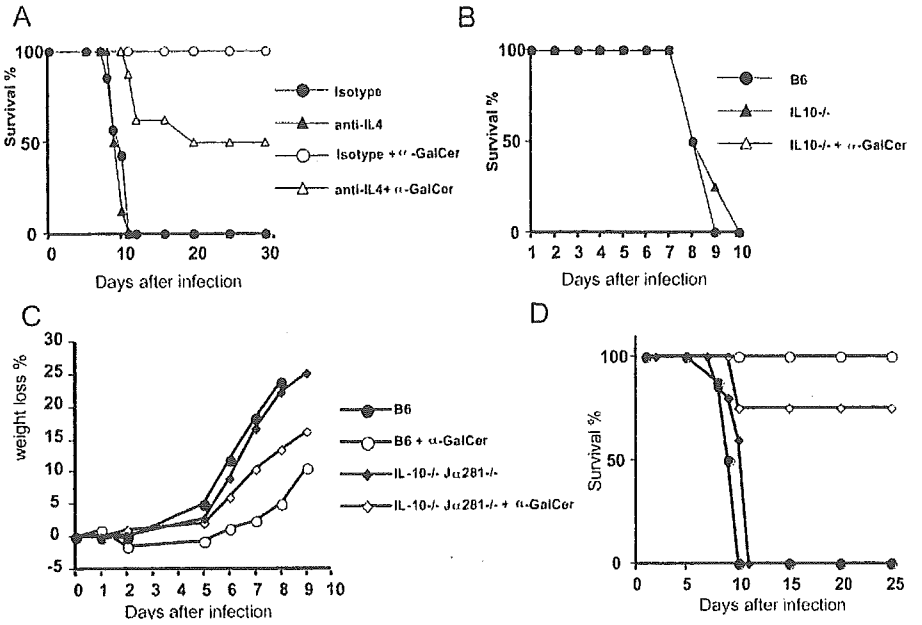

FIGURE 5. Roles of IL-4 and IL-10 in the protective process. *A*, Neutralization of IL-4 the day before α-GalCer treatment partially altered its protective effect. B6 mice were treated with 1 mg of anti-IL-4 Abs 24 h before α-GalCer treatment and 48 h before infection with *T. gondii*. Similarly infected α-GalCer-treated B6 mice and B6 mice treated with rat IgG were used as controls (eight mice per group). The survival rate of these mice was determined. Similar results were obtained in another separate experiment. *B*, α-GalCer treatment fails to protect IL-$10^{-/-}$ mice. IL-$10^{-/-}$ mice were treated (eight mice per group) with 5 μg of α-GalCer i.p. 24 h before infection. As a control, IL-$10^{-/-}$ mice and B6 mice sham-treated with DMSO alone were infected, and the survival rates of all mice were determined. *C* and *D*, IL-10 produced by NKT is partially responsible for the protective effect of α-GalCer. To assess the role of IL-10 produced by NKT cells, chimeric mice were generated. B6 mice were irradiated and then received i.v. 1×10^7 bone marrow cells recovered from femurs and tibias of donor mice. To generate mice in which only NKT cells were devoid of the IL-10 gene, a mixture (50/50%) of bone marrow cells from Jα$281^{-/-}$ mice and IL-$10^{-/-}$ mice was used for reconstitution (12 mice/group). Control mice were reconstituted with bone marrow from B6, Jα$281^{-/-}$, or IL-$10^{-/-}$ mice (six mice per group). Six weeks later, the efficiency of the reconstitution was determined. Chimeric mice were then infected, and weight loss (*C*) and survival rate (*D*) were recorded. This experiment has been performed twice with similar results.

Discussion

In contrast to B6 mice that develop acute lethal ileitis after oral infection with *T. gondii*, mice deficient in NKT cells, although permissive to parasite replication, are more resistant to this severe immunopathological manifestation, suggesting a critical role of these cells in the intestinal inflammation. NKT cells, present in the intestine at early stages after infection, can secrete IFN-γ that will initiate a Th1-like immune response mediating the lethal ileitis. The critical role of IFN-γ was confirmed by studies showing that mice deficient in IFN-γ production do not develop ileitis (27).

Results from this study show that the harmful effect of NKT cells can be neutralized by treatment with a single injection of α-GalCer. When intestinal NKT cells were stimulated by α-GalCer the day before infection, minor intestinal lesions developed, and the mice survived the infection. The beneficial effect of α-GalCer was accompanied by a shift in cytokine production by the intestinal NKT cells toward a Th2 profile (IL-4 and IL-10) and a dramatic increase in $CD4^+CD25^+Foxp3^+$ cells in MLNs. Depletion of regulatory T cells abrogated the protective effect of treatment with α-GalCer before the infection. This observation indicates that activation of NKT cells by α-GalCer triggers a regulatory T cell response that helps control the inflammatory intestinal disease observed after *T. gondii* infection.

We showed for the first time that conventional CD1d-restricted NKT cells are present in the small intestine of *T. gondii*-infected mice; more precisely, they are located within the lamina propria compartment. They are not associated with IELs in this model, contrary to what was described in previous studies that have identified NK-like T cells within the intraepithelial compartment of the mouse small intestine (35). The presence of unconventional NKT cells, non-CD1d-restricted cells, was also described in the large intestine (36). In this study it was observed that the purified NKT cells were mainly of the $CD4^+$ phenotype, with double-negative $CD4^-8^-$ cells making up the difference.

