity of α -ManCer toward V α 19 NKT cells is not apparently so significant compared with the activity of α -GalCer on V α 14 NKT cells. Thus, we performed a Student's t test to examine whether the immune responses of V α 19 Tg cells to α -ManCer were statistically significant. The results of the test indicated that the biological activity of α -ManCer to V α 19 Tg cells was more significant than the activity of controls (α -fucosyl ceramide [FucCer] or β -GalCer) with probability more than 96% (the α values were less than 0.04).

The immune responsiveness of V α 19 Tg cells to α -ManCer was dependent on the dose of the glycolipid. V α 19Tg cells but not C57BL/6 or β 2m $^{-/-}$ cells responded to α -ManCer in a dose-dependent manner (Figure 5B). The immune responses reached a maximum with the concentration of α -ManCer more than 2 μ g/ml, whereas α -FucCer did not induce any immune

responses of V α 19 Tg cells even with such concentration. These observations further support that α -ManCer specifically induces immune responses of V α 19 NKT cells.

MR1-dependent development of invariant V α 19 TCR-bearing cells has been reported [19]. This report well corresponds to the present result that immune responses of V α 19 Tg cells to α -ManCer are β 2m dependent. V α 19 Tg cells were stimulated with MR1 transfectants, and the immune responses were determined to examine whether the immune responsiveness of V α 19 NKT cells are MR1 dependent (Figure 6). V α 19 Tg cells but not C57BL/6 or β 2m $^{-/-}$ cells were activated during coculture with MR1 transfectants. This activation was enhanced when the MR1 transfectants were loaded with α -ManCer before coculture. Thus, it is strongly suggested that invariant V α 19 TCR $^+$ cells recognize and respond to α -ManCer presented by MR1.

We next focused on the antigenicity of α -glycosyl ceramides with an α -mannosyl-related sugar moiety. Assuming the importance of the 2-axial hydroxy group of the α -mannosyl residue, we tested for activity of α -altrosyl ceramide and α -talosyl ceramide (the 3-hydroxy group in α -altrose and 4-hydroxy group in α -talose are reversed from those in α -mannose). However, no significant immune responses of V α 19 NKT cells to these glycolipids were found (Figure 5A), suggesting a stringent recognition of the α -mannosyl residue by the invariant V α 19-J α 26 TCR. Because the natural occurrence of α -ManCer has not been reported yet, this glycolipid possibly mimics natural ligands for the NKT cells. We have recently found that 2,6- α -Man (α -Man)- and 6- α -Man- β -GlcNH $_2$ -phosphatidylinositol

