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 αGC has been shown to induce a superior T_h1-type response than αGC 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, αGC-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, αGC-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 αGC.

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 αGC *in vivo*, OCH may affect the functions of neighboring cell populations in a different manner from that of αGC. 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 μg ml^{-1}) and stored at −20°C until use. The following CpG ODN was synthesized: CpG ODN, 5′-GCATGACGTTGAGCT-3′.

Mice

C57BL/6 (B6) mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan). MHC class II-deficient I-A^bβ−/− 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 FcγIIIR/IIR 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–allophycoerythrin (APC). Then cells were washed twice with PBS and fixed in BD Cytofix/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^bβ−/− mice pre-treated with anti-asialo GM_1 antibody were injected with αGC or OCH (100 μg 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) GTAATTCAAACACTCCGCCC.

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 αGC 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. αGC 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 αGC or OCH. Again, αGC induced a larger population of IFN-γ-producing iNKT cells than OCH did (Fig. 1C). Interestingly, αGC induced a much larger population of IFN-γ-producing NK cells than

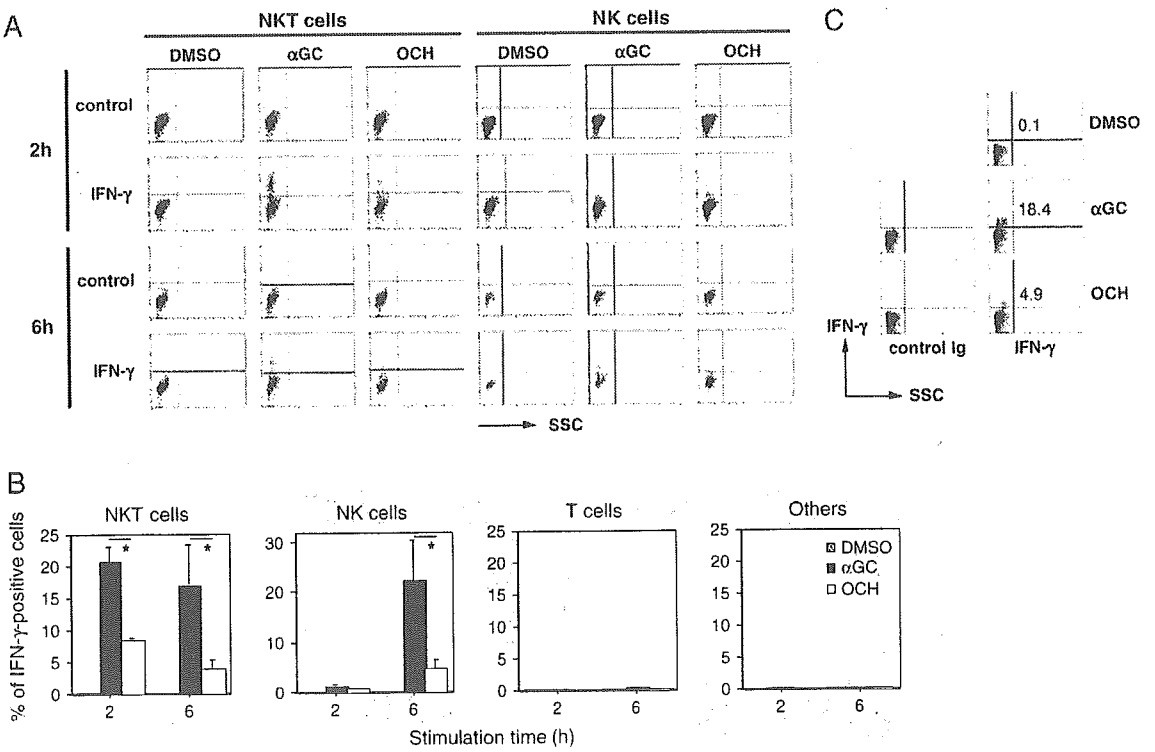


Fig. 1. Expression of IFN-γ by NKT cells and non-NKT cells after administration of glycolipid ligands. B6 mice were treated intra-peritoneally with 2 μg per mouse of either αGC or OCH, and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular 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 ± SD for triplicate samples) in the gated population after treatment with dimethyl sulfoxide (DMSO) (hatched bar), αGC (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 αGC 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, αGC-loaded DimerXI-stained cells were concentrated in the NK1.1+CD3+ population and <0.4% of cells were reactive to αGC-loaded DimerXI either in NK1.1+CD3− or NK1.1−CD3+ cell populations. Second, >95% of αGC-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.

αGC-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 αGC, 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 αGC 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 αGC treatment (Fig. 2, right panel). These results suggested that NKT cell-derived IFN-γ was involved in αGC-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 αGC administration *in vivo*.

OCH administration does not induce effective IL-12 production

As DCs were demonstrated to be activated after *in vivo* administration of αGC (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 αGC induced a rapid elevation of IL-4 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 αGC 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 αGC injection, OCH injection induced one-tenth amount of serum IL-12 level compared with αGC. In addition, freshly isolated liver NKT cells co-cultured with Flt3L-induced DCs produced significantly higher amount of IL-12 in the presence of αGC compared with OCH. Meanwhile, Flt3L-induced DCs loaded with either αGC 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 αGC, 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 αGC induced a superior IFN-γ production by NK cells than αGC does in an IL-12-dependent manner (14),

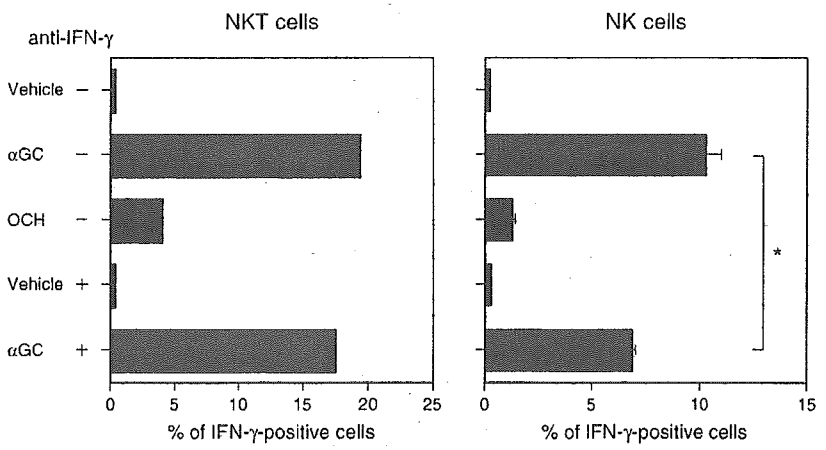


Fig. 2. αGC-induced IFN-γ production by NK cells is partly dependent on IFN-γ production by NKT cells. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids with or without 500 μg per mouse of anti-IFN-γ mAb. Four hours after treatment, spleen cells were harvested and subjected to intracellular cytokine staining. Plotted values represent the percentage of IFN-γ-positive cells (mean ± 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. *$P < 0.05$.

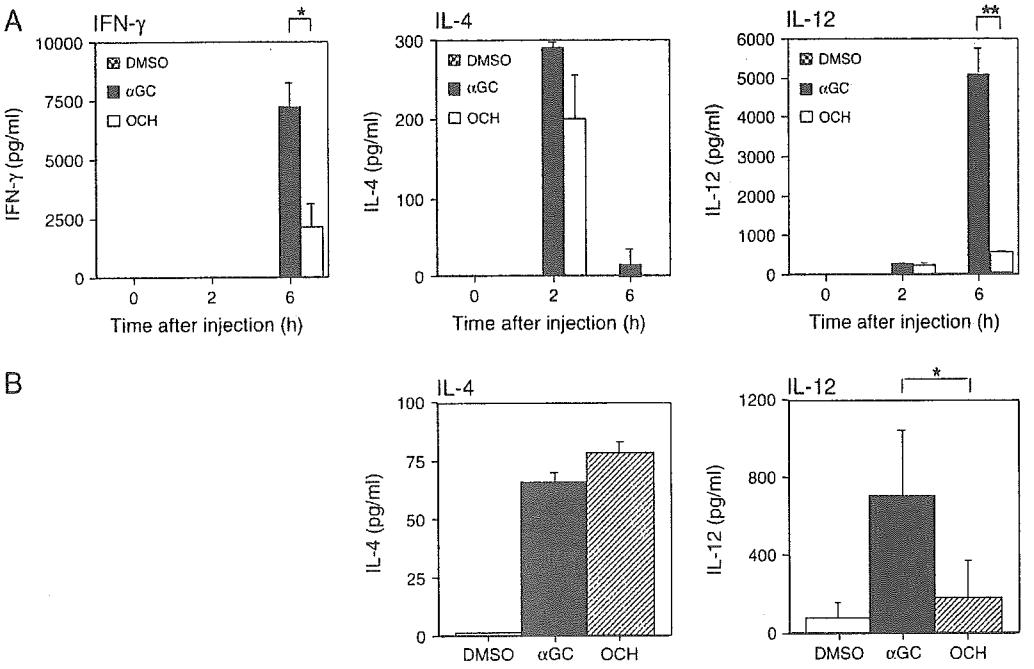


Fig. 3. OCH administration does not induce effective IL-12 production. (A) B6 mice were injected intra-peritoneally with vehicle alone, 2 μg per mouse of αGC or OCH and serum samples were collected at indicated times after injection. Serum levels of IFN-γ, IL-4 and IL-12 (mean ± SD) were determined by 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