Upon polyclonal or Ag-specific stimulation through the TCR, CD1d-restricted NKT cells have the capacity to produce IL-4 and IFN-γ (11). In this model of pathogen-driven ileitis, we observed that intestinal CD1d-restricted NKT cells promote an IFN-γ response, as reflected by the marked reduction of IFN-γ mRNA expression at serial time points after infection in Jα$281^{-/-}$ mice devoid of NKT cells compared with wild-type control mice. This early IFN-γ production by intestinal NKT cells may influence the Th1/Th2 balance and thus favor the switch toward a local inflammatory Th1 immune response. Secretion of IFN-γ by intestinal NKT cells may induce DC to secrete IL-12, resulting in an increased production of IFN-γ and TNF-α by lamina propria $CD4^+$ T cells that are important effector cells in the hyperinflammatory process associated with oral *T. gondii* infection. IFN-γ produced by NKT may activate other cell types, such as macrophages and neutrophils (37), that will act on NK cells and CD8 T cells to enhance their IFN-γ production. Our data confirmed the findings of previous studies in which $NK1.1^+$ cells were identified as a source of IFN-γ that is essential to limit parasite replication (32, 46) and also point out their role in triggering an exacerbated IFN-γ response leading to immunopathology.

NKT cells are certainly not the only source of IFN-γ. In Jα$281^{-/-}$ mice, characterized by the absence of NKT cells, a limited amount of IFN-γ was secreted after infection, followed by a

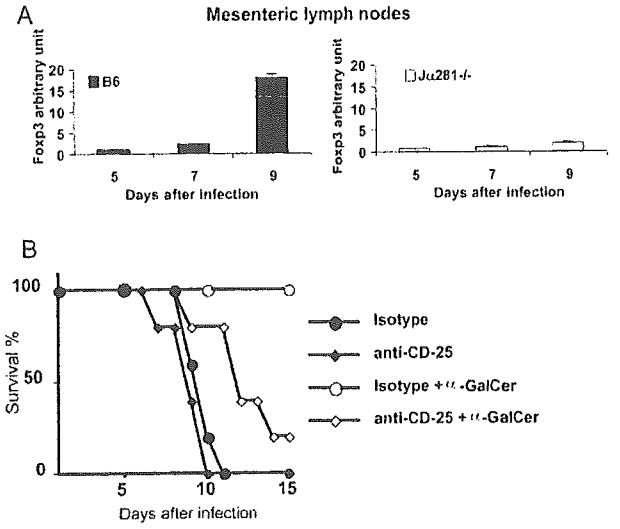

FIGURE 6. Implication of regulatory T cells after α-GalCer treatment. *A*, An increased number of Foxp3 regulatory T cells in MLNs from α-GalCer-treated mice was observed. cDNAs obtained from total MLNs of infected B6 and $J\alpha281^{-/-}$ mice treated, or not, with α-GalCer were submitted to quantitative real-time PCR with specific primers and probed for Foxp3 and actin (five mice per group). After normalization to actin expression, results were expressed as an increase in Foxp3 expression in α-GalCer-infected B6 or $J\alpha281^{-/-}$ mice compared with infected B6 or $J\alpha281^{-/-}$ mice. This experiment was repeated twice with similar results. *B*, B6 mice were depleted of $CD25^+$ cells with anti-CD25 mAb i.p. 3 days before α-GalCer treatment. Twenty-four hours later, all mice were infected. Similarly infected α-GalCer-treated B6 mice, anti-CD25-treated B6 mice, and B6 mice treated with isotype Abs were used as controls (five mice per group). Results are representative of two independent experiments.

significant increase in cytokine production with time (day 8). This late IFN-γ production indicates that other cells within the responding immune population (e.g., $CD4^+$ T cells from the lamina propria) are specifically activated and probably are responsible for the death of 75% of the $J\alpha281^{-/-}$ mice and the mild inflammation observed in the intestines of surviving mice.

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

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

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

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

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

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

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

The participation of IL-4 in this process cannot be ruled out. The role of IL-4 seems to be complex in toxoplasmosis. Our data indicate that neutralization of IL-4 cannot render α-GalCer-treated mice as susceptible as wild-type, infected, untreated mice, indicating the participation of other cytokine, such as IL-10. In addition, these experiments might indicate, as suggested by Nickdel et al. (54), that IL4-deficient mice are more resistant than wild-type mice to the development of ileitis. However, our data for IL-4 corroborate previous findings reporting that treatment with α-GalCer or OCH (a synthetic glycolipid that has shorter hydrophobic chain) improves mucosal Th1/Th2 cytokine balance by increasing IL-10 and IL-4 production and prevents experimental colitis in mice (55).

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

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

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

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Disclosures

The authors have no financial conflict of interest.