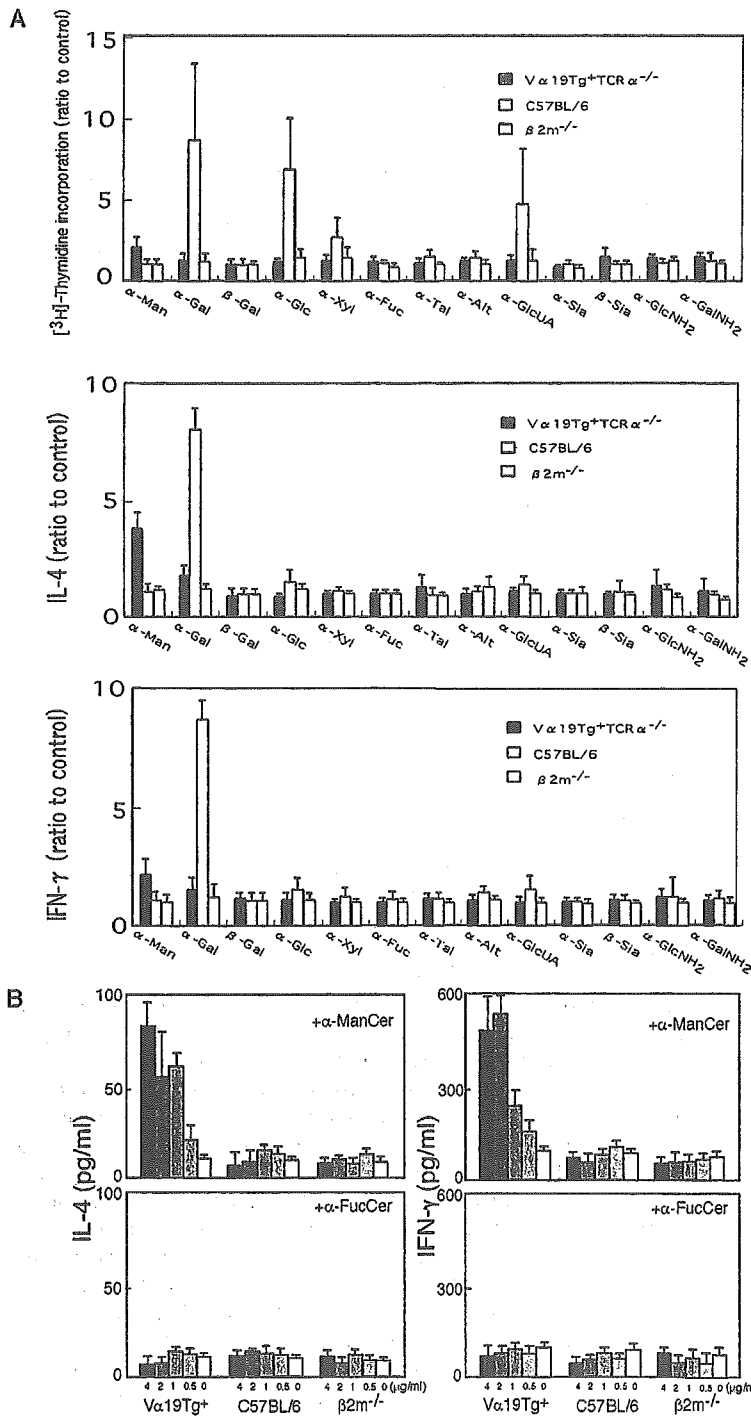


Figure 5. Activation of $V\alpha 19$ Tg⁺ Cells with Glycolipid Antigens in Culture

(A) Liver MNCs prepared from $V\alpha 19$ Tg⁺ TCR $\alpha^{-/-}$, C57BL/6, and $\beta 2m^{-/-}$ mice were cultured in the presence (1 μ g/ml) or absence of glycolipids. After 2 days, the immune responses were monitored by measuring cell proliferation (³H)-thymidine incorporation for 5 hr) and IL-4 and IFN- γ secretion in the culture supernatants. Results are shown as the fold increase relative to the control cultures with vehicle (1/200 v/v DMSO). The error bars indicate the standard deviations. Abbreviations: α -Gly, α -glycosyl ceramide; β -Gal, β -galactosyl ceramide; Sia, D-N-acetyl neuraminy; Tal, D-talosyl; and Alt, D-altrosyl.

(B) Dose-dependent activation of $V\alpha 19$ Tg⁺ cells with α -ManCer in culture. Liver MNCs prepared from $V\alpha 19$ Tg⁺ TCR $\alpha^{-/-}$, C57BL/6, and $\beta 2m^{-/-}$ mice were cultured in the presence of the indicated dose of glycolipids. After 2 days, the immune responses were monitored by measuring cytokine secretion into the culture supernatants. One of two independent experiments giving similar results is demonstrated.

have a similar degree of antigenicity to $V\alpha 19$ NKT cells as α -ManCer (M. Shimamura et al., submitted). These observations suggest the importance of the α -mannosyl residue in an appropriate location for the recognition by the invariant $V\alpha 19$ -J $\alpha 26$ TCR when the antigenic glycolipid is presented by MR1. It is possible that glycolipids with nonreducing end α -mannosyl residue(s) occur in mammalian cells that are more immunocompetent toward $V\alpha 19$ NKT cells than the glycolipids so far characterized. Another possibility is that modification of the

lipid structure in α -mannosylated glycolipids will alter the interaction with MR1 and improve the recognition of the α -mannose residue by the invariant TCR.

Significance

In this study, we accomplished the efficient chemical synthesis of a series of GSLs with azide sphingosine as a glycosyl acceptor. The methods we used will be applicable to the synthesis of related glycolipids.

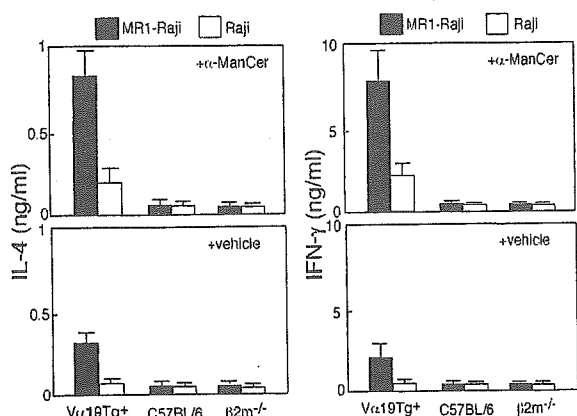


Figure 6. Stimulation of $V\alpha 19$ Tg⁺ Cells with α -ManCer in the Context of MR1

MR1-transfected or nontransfected Raji cells were incubated with α -ManCer (2 μ g/ml) or vehicle for 18 hr. They were irradiated (3,000 R), washed with medium, and then cultured with liver MNCs isolated from $V\alpha 19$ Tg⁺ TCR $\alpha^{-/-}$, C57BL/6, or $\beta 2m^{-/-}$ mice for 2 days. Cytokine production in the culture fluid was determined by ELISA. The error bars indicate the standard deviations. Experiments were repeated twice, and similar results were obtained.