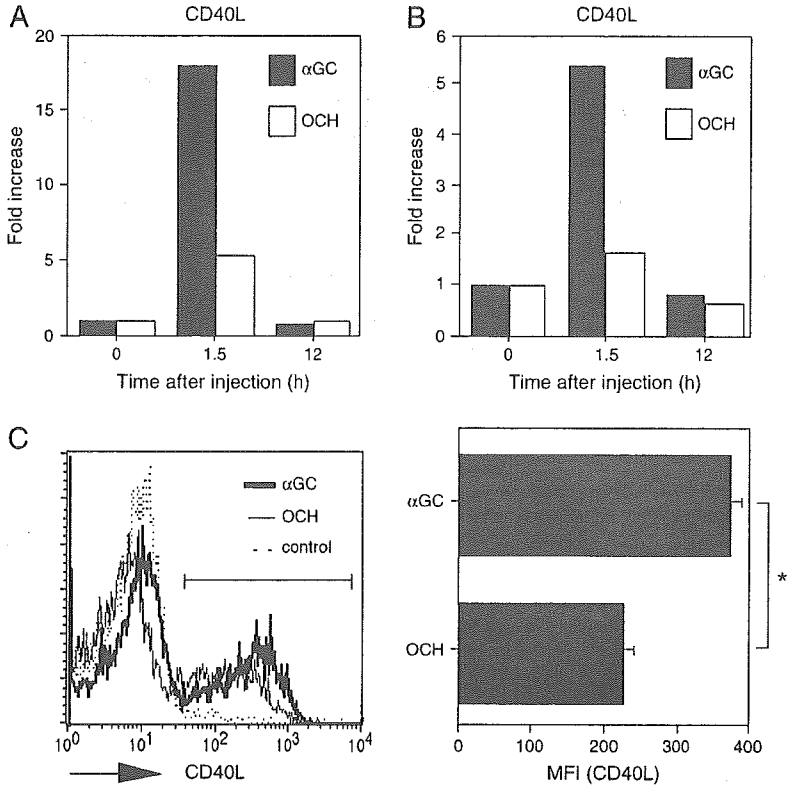


Fig. 4. Expression of CD40L on NKT cells stimulated with αGC or OCH. (A) Plotted values represent data of Affymetrix microarray analysis for indicated genes. The αGC- or OCH-stimulated liver NKT cells (purified as CD3+ NK1.1+ cells) as well as unstimulated NKT cells were analyzed at the indicated time 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 αGC-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 αGC 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 ± 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 μg per mouse of CpG ODN plus OCH and 14-fold induction with 100 μg per

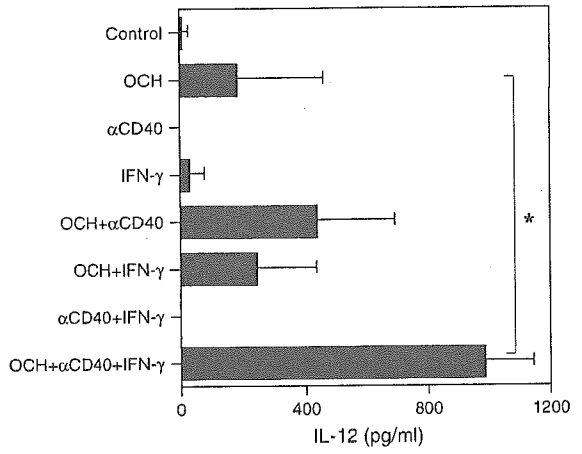


Fig. 5. Co-administration of IFN-γ and stimulatory anti-CD40 mAb augments IL-12 production after OCH administration *in vivo*. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids in combination with murine IFN-γ (1 μg per mouse) and/or stimulatory anti-CD40 mAb (100 μg per mouse) 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 μg per mouse of CpG ODN plus OCH and 2.1-fold induction with 100 μg per mouse of CpG ODN plus OCH). Accordingly, co-administration of OCH and 10 μg 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 pro-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 αGC administration. NKT cell-derived IFN-γ was partially involved in inducing IFN-γ production by NK cells after αGC administration, implying that an IFN-γ-independent mechanism is also important for indirect up-regulation of IFN-γ production by NK cells after αGC administration *in vivo*. OCH administration induced lower CD40L expression by NKT cells compared with αGC 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 αGC in iNKT cells. Supporting these observation is another report in which truncation of the phytosphingosine lipid chain of αGC 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 αGC. 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 αGC 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 αGC 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 αGC 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, αGC-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). αGC 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

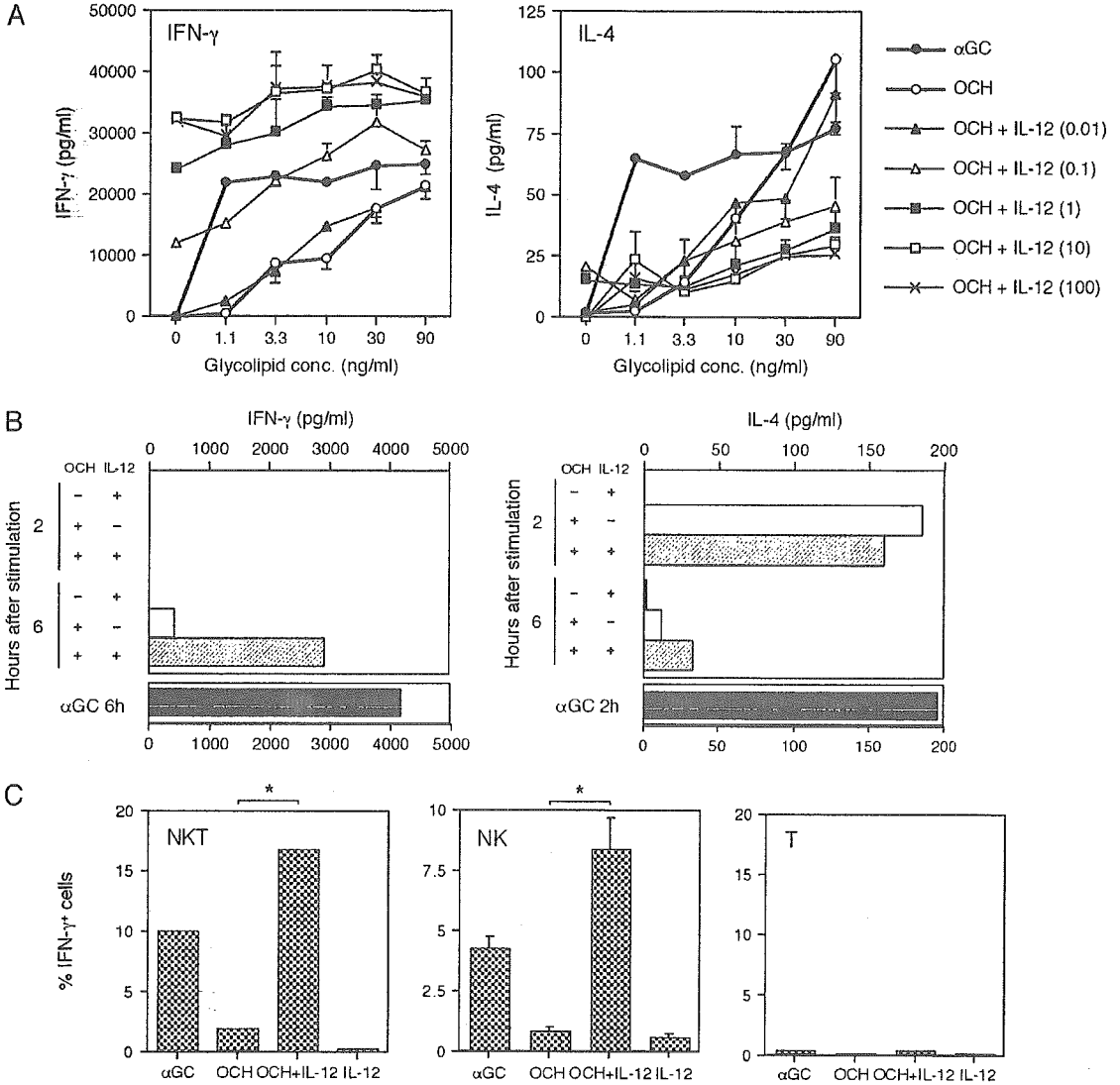


Fig. 6. Co-administration of IL-12 augments IFN-γ production by OCH. (A) Effects of IL-12 on cytokine production of splenocytes stimulated with glycolipids *in vitro*. Splenocytes were stimulated with various concentration of αGC or OCH in the presence or absence of IL-12 (with concentrations from 0.01 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 ± 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 μg 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 μg 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 ± 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 αGC 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