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Preferential T_h2 polarization by OCH is supported by incompetent NKT cell induction of CD40L and following production of inflammatory cytokines by bystander cells *in vivo*

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Abstract

The altered glycolipid ligand OCH is a selective inducer of T_h2 cytokines from NKT cells and a potent therapeutic reagent for T_h1-mediated autoimmune diseases. Although we have previously shown the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells, little is known about the extrinsic regulatory network for IFN-γ production. Here we demonstrate that OCH induces lower production of IFN-γ, not only by NKT cells but also by NK cells compared with α-galactosylceramide. OCH induced lower IL-12 production due to ineffective primary IFN-γ and CD40 ligand expression by NKT cells, and resulted in lower secondary IFN-γ induction. Co-injection of a sub-optimal dose of IFN-γ and stimulatory anti-CD40 mAb compensates for the lower induction of IL-12 by OCH administration. IL-12 converts OCH-induced cytokine expression from IL-4 predominance to IFN-γ predominance. Furthermore, CpG oligodeoxynucleotide augmented IL-12 production when co-administrated with OCH, resulting in increased IFN-γ production. Taken together, the lower IL-12 production and subsequent lack of secondary IFN-γ burst support the effective T_h2 polarization of T cells by OCH. In addition, highlighted in this study is the characteristic property of OCH that can induce the differential production of IFN-γ or IL-4 according to the availability of IL-12.

Introduction

NKT cells are a unique subset of CD1d-restricted T lymphocytes that express TCR and some NKR. NKT cells recognize glycolipid antigens such as α-galactosylceramide (αGC) by an invariant TCRα chain composed of Vα14-Jα18 segments in mice and Vα24-Jα18 segments in humans, associated with TCRβ chains using a restricted set of Vβ genes (1, 2). NKT cells rapidly secrete large amounts of cytokines including IL-4 and IFN-γ upon antigen stimulation and are effective regulators of T_h1/T_h2 balance *in vivo* (3–5). We have previously demonstrated that *in vivo* administration to mice of altered glycolipid ligand, OCH, ameliorates experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA) and type I diabetes by enhancing IL-4-dependent T_h2 responses without inducing IFN-γ production and pathogenic T_h1 responses (6–8).

Recently, we have clarified the intrinsic molecular mechanism of preferential IL-4 production by OCH-stimulated NKT cells (9). IFN-γ production by NKT cells was more susceptible to the sphingosine length of glycolipid ligand than that of IL-4, and the length of sphingosine chain determined the half-life of NKT cell stimulation by CD1d-associated glycolipids. IFN-γ production by NKT cells required longer T cell stimulation than did IL-4 production and the transcription of the IFN-γ gene required *de novo* protein synthesis by activated NKT cells. The NF-κB family member transcription factor c-Rel was preferentially transcribed in αGC-stimulated, but not in OCH-stimulated, NKT cells and was identified as essential for IFN-γ production by activated NKT cells. Therefore, the differential duration of NKT cell stimulation, due to the binding stability of individual glycolipid antigens to CD1d molecules, determines whether signaling leads to effective c-Rel transcription and IFN-γ production by activated NKT cells.

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

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and NK cells has been demonstrated, suggesting that activated NKT cells may influence further IFN-γ production by other cells including NK cells (3, 10). A C-glycoside analog of 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

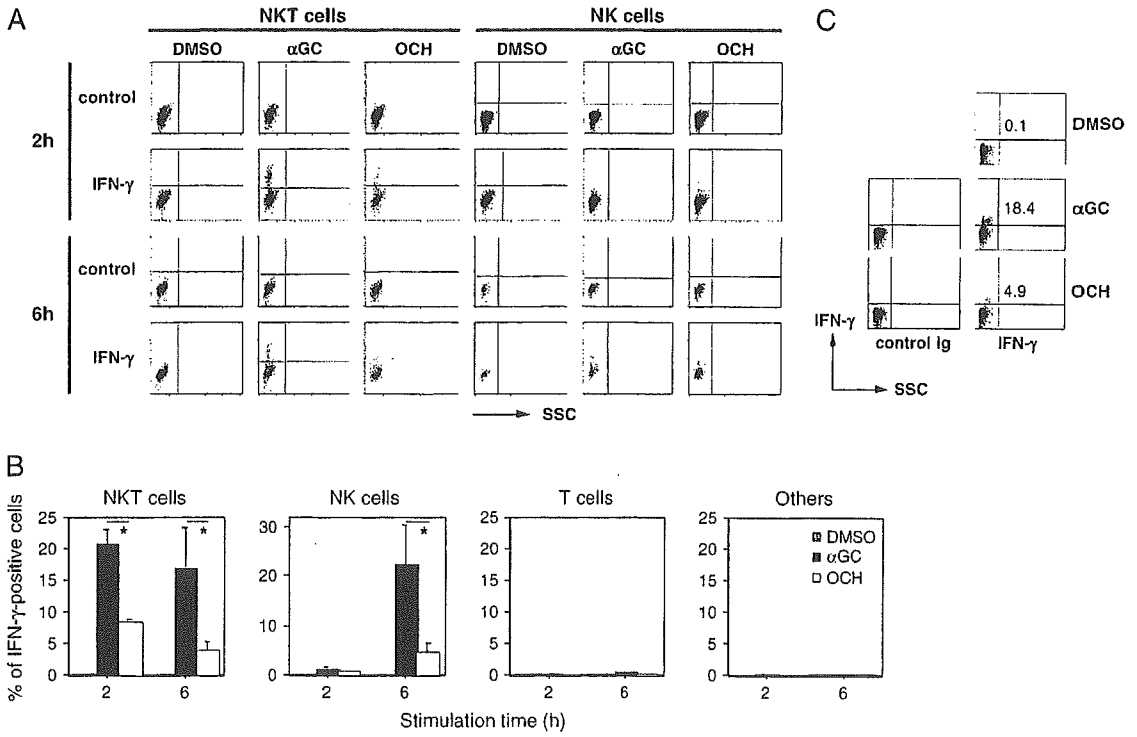

Fig. 1. Expression of IFN-γ by NKT cells and non-NKT cells after administration of glycolipid ligands. B6 mice were treated intra-peritoneally with 2 μg per mouse of either αGC or OCH, and spleen cells were harvested at various time points after glycolipid administration and subjected to intracellular 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),