The biological data on the GSLs demonstrate that α -ManCer is a potent stimulant for $V\alpha 19$ NKT cells. The present study serves as the second example of the recognition of GSLs in the context of nonpolymorphic MHC class-I-like molecules by invariant TCRs. Such an immune recognition system, namely the specific recognition of α -ManCer with MR1 or α -GalCer with CD1 by the invariant $V\alpha 19$ or $V\alpha 14$ TCR, may be of fundamental importance for the regulation of the immune system presumably by linking the natural and adoptive immune systems.

Experimental Procedures

Synthesis of GSLs

Outlines of the methods used to synthesize GSLs are shown in Table 1 and the Supplemental Data available with this article online. For data on the products and the procedures, see the Supplemental Data.

Mice

C57BL/6 mice were purchased from Sankyo Service (Tokyo). Recombination activating gene (Rag)-2-deficient mice and $\beta 2m$ -deficient mice were obtained from Jackson Laboratory (Bar Harbor, Maine). TCR $C\alpha$ -deficient mice with the C57BL/6 background [35] were provided by Drs. H. Ishikawa (Keio University) and M. Nanno (Yakult Co.). All experiments on mice were performed in accordance with the guidelines of Mitsubishi Kagaku Institute of Life Sciences.

Establishment of $V\alpha 19$ Transgenic Mice

A $V\alpha 19$ -J $\alpha 26$ (AV19-AJ33) transgene with the endogenous TCR α promoter and the enhancer was injected into TCR $C\alpha$ -deficient fertilized eggs, and transgenic (Tg) mouse lines were established (M. Shimamura et al., submitted).

Cell Preparations

Mononuclear cells (MNCs) were prepared from single-cell suspension of mouse organs by density gradient centrifugation with Lymphosepar II (IBL, Gunma, Japan; $d = 1.090$) for spleen and bone

marrow and Percoll (Pharmacia, Uppsala, Sweden) for liver-cell preparations as described previously [17].

MR1 Transfectants

Mouse MR1 A cDNA [36] was amplified from C57BL/6 spleen cells with the following PCR primers: 5'-MR1, 5'-ATGATGCTCCTGGT TACCTGG-3'; FLAG-3'-MR1, 5'-CTACTTGTATCGTCATCCTTGTA GTC(FLAG)-AGAGGGAGAGCTTCCCTCAT-3'.

MR1 transfectants were established as described elsewhere (M. Shimamura et al., submitted). Briefly, the cloned PCR product was recombined into a eukaryotic expression vector, (pCXN) [37] (provided by Dr. J. Miyazaki, Osaka University), and the expression vector was transfected into a human Burkitt's B lymphoma, Raji [38] (obtained from ATCC). The transfectants were selected in the culture medium containing G418 (1 mg/ml) for 1 month. The expression of FLAG (Asp-Tyr-Lys-Asp-Asp)-MR1 in the transfectants was analyzed by Western blot with anti-FLAG antibody and HRP-labeled anti-mouse immunoglobulin (Sigma, St. Lewis).

Stimulation of $V\alpha 19$ Tg⁺ Cells with MR1 Transfectants

MR1-transfectants or their parental cells (1×10^5 per well in DMEM, 10% FCS) were irradiated (3,000 R). They were incubated in the medium with glycolipids (2 μ g/ml) for 18 hr and washed twice with DMEM. These cells were cocultured with liver MNCs (1×10^6 per well) from $V\alpha 19$ Tg or non-Tg mice (2–4 months of age) in 200 μ l DMEM (10% FCS) for 2 days. Immune responses by the liver MNCs in the mixed lymphocyte reactions were monitored by measuring cytokines in the culture fluid.

Antigen Assay

Liver MNCs (10^6) from indicated mouse strains (2 to 4 months of age) were cultured in 200 μ l of DMEM (10% FCS) in the presence of glycolipids (1 μ g/ml). In some experiments, concentration of glycolipids in culture was serially altered. Concentration of cytokines in the supernatants was determined by ELISA after 2 days. Cell proliferation was assessed at day 2 of culture by measuring the incorporation of [³H]-thymidine (0.5 μ Ci/ml, Amersham, Buckinghamshire, United Kingdom) for 5 hr.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at <http://www.chembiol.com/cgi/content/full/12/6/677/DC1/>.

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