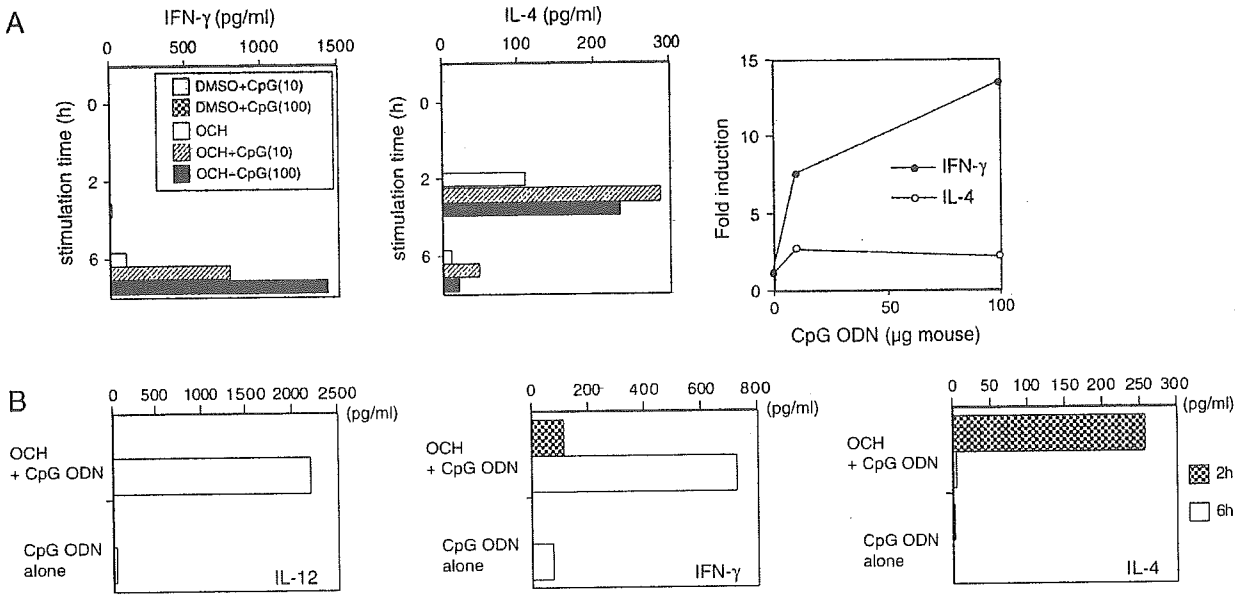


Fig. 7. Co-administration of CpG ODN augments IFN-γ production by OCH stimulation *in vivo*. (A) B6 mice were injected with 10 μg per mouse or 100 μg per mouse of CpG ODN alone or in combination with 2 μg per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels 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 μg per mouse of CpG ODN alone or in combination with 2 μg 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.

Acknowledgements

We thank Miho Mizuno for technical assistance and Yuki Kikai for cell sorting. We are grateful to John Ludovic Croxford for critical reading of the manuscript. This work was supported by the Pharmaceutical and Medical Devices Agency, Grant-in-Aid for Scientific Research (B) 14370169 from Japan Society for the Promotion of Science, Kato Memorial Bioscience Foundation and Uehara Memorial Foundation.

Abbreviations

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

References

1 Kronenberg, M. and Gapin, L. 2002. The unconventional lifestyle of NKT cells. *Nat. Rev. Immunol.* 2:557.
2 Taniguchi, M., Harada, M., Kojo, S., Nakayama, T. and Wakao, H. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 21:483.
3 Smyth, M. J., Crowe, N. Y., Pellicci, D. G. *et al.* 2002. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* 99:1259.
4 Singh, N., Hong, S., Scherer, D. C. *et al.* 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163:2373.
5 Hayakawa, Y., Takeda, K., Yagita, H., Van Kaer, L., Saiki, I. and Okumura, K. 2001. Differential regulation of Th1 and Th2 functions of NKT cells by CD28 and CD40 costimulatory pathways. *J. Immunol.* 166:6012.
6 Mizuno, M., Masumura, M., Tomi, C. *et al.* 2004. Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. *J. Autoimmun.* 23:293.
7 Chiba, A., Oki, S., Miyamoto, K., Hashimoto, H., Yamamura, T. and Miyake, S. 2004. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. *Arthritis Rheum.* 50:305.
8 Miyamoto, K., Miyake, S. and Yamamura, T. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 413:531.
9 Oki, S., Chiba, A., Yamamura, T. and Miyake, S. 2004. The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells. *J. Clin. Investig.* 113:1631.
10 Carnaud, C., Lee, D., Donnars, O. *et al.* 1999. Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163:4647.
11 Fujii, S. I., Shimizu, K., Smith, C., Bonifaz, L. and Steinman, R. M. 2003. Activation of natural killer T cells by {alpha}-galactosylceramide rapidly induces the full maturation of dendritic cells *in vivo* and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med.* 198:267.
12 Hermans, I. F., Silk, J. D., Gileadi, U. *et al.* 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen *in vivo* through direct interaction with dendritic cells. *J. Immunol.* 171:5140.
13 Kitamura, H., Ohta, A., Sekimoto, M. *et al.* 2000. Alpha-galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell Immunol.* 199:37.
14 Schmieg, J., Yang, G., Franck, R. W. and Tsuji, M. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-galactosylceramide. *J. Exp. Med.* 198:1631.
15 Vincent, M. S., Leslie, D. S., Gumperz, J. E., Xiong, X., Grant, E. P. and Brenner, M. B. 2002. CD1-dependent dendritic cell instruction. *Nat. Immunol.* 3:1163.
16 Tomura, M., Yu, W. G., Ahn, H. J. *et al.* 1999. A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J. Immunol.* 163:93.
17 Brigl, M., Bry, L., Kent, S. C., Gumperz, J. E. and Brenner, M. B. 2003. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat. Immunol.* 4:1230.
18 Gilliet, M., Boonstra, A., Paturel, C. *et al.* 2002. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 195:953.
19 Chiba, A., Kaieda, S., Oki, S., Yamamura, T. and Miyake, S. 2005. The involvement of V(alpha)14 natural killer T cells in the pathogenesis of arthritis in murine models. *Arthritis Rheum.* 52:1941.
20 Pal, E., Tabira, T., Kawano, T., Taniguchi, M., Miyake, S. and Yamamura, T. 2001. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells. *J. Immunol.* 166:662.
21 Kitamura, H., Iwakabe, K., Yahata, T. *et al.* 1999. The natural killer T (NKT) cell ligand alpha-galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J. Exp. Med.* 189:1121.
22 Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3:133.
23 Schulz, O., Edwards, A. D., Schito, M. *et al.* 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells *in vivo* requires a microbial priming signal. *Immunity* 13:453.
24 Hilkens, C. M., Kalinski, P., de Boer, M. and Kapsenberg, M. L. 1997. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* 90:1920.
25 Vieira, P. L., de Jong, E. C., Wierenga, E. A., Kapsenberg, M. L. and Kalinski, P. 2000. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J. Immunol.* 164:4507.
26 Snijders, A., Kalinski, P., Hilkens, C. M. and Kapsenberg, M. L. 1998. High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* 10:1593.
27 Klinman, D. M. 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat. Rev. Immunol.* 4:249.
28 Krug, A., Towarowski, A., Britsch, S. *et al.* 2001. Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. *Eur. J. Immunol.* 31:3026.
29 Parekh, V. V., Singh, A. K., Wilson, M. T. *et al.* 2004. Quantitative and qualitative differences in the *in vivo* response of NKT cells to distinct alpha- and beta-anomeric glycolipids. *J. Immunol.* 173:3693.
30 Goff, R. D., Gao, Y., Mattner, J. *et al.* 2004. Effects of lipid chain lengths in alpha-galactosylceramides on cytokine release by natural killer T cells. *J. Am. Chem. Soc.* 126:13602.
31 Smyth, M. J., Thia, K. Y., Street, S. E. *et al.* 2000. Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* 191:661.
32 Whiteside, T. L. and Herberman, R. B. 1995. The role of natural killer cells in immune surveillance of cancer. *Curr. Opin. Immunol.* 7:704.
33 Ortaldo, J. R., Young, H. A., Winkler-Pickett, R. T., Bere, E. W., Jr, Murphy, W. J. and Wiltrout, R. H. 2004. Dissociation of NKT stimulation, cytokine induction, and NK activation *in vivo* by the use of distinct TCR-binding ceramides. *J. Immunol.* 172:943.