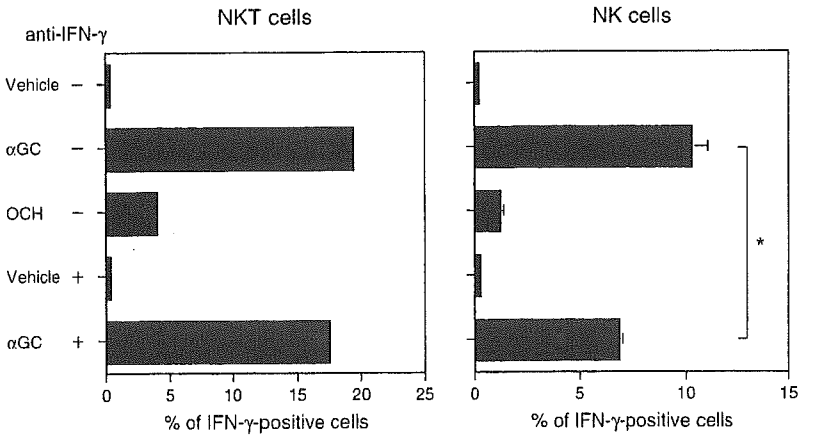

Fig. 2. αGC-induced IFN-γ production by NK cells is partly dependent on IFN-γ production by NKT cells. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids with or without 500 μg per mouse of anti-IFN-γ mAb. Four hours after treatment, spleen cells were harvested and subjected to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

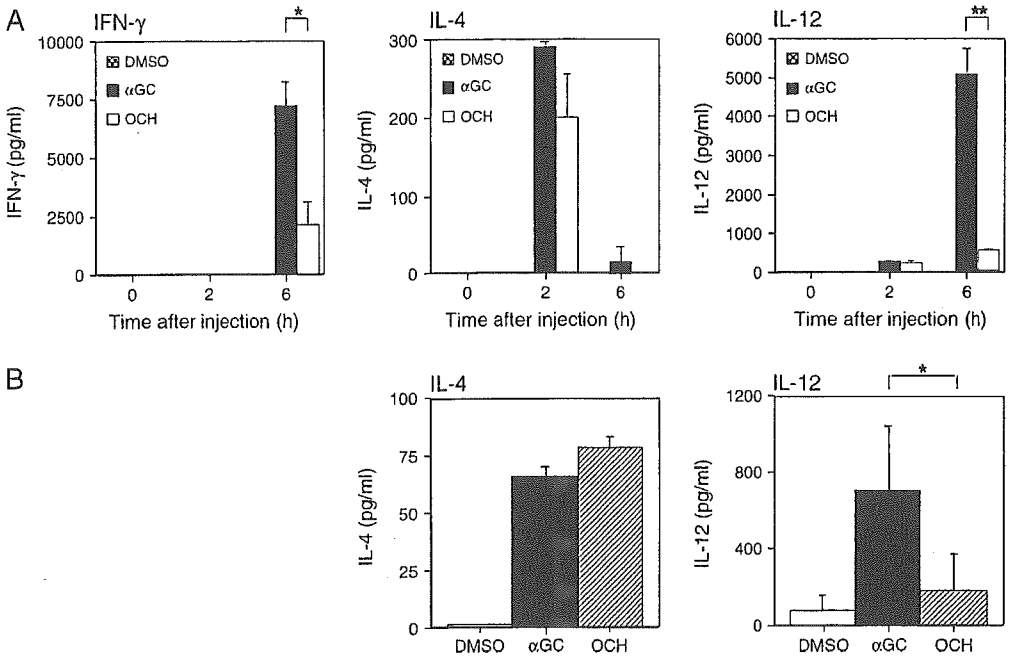

Fig. 3. OCH administration does not induce effective IL-12 production. (A) B6 mice were injected intra-peritoneally with vehicle alone, 2 μg per mouse of αGC or OCH and serum samples were collected at indicated times after injection. Serum levels of IFN-γ, IL-4 and IL-12 (mean ± SD) were determined by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