34 Ueno, Y., Tanaka, S., Sumii, M. *et al.* 2005. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice. *Inflamm. Bowel Dis.* 11:35.
35 Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A. and Alber, G. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* 184:747.
36 Quezada, S. A., Jarvinen, L. Z., Lind, E. F. and Noelle, R. J. 2004. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu. Rev. Immunol.* 22:307.
37 Hayes, M. P., Murphy, F. J. and Burd, P. R. 1998. Interferon-gamma-dependent inducible expression of the human interleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from the CpG-rich promoter active in Epstein–Barr virus-transformed lymphoblastoid cells. *Blood* 91:4645.
38 Ma, X., Chow, J. M., Gri, G. *et al.* 1996. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J. Exp. Med.* 183:147.
39 Grohmann, U., Belladonna, M. L., Bianchi, R. *et al.* 1998. IL-12 acts directly on DC to promote nuclear localization of NF-kappaB and primes DC for IL-12 production. *Immunity* 9:315.
40 Gumperz, J. E. 2004. CD1d-restricted NKT cells and myeloid IL-12 production: an immunological crossroads leading to promotion or suppression of effective anti-tumor immune responses? *J. Leukoc. Biol.* 76:307.

Chemistry & Biology, Vol. 12, 677–683, June, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.04.014

Synthetic α-Mannosyl Ceramide as a Potent Stimulant for an NKT Cell Repertoire Bearing the Invariant Vα19-Jα26 TCR α Chain

Naoki Okamoto,[1] Osamu Kanie,[2] Yi-Ying Huang,[1] Rei Fujii,[1] Hiroko Watanabe,[1] and Michio Shimamura[1,*]
[1]Developmental Immunology Unit
[2]Glycotechnology Unit
Mitsubishi Kagaku Institute of Life Sciences
Tokyo 194-8511
Japan

Summary

A NKT cell repertoire is characterized by the expression of the Vα19-Jα26 invariant TCR α chain (Vα19 NKT cell). This repertoire, as well as a well-established Vα14-Jα281 invariant TCR α⁺ NKT cell subset (Vα14 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 Vα19 NKT cells. Vα19 as well as Vα14 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 Vα19 NKT cells was determined by measuring their immune responses in culture with glycolipids. Comprehensive examinations revealed substantial antigenic activity for Vα19 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 Vα14-Jα18 TCR α chain (Vα14 NKT cells) [3, 4] is positively selected by the nonpolymorphic MHC class-I-like CD1d molecule in association with β2-microglobulin (β2m) [5–9]. Vα14 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 Vα19 NKT cell) that was characterized by the expression of the Vα19-Jα26 (AV19-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 Vα19-Jα26 TCR-bearing cells by the nonclassical MHC class I molecule MR1 has also been shown [19]. Similar to Vα14 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 Vα19NKT 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 stimulus to the mammalian immune system by specifically activating Vα14 NKT cells, prompted us to investigate unnatural GSLs as agents for stimulating Vα19 NKT cells.

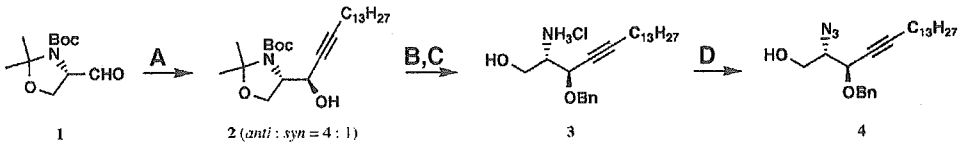
In the current study, we focused on GSLs related to α-GalCer as potential stimulants for Vα19 NKT cells because Vα19 as well as Vα14 NKT cells are suggested to recognize antigens presented by nonclassical MHC class I molecules, presumably MR1. Therefore, an efficient method of producing α-GalCer-related 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 Vα19 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 precurser. 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 ^{1}H 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.

*Correspondence: michio@libra.ls.m-kagaku.co.jp



1 2 (*anti* : *syn* = 4 : 1) 3 4

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%.

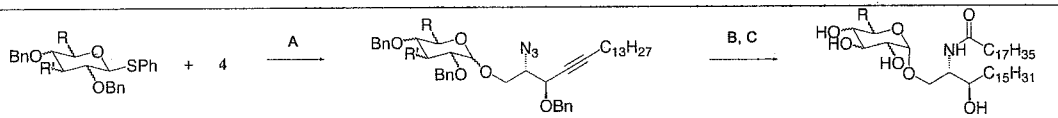
Next, the appropriate glycosyl donors to be coupled with **4** were considered. We decided to use 2-*O*-benzylated glycosyl donors for coupling a monosaccharides with a 2-OH group and 2-azido-sugars for coupling glucosamine or galactosamine to preferentially obtain α-glycosides while taking into account 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 (α : β = 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-$CaCO_3$, stearic anhydride, AcOEt, room temperature. (C) H_2, $Pd(OH)_2$, MeOH/AcOEt (2/1), room temperature.

[a]After deacetylation with NaOMe/MeOH.

[b]At 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.

[c]Yield for a mixture of anomers.

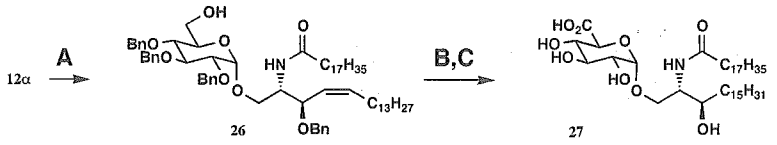


Figure 2. Synthesis of α-Glycosylceramide Containing Glucuronic Acid

Reagents and conditions. (A) H_2, Pd-$CaCO_3$, stearic anhydride, AcOEt, rt. (B) Jones reagent, acetone, 60°C. (C) H_2, $Pd(OH)_2$, MeOH:AcOEt=2:1, rt, 69% yield from 12α.

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% (α/β = 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 glycosamynyl and galactosaminyl 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 $BF_3 \cdot Et_2O$ as a promoter. In this case, α-anomer was formed as the sole product. **35α** 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 Vα14 NKT cells, was tested for its potential to activate Vα19 NKT cells in a continuous search for specific antigens for this novel NKT cell repertoire. Total MNCs were prepared from livers of invariant Vα19-Jα26 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 Vα19 Tg^+ cells are generated as TCR^+ cells in Vα19 Tg^+ TCR $\alpha^{-/-}$ mice, (2) the liver is the organ with the largest proportion of NKT cells among MNCs (Vα19 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, Vα19 Tg^+ cells seemed to have some responsiveness to α-GalCer. In contrast, both C57BL/6 and Vα19 Tg^+ cells showed no detectable responsiveness to β-GalCer. Vα19 NKT cells are the sole component of the NKT cell population in Vα19 Tg^+ $TCR\alpha^{-/-}$ mice, whereas Vα14 NKT cells are the major subset in C57BL/6 mice. Therefore, these findings strongly suggest that Vα19 NKT cells as well as Vα14 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). Vα19 $Tg^+TCR\alpha^{-/-}$ but not C57BL/6 cells

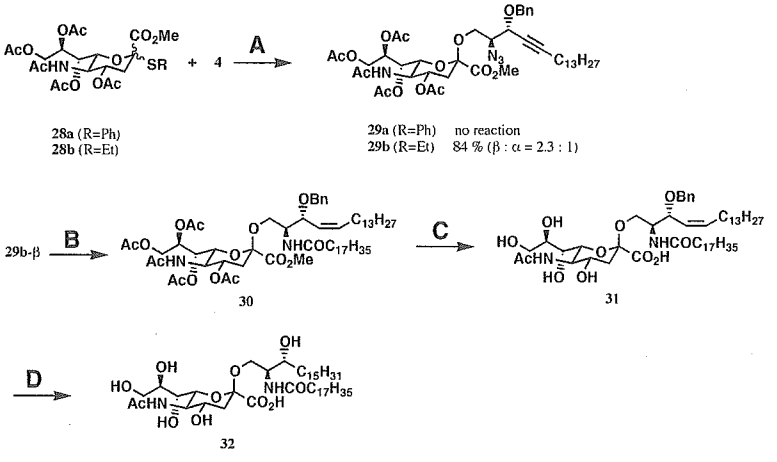


Figure 3. Synthesis of β-Sialyl Ceramide

Reagents and conditions. (A) NIS, TfOH, MS 4 Å, CH_2Cl_2:$(CH_2Cl)_2$=2:1, −40°C to −30°C. (B) H_2, Pd-$CaCO_3$, stearic, anhydride, AcOEt, rt. (C) LiOH, MeOH:H_2O=4:1, 0°C to rt. (D) H_2, $Pd(OH)_2$, MeOH, rt, 82% yield from **29b-β**.

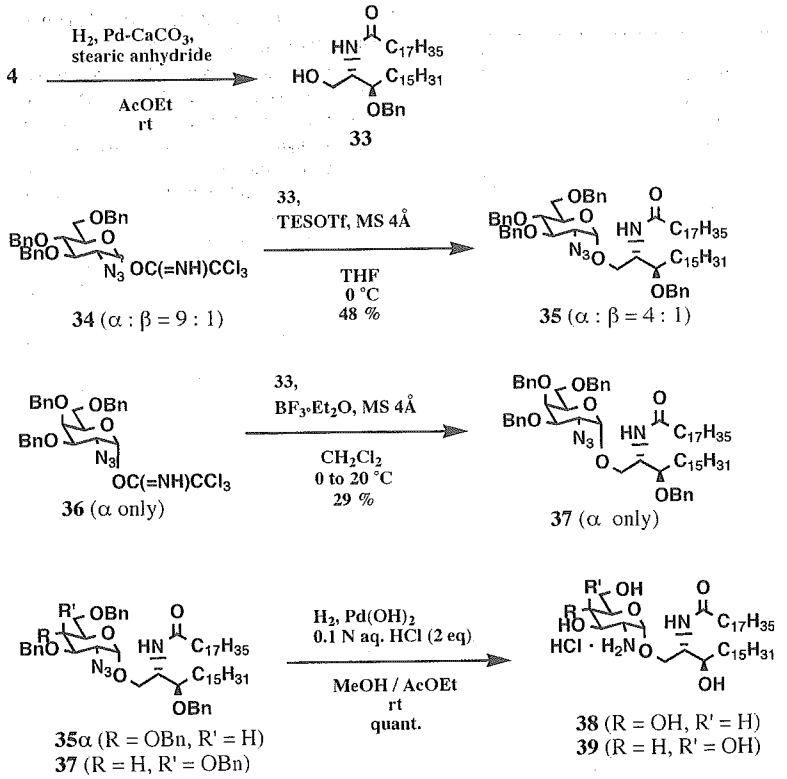


Figure 4. Synthesis of α-Glucosaminyl and α-Galactosaminyl Ceramides

For the synthesis of glucosaminyl and galactosaminyl 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 Vα19 NKT cells recognize α-ManCer as a specific antigen in contrast to Vα14 NKT cells reactive to α-GalCer [10], α-GlcCer [10], and α-GlcUACer [33, 34]. Few NKT cells are generated in $\beta2m^{-/-}$ mice because the expression of β2m associated with nonclassical MHC class I molecules is required for the positive selection of these NKT cell repertoires. Less reactivity by $\beta2m^{-/-}$ cells to α-ManCer further supports that the immune responses to α-ManCer were conferred to Vα19 NKT cells in Vα19 Tg^{+} $TCR\alpha^{-/-}$ mice.

The biological activity of α-ManCer toward Vα19 NKT cells is not apparently so significant compared with the activity of α-GalCer on Vα14 NKT cells. Thus, we performed a Student's t test to examine whether the immune responses of Vα19 Tg cells to α-ManCer were statistically significant. The results of the test indicated that the biological activity of α-ManCer to Vα19 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 Vα19 Tg cells to α-ManCer was dependent on the dose of the glycolipid. Vα19Tg cells but not C57BL/6 or $\beta2m^{-/-}$ 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 μg/ml, whereas α-FucCer did not induce any immune responses of Vα19 Tg cells even with such concentration. These observations further support that α-ManCer specifically induces immune responses of Vα19 NKT cells.

MR1-dependent development of invariant Vα19 TCR-bearing cells has been reported [19]. This report well corresponds to the present result that immune responses of Vα19 Tg cells to α-ManCer are β2m dependent. Vα19 Tg cells were stimulated with MR1 transfectants, and the immune responses were determined to examine whether the immune responsiveness of Vα19 NKT cells are MR1 dependent (Figure 6). Vα19 Tg cells but not C57BL/6 or $\beta2m^{-/-}$ 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 Vα19 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 Vα19 NKT cells to these glycolipids were found (Figure 5A), suggesting a stringent recognition of the α-mannosyl residue by the invariant Vα19-Jα26 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

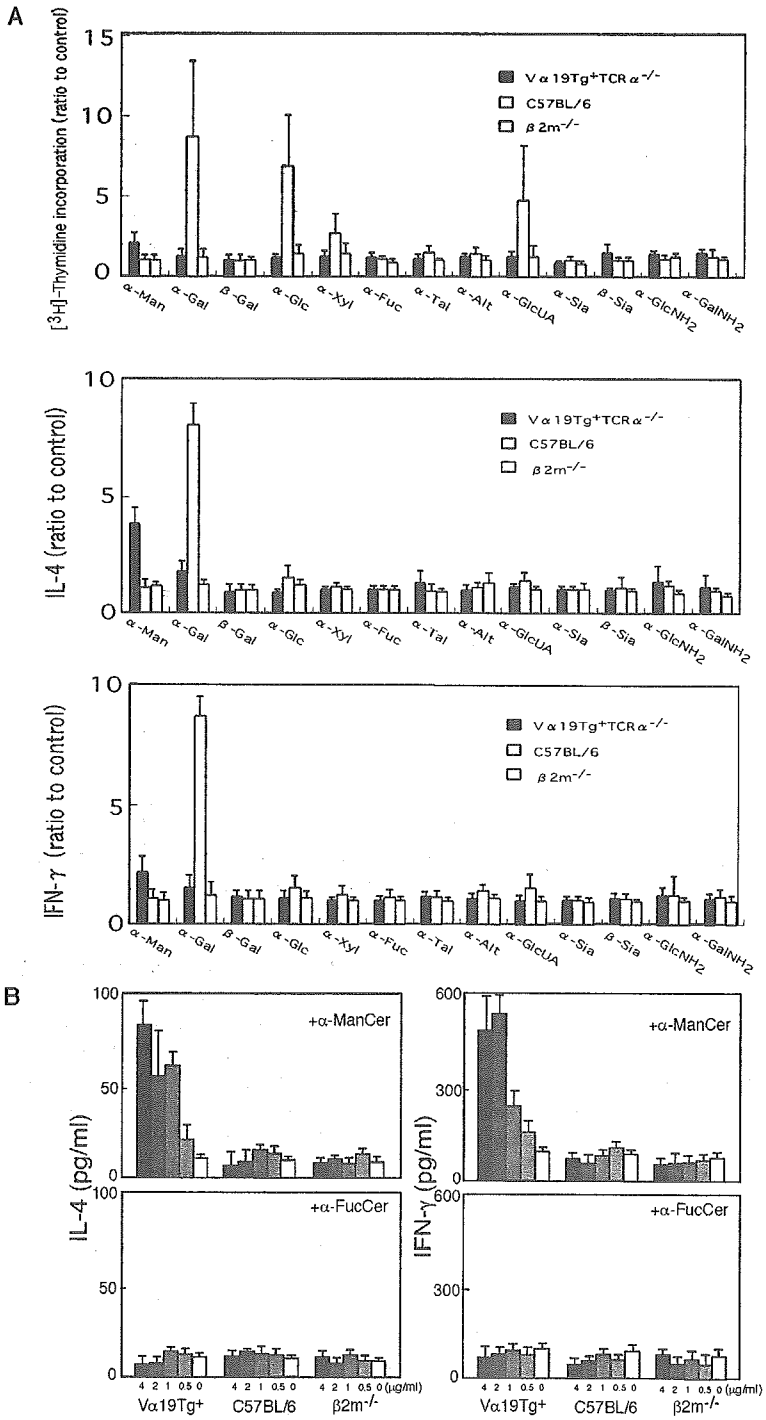


Figure 5. Activation of Vα19 Tg+ Cells with Glycolipid Antigens in Culture

(A) Liver MNCs prepared from Vα19 Tg+ TCRα$^{-/-}$, C57BL/6, and β2m$^{-/-}$ mice were cultured in the presence (1 μg/ml) or absence of glycolipids. After 2 days, the immune responses were monitored by measuring cell proliferation ([^{3}H]-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 neuraminyl; Tal, D-talosyl; and Alt, D-altrosyl.