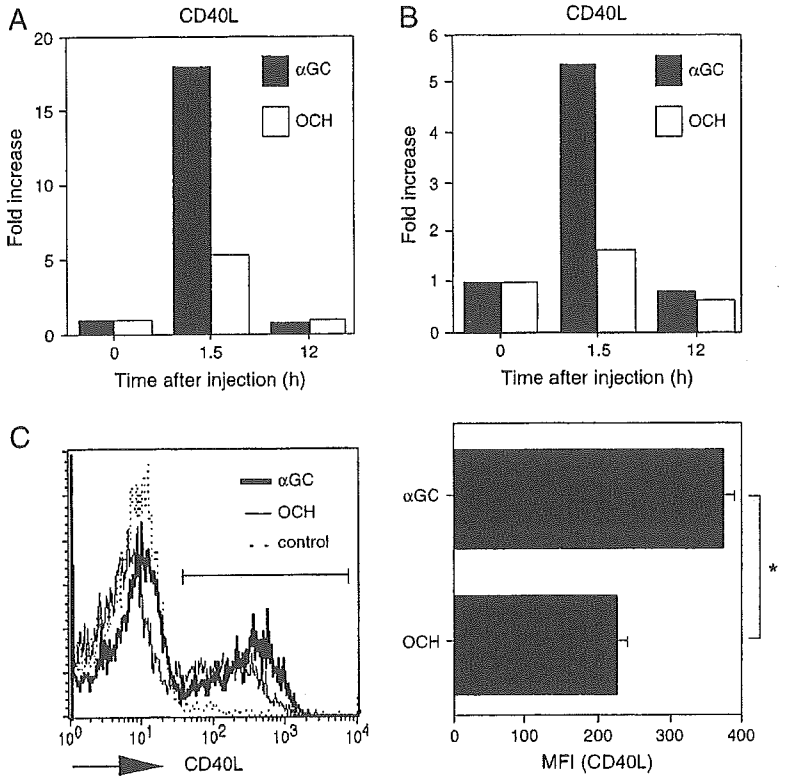

Fig. 4. Expression of CD40L on NKT cells stimulated with αGC or OCH. (A) Plotted values represent data of Affymetrix microarray analysis for indicated genes. The αGC- or OCH-stimulated liver NKT cells (purified as CD3+ NK1.1+ cells) as well as unstimulated NKT cells were analyzed at the indicated time 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

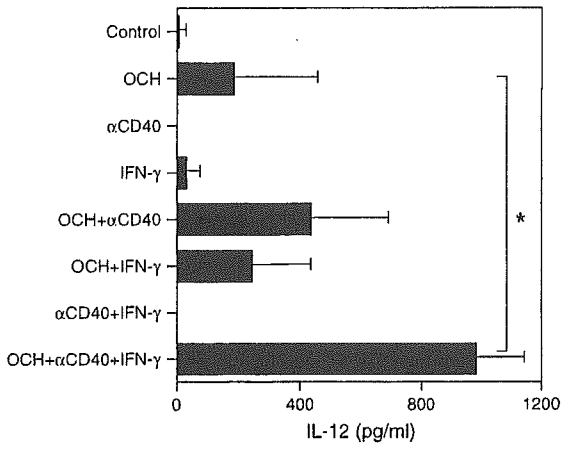

Fig. 5. Co-administration of IFN-γ and stimulatory anti-CD40 mAb augments IL-12 production after OCH administration *in vivo*. B6 mice were treated intra-peritoneally with 2 μg per mouse of glycolipids in combination with murine IFN-γ (1 μg per mouse) and/or stimulatory anti-CD40 mAb (100 μg per mouse) 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