(B) Dose-dependent activation of Vα19 Tg+ cells with α-ManCer in culture. Liver MNCs prepared from Vα19 Tg+ TCRα$^{-/-}$, C57BL/6, and β2m$^{-/-}$ 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 Vα19 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 Vα19-Jα26 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 Vα19 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.

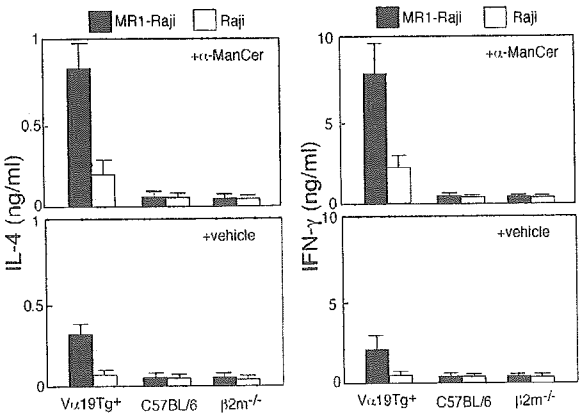


Figure 6. Stimulation of Vα19 Tg+ Cells with α-ManCer in the Context of MR1

MR1-transfected or nontransfected Raji cells were incubated with α-ManCer (2 μg/ml) or vehicle for 18 hr. They were irradiated (3,000 R), washed with medium, and then cultured with liver MNCs isolated from Vα19 Tg+ TCRα−/−, C57BL/6, or β2m−/− 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 Vα19 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 Vα19 or Vα14 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 β2m-deficient mice were obtained from Jackson Laboratory (Bar Harbor, Maine). TCR Cα-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 Vα19 Transgenic Mice

A Vα19-Jα26 (AV19-AJ33) transgene with the endogenous TCR α promoter and the enhancer was injected into TCR Cα-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′-CTACTTGTCATCGTCATCCTTGTA 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-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 Vα19 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 μg/ml) for 18 hr and washed twice with DMEM. These cells were cocultured with liver MNCs (1×10^6 per well) from Vα19 Tg or non-Tg mice (2–4 months of age) in 200 μl 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 μl of DMEM (10% FCS) in the presence of glycolipids (1 μg/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 [^{3}H]-thymidine (0.5 μCi/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/.

Acknowledgments

The authors thank Drs. H. Ishikawa and M. Nanno for providing TCR Cα-deficient mice. They also thank Mr. S. Kamijo and his group for taking care of the mice. This work was supported by a grant from the Ministry of Health, Welfare, and Labor of Japan.

Received: December 24, 2004
Revised: April 12, 2005
Accepted: April 13, 2005
Published: June 24, 2005

References

1. MacDonald, H.R. (1995). NK1.1+ T cell receptor α/β cells: new clues to their origin, specificity and function. J. Exp. Med. *182*, 630–638.
2. Bendelac, A., Rivera, M.N., Park, S.H., and Roark, J.H. (1997). Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu. Rev. Immunol. *15*, 535–562.
3. Lantz, O., and Bendelac, A. (1994). An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4−8− T cells in mice and humans. J. Exp. Med. *180*, 1097–1106.
4. Makino, Y., Kanno, R., Ito, T., Higashino, K., and Taniguchi, M. (1995). Predominant expression of invariant Vα14+ TCR α chain in NK1.1+ T cell populations. Int. Immunol. *7*, 1157–1161.
5. Bendelac, A., Killeen, N., Littman, D.R., and Schwarz, R.H.

(1994). A subset of CD4+ thymocytes selected by MHC class I molecules. Science *263*, 1774–1778.
6. Bendelac, A., Lantz, O., Quimby, M.E., Yewdell, J.W., Bennink, J.R., and Brutkiewicz, R.R. (1995). CD1 recognition by mouse NK1+ T lymphocytes. Science *268*, 863–865.
7. Smiley, S.T., Kaplan, M.H., and Grusby, M.J. (1997). Immunoglobulin E production in the absence of interleukin 4-secreting CD1-dependent cells. Science *275*, 977–979.
8. Chen, Y.-H., Chiu, M.N., Mandel, M., Wang, N., and Wang, C.-R. (1997). Impaired NK1+ T cell development and early IL-4 production in CD1-deficient mice. Immunity *6*, 459–467.
9. Mendiratta, S.K., Martin, W.D., Hong, S., Boesteanu, A., Joyce, S., and Kaer, L.V. (1997). CD1d mutant mice are deficient in natural T cells that promptly produce IL-4. Immunity *6*, 469–477.
10. Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., Ueno, H., Nakagawa, R., Sato, H., Kondo, E., et al. (1997). CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides. Science *278*, 1626–1629.
11. Zlotnik, A., Godfrey, D.I., Fischer, M., and Suda, T. (1995). Cytokine production by mature and immature CD4−CD8− T cells. αβTCR+ CD4−CD8− cells produce IL-4. J. Immunol. *149*, 1211–1215.
12. Arase, H., Arase, N., Nakagawa, K., Good, R.A., and Onoé, K. (1993). NK1.1+ CD4+ CD8− thymocytes with specific lymphokine secretion. Eur. J. Immunol. *23*, 307–310.
13. Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., and Paul, W.E. (1995). Role of NK1.1+ T cells in a T_H2 response and in immunoglobulin E production. Science *270*, 1845–1847.
14. Arase, H., Arase, N., and Saito, T. (1996). Interferon γ production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. J. Exp. Med. *183*, 2391–2396.
15. Terabe, M., Matsui, S., Noben-Trauth, N., Chen, H., Watson, C., Donaldson, D.D., Carbone, D.P., Paul, W.E., and Brezofsky, J.A. (2000). NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat. Immunol. *1*, 515–520.
16. Moodycliffe, A.M., Nghiem, D., Clydesdale, G., and Ulrich, S.E. (2000). Immune suppression and skin cancer development: regulation by NKT cells. Nat. Immunol. *1*, 521–525.
17. Shimamura, M., and Huang, Y.-Y. (2002). Presence of a novel subset of NKT cells bearing an invariant Vα19.1-Jα26 TCR α chain. FEBS Lett. *516*, 97–100.
18. Tilloy, F., Treiner, E., Park, S.-H., Garcia, G., Lemonnier, F., de la Salle, H., Bendelac, A., Bonneville, M., and Lantz, O. (1999). An invariant T cell receptor α chain defines a novel TAP-independent major hitocompatibiity complex class Ib-restricted α/β T cell subpopulation in mammals. J. Exp. Med. *189*, 1907–1921.
19. Treiner, E., Duban, L., Bahram, S., Radosavijevic, M., Wanner, V., Tilloy, F., Affaticati, P., Gilfillan, S., and Lantz, O. (2003). Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature *422*, 164–169.
20. Hakomori, S. (1986). Glycosphingolipids. Sci. Am. *254*, 32–41.
21. Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. Glycobiology *3*, 97–130.
22. Natori, T., Koezuka, Y., and Higa, T. (1993). Agelasphins, novel α-galactosylceramides from the marine sponge *Agelas mauritianus*. Tetrahedron Lett. *34*, 5591–5592.
23. Morita, M., Motoki, K., Akimoto, K., Natori, K., Sakai, T., Sawa, E., Yamaji, K., Koezuka, Y., Kobayashi, E., and Fukushima, E. (1995). Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. J. Med. Chem. *38*, 2176–2187.
24. Figueroa-Perez, S., and Schmidt, R.R. (2000). Total synthesis of α-galactosyl cerebroside. Carbohydr. Res. *328*, 95–102.
25. He, L., Byun, H.-S., and Bittman, R. (2000). Stereoselective preparation of ceramide and its skeleton backbone modified analogues via cyclic thionocarbonate intermediates derived by catalytic asymmetric dihydroxylation of α,β-unsaturated ester precursors. J. Org. Chem. *65*, 7627–7633.
26. Plettenburg, O., Bodmer-Narkevitch, V., and Wong, C.-H. (2002). Synthesis of α-galactosyl ceramide, a potent immunostimulatory agent. J. Org. Chem. *67*, 4559–4564.
27. Sawatzki, P., and Kolter, T. (2004). Syntheses of 3-C-methylceramides. Eur. J. Org. Chem. *1998*, 3693–3700.
28. Koskinen, P.M., and Koskinen, A.M.P. (1998). Sphingosine, an enigmatic lipid: a review of recent literature syntheses. Synthesis (Mass.) *1998*, 1075–1091.
29. Herold, P. (1988). Synthesis of D-erythro- and D-threo-sphingosine derivartives from L-serine. Helv. Chim. Acta *71*, 354–362.
30. Garner, P., Park, J.M., and Malecki, E. (1988). A stereodivergent synthesis D-erythro-sphingosine and D-threo-sphingosine from L-serine. J. Org. Chem. *53*, 4395–4397.
31. Vasella, A., Witzig, C., Chiara, J.-L., and Martin-Lomas, M. (1991). Convinient synthesis of 2-azido-2-deoxy-aldoses by diazotransfer. Helv. Chim. Acta *74*, 2073–2077.
32. Buskas, T., Garegg, P.J., Konradsson, P., and Maloisel, J.-L. (1994). Facile preparation of glycosyldonors for oligosaccharide synthesis: 2-azido-2-deoxyhexopyranosyl building blocks. Tetrahedron Asymmetry *5*, 2187–2194.
33. Kinjo, Y., Wu, D., Kim, G., Xing, G.-W., Poles, M.A., Ho, D.D., Tsuji, M., Kawahara, K., Wong, C.-H., and Kronenberg, M. (2005). Recognition of bacterial glycosphingolipids by natural killer T cells. Nature *434*, 520–525.
34. Mattner, J., Debord, K.L., Ismail, N., Goff, R.D., Cantu, C., 3rd, Zhou, D., Saint-Mezard, P., Wang, V., Gao, Y., Yin, N., et al. (2005). Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature *434*, 525–529.
35. Mombaerts, P., Clarke, A.R., Rudnicki, M.A., Iacomini, J., Itohara, S., Lafáille, J.J., Wang, L., Ichikawa, Y., Jaenisch, R., Hooper, M.L., et al. (1992). Mutations in T cell receptor genes α and β block thymocyte development at different stages. Nature *360*, 225–231.
36. Yamaguchi, H., Hirai, M., Kurokawa, Y., and Hashimoto, K. (1997). A highly conserved major histocompatibility complex class I-related gene in mammals. Biochem. Biophys. Res. Commun. *238*, 697–702.
37. Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection for high- expression transfectants with a novel eukaryotic vector. Gene *108*, 193–199.
38. Pulvertaft, J.V. (1964). Cytology of Burkitt's tumor (African lymphoma). Lancet *1*, 238–240.

Therapeutic Potential of Glycolipid Ligands for Natural Killer (NK) T Cells in the Suppression of Autoimmune Diseases

Sachiko Miyake* and Takashi Yamamura

Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan

Abstract: NKT cells emerge as important regulatory cells in autoimmune responses. Abnormalities in the numbers and functions of natural killer T (NKT) cells have been observed in patients with autoimmune diseases as well as in a variety of mouse strains that are genetically predisposed for development of autoimmune diseases. Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens presented by the non-polymorphic MHC class I-like protein, CD1d. Recently, we and other groups have demonstrated that administration of glycolipid ligands such as α-galactosylceramide (α-GC) or its sphingosine truncated derivative, OCH suppressed autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), diabetes in NOD mice and collagen-induced arthritis (CIA) by inducing T helper (Th) 2 bias of autoimmune T cells. OCH is a unique ligand to stimulate NKT cells to selectively produce Th2 cytokines whereas α-GC induces both interleukin (IL)-4 and interferon (IFN)-γ, and is more beneficial for treatment of a wide variety of Th1-mediated autoimmune diseases. The lack of polymorphism of CD1d and cross-reactive responses of mouse and human NKT cells to the same ligand indicates that targeting NKT cells with this ligand may be an attractive means for intervening in human autoimmune diseases such as type I diabetes (T1D), multiple sclerosis (MS) and rheumatoid arthritis (RA).

The present review will focus on the potential roles of NKT cells in the pathogenesis of autoimmune diseases and the recent advances in glycolipid therapy for autoimmune disease models. The molecular mechanism of OCH-induced Th2-selective cytokine secretion will also be discussed.

Key Words: NKT cell, CD1, α-galactosylceramide, OCH, autoimmune disease, therapy, Th1/Th2.

INTRODUCTION

Autoimmunity has been studied for more than four decades, but its pathogenesis has remained a mystery. Despite that, potent new biologic therapeutics including cytokines and anti-cytokine reagents show remarkable clinical efficacy in several autoimmune diseases such as MS and RA. However, these drugs have limited value at best, and sometimes are accompanied by serious side effects. Thus drug development for these autoimmune diseases is a fundamental challenge in the 21st century.

It is well-established that central tolerance, the deletion of T cells with high avidity for self-antigens restricts the repertoire of peripheral auto-reactive T cells. However, this process is incomplete. Lymphocytes with lower avidity for self-antigens, or with high avidity for determinants that are not expressed in the thymus (self or foreign), are found in the periphery. The presence of peripheral T cells that react with self-antigens in healthy individuals indicates the existence of physiological regulatory mechanisms that prevent pathological autoimmunity. Such control is referred to as peripheral tolerance, and peripheral tolerance comprises pathways that act directly on auto-reactive cells (intrinsic tolerance: ignorance, anergy, phenotypic skewing) or indirectly through cells such as $CD4^+CD25^+$ T cells and NKT cells [1]. Disruption of these tolerance mechanisms could lead to autoimmune disease. Conversely, maintenance or re-establishment of peripheral tolerance is a therapeutic strategy to restrain destructive autoimmune processes. Thus it seems attractive to induce or stimulate regulatory cells to control harmful autoimmunity [2,3]. Among several different regulatory cells, we would like to focus on targeting NKT cells, because a number of recent studies suggest NKT cells are involved in the pathogenesis of autoimmunity. In addition, several glycolipid ligands can selectively stimulate NKT cells and have been shown to prevent autoimmune disease models.

NKT CELLS AND THEIR ANTIGENS

NKT cells are usually defined as cells co-expressing of the natural killer receptors such as NK1.1 or NKR-P1A (CD161) and a αβ T cell receptor (TCR). Although $NK1.1^+$ TCR^+ lymphocytes are heterogeneous, most NKT cells express an invariant TCRα chain composed of Vα14-Jα18 segments in mice and Vα24-Jα18 segments in humans, which is associated with TCR β chains using a restricted set of Vβ genes. These Vα14 invariant NKT cells recognize glycolipid antigens such as α–GC presented by a nonpolymorphic MHC class I-like molecule, CD1d [4,5]. As little is known about CD1d non-restricted NKT cells or α-GC independent CD1d restricted NKT cells, in this review we focus on the α-GC responsive NKT cells, and "NKT cells" will be used for α-GC responsive NKT cells.

*Address correspondence to this author at the Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan; Tel: +81-42-341-2711; Fax: +81-42-346-1753; E-mail: miyake@ncnp.go.jp

1568-0088/05 $50.00+.00

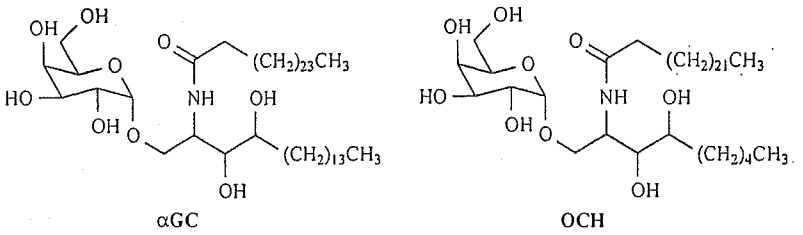


Fig. (1). Structure of α-galactosylceramide (α-GC) and OCH. The α-anomeric conformation of sugar moiety, the configuration of the 2-hydroxyl group on the sugar moiety, 3,4 –hydroxyl groups of the phytosphingoshine are important for NKT cell recognition of α-GC [10]. The OCH analogs has a shorter shpingosine chain.

NKT cells are comprised of two subsets; $CD4^+$ or $CD4^-$ $CD8^-$ (double negative DN). Although the tissue distribution of NKT cells varies, they are most frequent in the liver and bone marrow, and less abundant in the spleen. Whereas human and mouse NKT cells share many characteristics, the frequency is much lower in humans [2]. Moreover, $CD4^+$ and DN NKT cells appear different in terms of cytokine production in humans but not in mice [5,6]. The $CD4^+$ subset of human NKT cells produces both Th1 and Th2 cytokines upon antigen stimulation, whereas the DN subset produces Th1 cytokines and upregulates production of perforin after exposure to cytokines [6].

NKT cells are selected by, and restricted to CD1d. This unique class of antigen-presenting molecules has been highly conserved through mammalian evolution. It is speculated that self glycolipid antigens probably function as activating ligands for NKT cells due to the self-reactivity of NKT cells and the activated memory phenotype of NKT cells isolated from human umbilical-cord blood [7,8] and germ-fee mice [9].