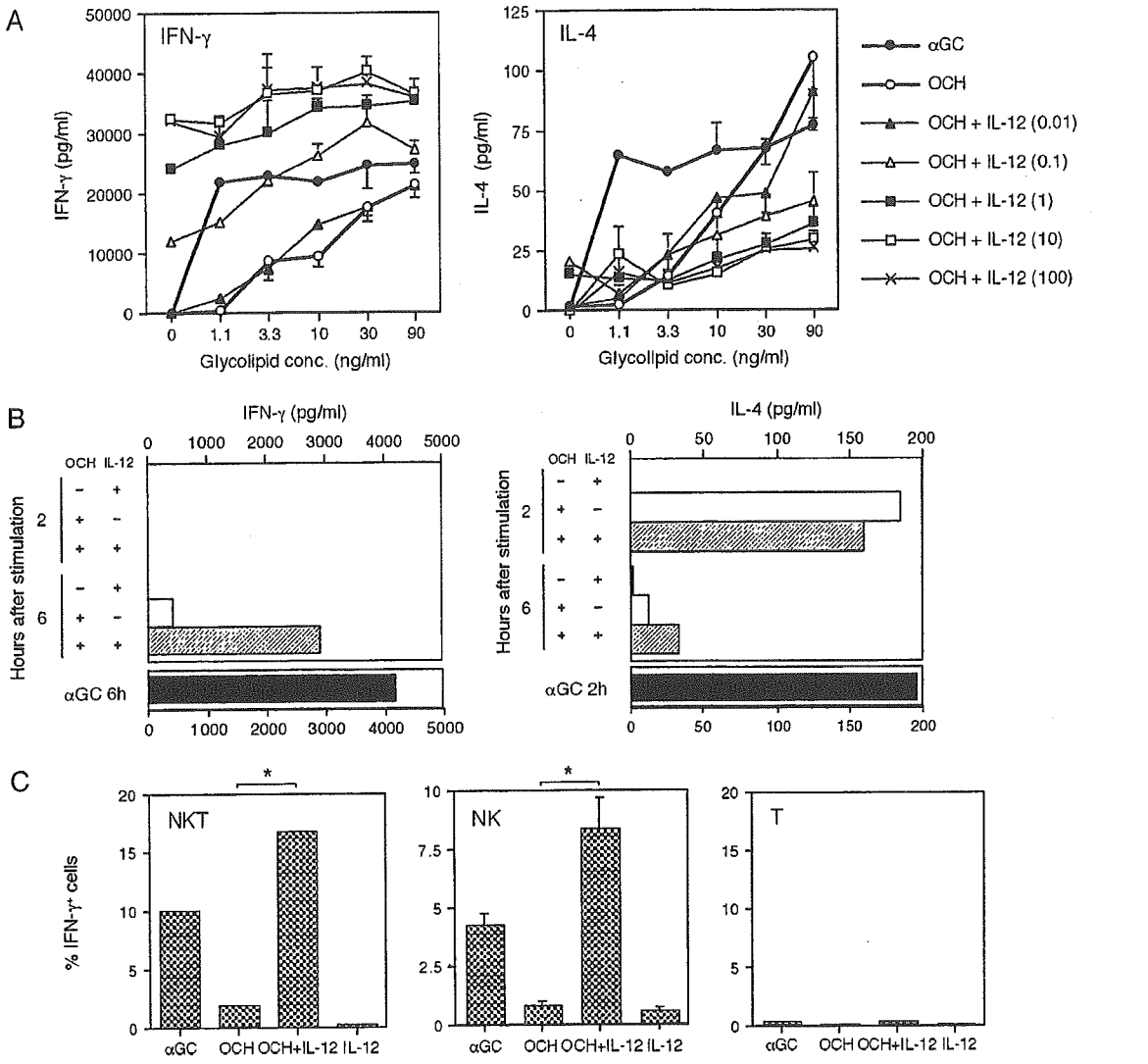

Fig. 6. Co-administration of IL-12 augments IFN-γ production by OCH. (A) Effects of IL-12 on cytokine production of splenocytes stimulated with glycolipids *in vitro*. Splenocytes were stimulated with various concentration of αGC or OCH in the presence or absence of IL-12 (with concentrations from 0.01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

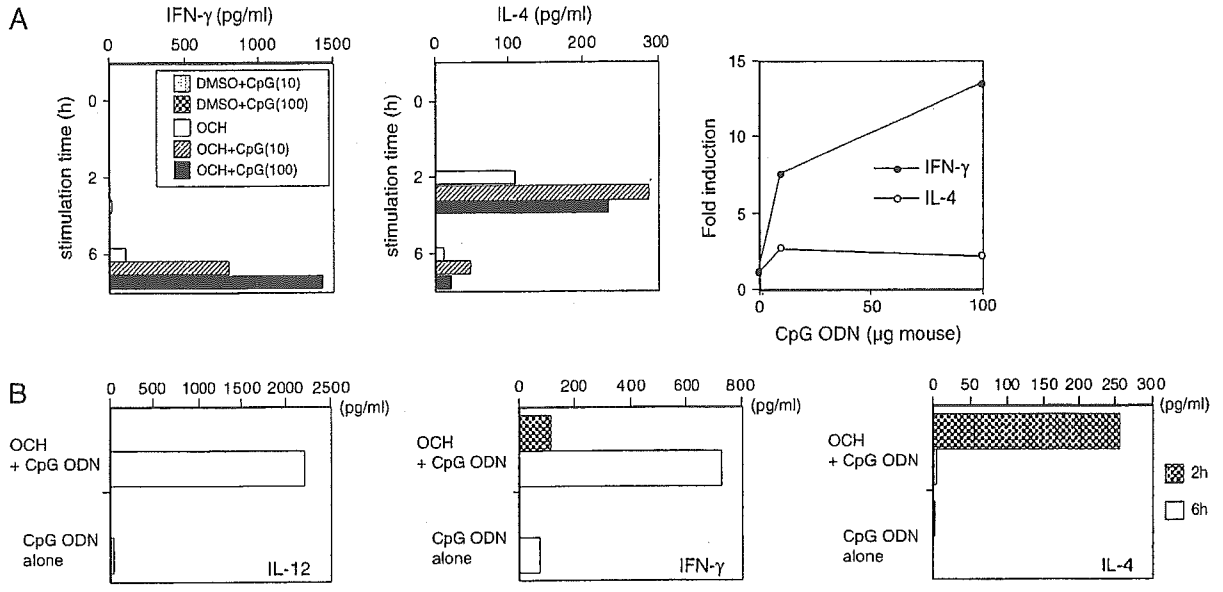

Fig. 7. Co-administration of CpG ODN augments IFN-γ production by OCH stimulation *in vivo*. (A) B6 mice were injected with 10 μg per mouse or 100 μg per mouse of CpG ODN alone or in combination with 2 μg per mouse of OCH and serum samples were collected at indicated times after injection. Serum levels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

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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

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

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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.

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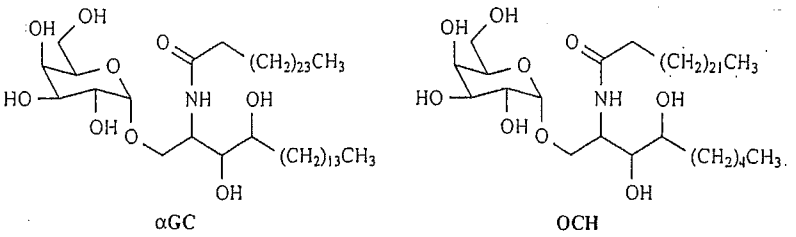

Fig. (1). Structure of α-galactosylceramide (α-GC) and OCH. The α-anomeric conformation of sugar moiety, the configuration of the 2-hydroxyl group on the sugar moiety, 3,4 –hydroxyl groups of the phytosphingoshine are important for NKT cell recognition of α-GC [10]. The OCH analogs has a shorter shpingosine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