α-GC is a synthetic glycolipid originally isolated from marine sponges *Agelas mauritanius*, and later, a synthetic analog of this compound was developed for experimental studies and clinical trials (Fig. (1)) [10]. α-GC has been shown to be a potent stimulator of both murine and human NKT cells [10-12]. NKT cells respond to sphingolipids substituted with an α-linked galactose or glucose, but not α-linked mannose and sphingolipids containing β-linked galactose or glucose [10]. Sphingolipids containing β-linked sugars resemble common mammalian lipids, whereas α-glycosyl sphingolipids have not been found in normal mammalian tissues. Recently, GD3, a ganglioside expressed on human tumors of neuroectodermal origin has been reported to be recognized by NKT cells [13]. Similar to α-GC, GD3 is not expressed or expressed at low levels on normal tissues.

REGULATION OF CYTOKINE PRODUCTION BY NKT CELLS

NKT cells are characterized by exhibiting a pre-activated phenotype in physiological conditions, being $CD69^+$, $CD62L^{low}$, and $CD44^{high}$. Consistent with the pre-activation status, NKT cells release large amounts of cytokines including IL-4 and IFN-γ promptly upon antigen stimulation and affect the functions of neighboring cell populations such as T cells, B cells, NK cells and dendritic cells (Fig. (2)). [2,5,6]. The mechanisms underlying their rapid cytokine production or their distinct cytokine patterns remain unknown. Recently, Stetson DB *et al.* reported that NKT cells contained 1,000-fold more IL-4 message and 200-fold more IFN-γ message than naive $CD4^+$T cells and levels of H3 acetylation at both the IL-4 and IFN-γ promoters [14]. These chromatin modifications at cytokine genes that correlated with the presence of abundant cytokine mRNAs are similar to differentiated helper T cells such as Th1 or Th2 cells. During differentiation, one set of genes is epigenetically activated and the other is silenced in Th1 or Th2 cells. It is thought that lineage-specific transcription factors such as GATA-3 and T-bet function to maintain and increase the accessibility of one cytokine locus while suppressing or silencing the other in the differentiated cells [15]. Interestingly, NKT cells express both GATA-3 and T-bet allowing hyperacetylation at the IL-4 and IFN-γ promoters (Oki S and Miyake S, unpublished observations).

α-GC induces a variety of cytokines including IFN-γ, IL-2, tumor necrotic factor-α, IL-4 and IL-13 from NKT cells. In contrast, a sphingosine-truncated analogs of α-GC, such as OCH, stimulates NKT cells to preferentially produce IL-4, IL-13. It is important to understand the mechanisms how OCH can stimulate NKT cells to produce Th2 cytokines selectively. IFN-γ production by NKT cells seems to correlate with the stability of glycolipid ligands to bind to the CD1d molecule, and the binding stability correlates with the length of sphingosine chains. Thus OCH binds to CD1d molecule less stably compared to α-GC because of the truncation of sphingosine chain and is therefore not able to sustain TCR stimulation, resulting in preferential production of IL-4 from NKT cells [16]. Given that IL-4 secretion consistently precedes IFN-γ production by NKT cells after TCR ligation, we speculated a critical difference in the upstream transcriptional requirements for the IFN-γ and the IL-4 genes in NKT cells. In support of this speculation, cyclohexamide treatment inhibited the transcription of IFN-γ, but not that of IL-4. In contrast, transcription of both cytokines was abolished by cyclosporine A treatment, indicating that TCR-mediated activation of nuclear factor of activated T cells (NF-AT) is essential for the production of both cytokines. Interestingly, IFN-γ production by NKT cells requires longer TCR stimulation than required for IL-4 when stimulated with immobilized anti-CD3 antibody. TCR stimulation-dependent NF-AT activation is regulated in a manner quite sensitive to change of Ca^{2+} concentration [17]. Thus activated NF-AT might be no longer available for effective IFN-γ transcription due to its quick export from the nucleus after the short duration of TCR stimulation by OCH.

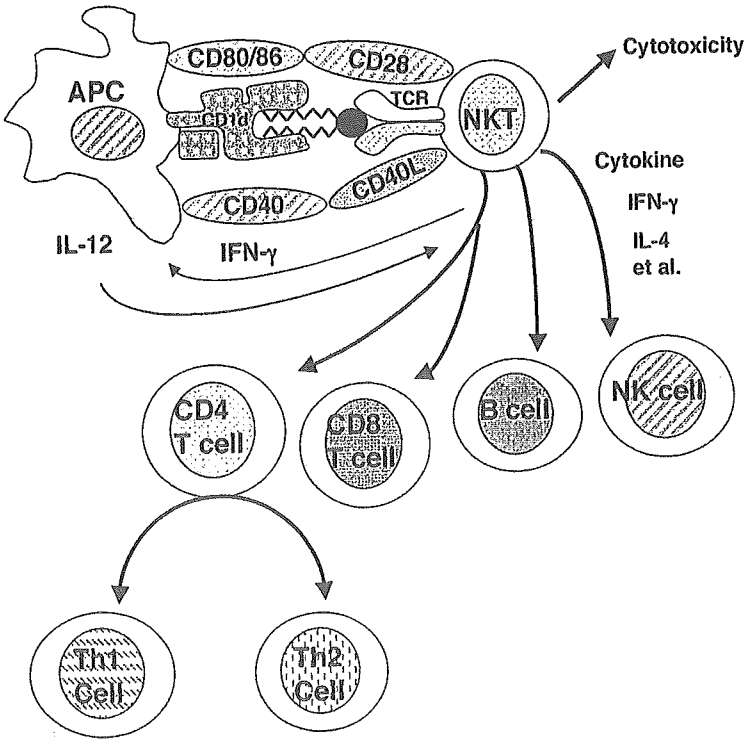


Fig. (2). A model of NKT cell activation and their interactions with other subsets of cells. NKT cells recognize glycolipid ligand presented by CD1d molecule. After stimulation, NKT cells produce a variety of cytokines and exert effector functions. NKT cells might be important for the differentiation of $CD4^+$ T cells into Th1 or Th2 cells, maturation of dendritic cells and activation of B cells and natural killer cells.

Given that *in vivo* administration of soluble OCH and α–GC induces cytokine production by NKT cells within 90 m, stimulation of NKT cells after *in vivo* injection of α–GC or OCH probably occurs without intracellular processing. In fact, when it is presented by antigen presenting cells (APCs) expressing a cytoplasmic tail mutant of the CD1d molecule which is unable to undergo endosomal/lysosomal sorting, the stability of glycolipid antigen binding to CD1d correlated with its length of sphingosine chain. However, when we used APCs expressing wild type CD1d and pulsed antigens for longer time period, the uptake of glycolipids and subsequent endosomal/lysosomal assembly with CD1d, strengthened the interaction of glycolipids with CD1d and abolished the correlation of the binding stability to CD1d and lipid tail length. When we used bone marrow-derived mature dendritic cells as APCs, there was no significant difference between long-term pulsed OCH and α–GC in the ability to induce IFN-γ by freshly isolated NKT cells *in vitro* (Oki S. and Miyake S., unpublished observation).

GLYCOLIPID THERAPIES FOR AUTOIMMUNE DISEASE MODELS

Experimental Autoimmune Encephalomyelitis

EAE is an autoimmune inflammatory disease affecting the central nervous system (CNS) that serves as a model for MS. EAE can be induced in susceptible mouse strains by immunization with CNS proteins or peptides in adjuvant or by the passive transfer of T cells reactive against such CNS antigens. Studies with animal models has suggested that myelin-specific Th1 cells secreting IFN-γ, tumor necrotic factor-α and IL-2 mediate EAE, whereas myelin-specific Th2 cells producing IL-4 and Il-10 play a protective role [18]. Therefore administration of Th2 cytokines to control the disease was considered for clinical use. However, clinical trails of recombinant cytokines, except for IFN-β, have mostly failed because of accompanying side effects. Recently, local delivery of Th2 cytokines, using autoimmune T cells, using hybridomas or fibroblasts transfected with genes encoding anti-inflammatory cytokines was found to be effective in the suppression of EAE [19,20]. However, this strategy seems to be difficult for clinical treatment without major technical advances in introducing particular genes into these cells and in culturing autoimmune T cells. Since NKT cells are known to rapidly invade and accumulate in inflammatory lesions in a manner similar to inflammatory cells and produce cytokines, the stimulation of NKT cells to produce Th2 cytokines would be a powerful strategy to deliver protective cytokines to autoimmune-mediated inflammatory lesions. Nevertheless we observed only a marginal effect of α-GC on the clinical course of EAE induced in C57BL/6 (B6) mice with myelin oligodendrocyte glycoprotein (MOG) derived peptides even though we tried protocols with varying doses of α-GC or different timing of injection [21,22]. Since NKT cells produce both IFN-γ and IL-4 upon stimulation with α-GC, we postulated that α-GC could not prevent EAE because NKT cell-derived IFN-γ would mask the protective effect of the IL-4 simultaneously produced by NKT