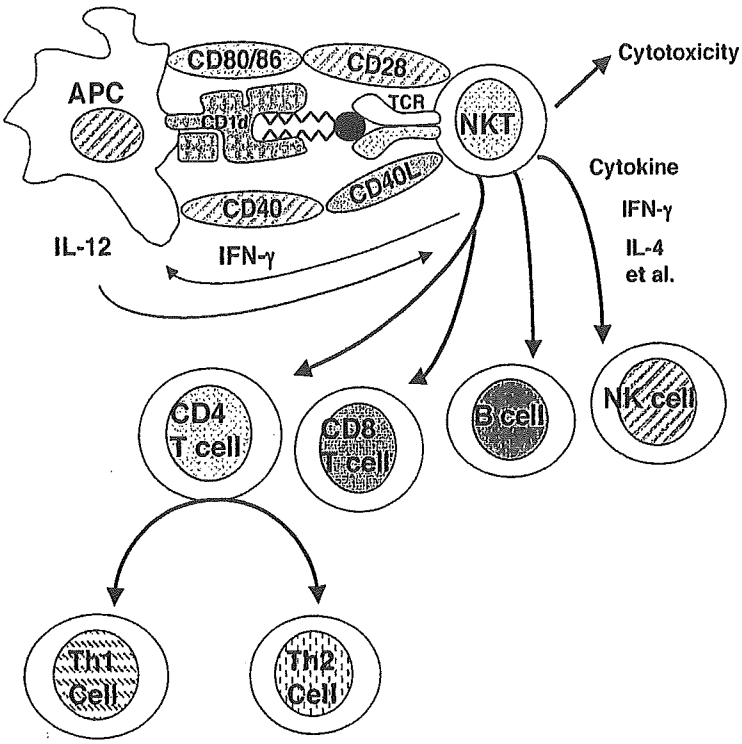

Fig. (2). A model of NKT cell activation and their interactions with other subsets of cells. NKT cells recognize glycolipid ligand presented by CD1d molecule. After stimulation, NKT cells produce a variety of cytokines and exert effector functions. NKT cells might be important for the differentiation 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

cells. We showed several lines of evidence supporting this idea [21]. First, α-GC treatment inhibited EAE induced in IFN-γ knockout mice. Secondly, α-GC treatment augmented the clinical sings of EAE induced in IL-4 knockout mice. Thirdly, blockade of CD86 polarized NKT cells toward a Th2-like phenotype with concomitant suppression of EAE, and activation of APCs by treatment with stimulatory anti-CD40 mAb biased them towards a Th1-like phenotype and exacerbated EAE. As such, EAE could be prevented when ligand stimulation would lead to selective production of Th2 cytokines by NKT cells *in vivo*. Thus we synthesized several analogs of α-GC and found that a sphingosine-truncated analog, OCH, induced selective IL-4 production by NKT cells (Fig. (3)). As expected, administration of OCH prevented development of EAE in both clinical and pathological parameters. The inhibitory effect of OCH was not observed for EAE induced either in NKT cell deficient or IL-4 knockout mice, confirming that IL-4 produced by NKT cells is critical for OCH-mediated suppression on EAE [22].

By contrast, two more reports have shown that α-GC protects mice against EAE when delivered in the immunization protocol (MOG_{35-55} and complete Freund's adjuvant [CFA]) with subsequent multiple intraperitoneal injection or by using a single injection at the day of induction of EAE [23,24]. More recently, Furlan R *et al.* showed that EAE was suppressed only when α-GC was administered at the time of immunization subcutaneously mixed with CFA but not administered intraperitoneally [25]. Although it is not clear the difference among these studies, the role of NKT cells in the pathogenesis or prevention of autoimmunity in CNS may depend on the stage of disease and the associated cytokine milieu, the timing or the route of administration. These parameters are critical to modulate diseases.

In addition to B6 mice, SJL mice are highly susceptible to EAE and EAE induced by immunization with proteolipid protein derived peptides $PLP_{139-151}$ is used as a remitting-relapsing MS model. In the context of NKT cells, SJL mice have been reported to be markedly diminished in number and cytokine production upon activation [26]. Singh AK *et al.* reported that SJL mice responded poorly to treatment with α-GC [24]. When SJL mice were treated with α–GC, the morbidity and mortality were exacerbated although the onset of disease was delayed. By contrast, a multiple injection of OCH protected SJL mice against EAE (Miyake S and Yamamura T, unpublished observation). Furthermore, OCH protected SJL mice against the relapse of EAE, suggesting

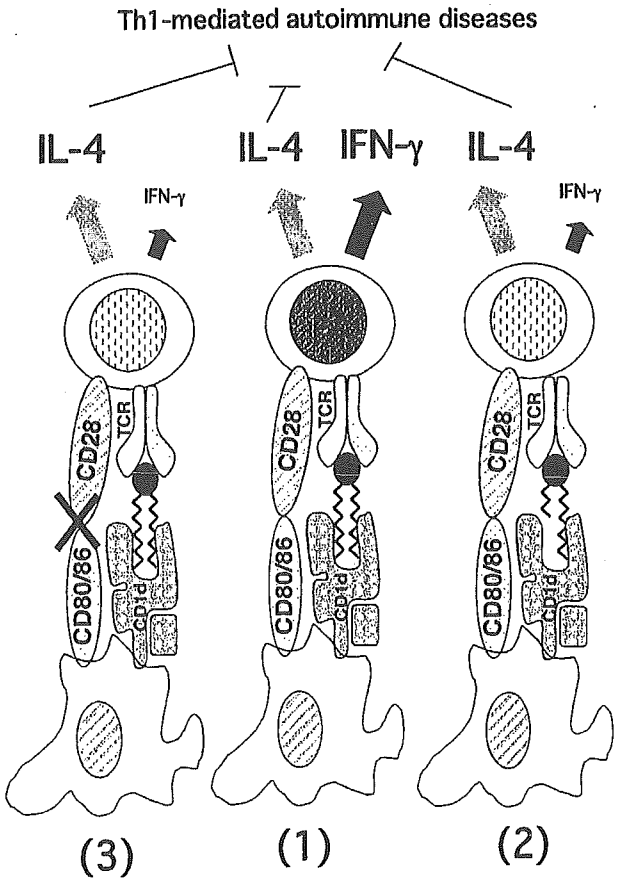

Fig. (3). Modulation of NKT cell cytokine production by an altered ligand or by co-stimulator blockade. 1) α-GC stimulates NKT cells to produce both anti-inflammatory (e.g. IL-4 and IL-10) and pro-inflammatory (e.g. IFN-γ) factors. This response can be modified by 2) stimulation with an altered ligand such as OCH or 3) stimulation in the absence of CD28/B7.2 co-stimulation. These modifications are potentially important therapeutic approach to suppress Th1-mediated autoimmune diseases.

that OCH holds possibilities as a therapeutic agent to prevent relapses for MS.

Glycolipid Therapy for Collagen-induced Arthritis

RA is an autoimmune disease characterized by persistent inflammation of joints resulting progressive destruction of cartilage and bone. Although its precise etiology is not clearly understood, cumulative evidence suggests that Th1 cells exacerbate disease, whereas Th2 cells suppress arthritis [27]. Given that NKT stimulation with OCH suppressed Th1-mediated diseases such as EAE, OCH might be an effective therapeutic reagent for CIA which serves as an animal model for RA. We have demonstrated that OCH administration inhibited the clinical course of CIA induced in B6 mice by immunization with the chicken type II collagen [28]. Histological analysis revealed that OCH treatment protected against infiltration of inflammatory cells and destruction of cartilage and bone. The suppressive effect of OCH was not observed for CIA induced either in CD1d knockout mice or in Jα18 knockout mice deficient in NKT cells. We also observed OCH suppressed CIA induced in DBA/1J mice immunized with bovine type II collagen. Moreover, injection of OCH strongly suppressed CIA in SJL mice even though these mice have defects in numbers and functions of NKT cells, and even after the arthritis had already developed. By contrast, administration of α-GC didn't suppress arthritis in any of these three models. Suppression of arthritis was associated with the elevation of IgG1:IgG2a ratio indicating the Th2 bias of type II collagen-reactive T cells. Injection of neutralizing antibody to either IL-10 or IL-4 reversed the beneficial effect of OCH treatment. These results imply that IL-10 and IL-4 are critical in the OCH-mediated suppression of CIA and are consistent with our idea that OCH modulated CIA by stimulating the production of Th2 cytokines from NKT cells although the source of IL-10 remains to be elucidated. Since OCH seems a potential therapeutical tool to suppress arthritis, the role of NKT cells in the natural course of arthritis should be clarified in the future.

Glycolipid Therapy for Autoimmune Diabetes in NOD Mice

Nonobese diabetic (NOD) mice develop a spontaneous autoimmune diabetes similar to the human T1D. Autoimmune destruction of β cells is preceded by infiltration of pancreatic islets by macrophages, B cells and T lymphocytes [29,30]. Many studies have indicated that Th1 type $CD4^+$ cells and $CD8^+$ T cells have been implicated in the development of diabetes in the NOD mouse. In parallel with these effector cells, the regulatory cells including NKT cells have been suggested to inhibit the development of diabetes. Although the mechanisms of suppressive effect of these regulatory T cells are not fully understood, it is believed that an imbalance between autoreactive effector T cells and regulatory T cells may trigger the development of destructive insulitis and diabetes [28].

Studies have indicated that NOD mice were deficient in the number and function of NKT cells [31]. Although the correlation between a defect in NKT cells and the susceptibility of diabetes in NOD mice is still debated [3,32,33], the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by prevention of diabetes following infusion of NKT cell enriched thymocytes preparations [34] and by the increase of NKT cells in Vα14Jα281 transgenic NOD mice [35].

Several recent papers investigated the effect of treating NOD mice with α-GC [33,35-38]. When started around three or four weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes. After treatment, splenocytes from NOD mice produced a greater amount of IL-4 in response to islet antigens and the IgG1/IgG2a (Th2/Th1) ratio of anti-GAD antibody increased. Thus it appears that the mechanism of protection is similar to that observed by increasing the numbers of NKT cells in NOD mice and by α–GC treatment in other autoimmune disease models such as EAE and CIA. This effect was auto-antigen specific as no difference was observed in the immune response to ovalbumin [36]. However, the mechanism in which glycolipid treatment induces an auto-antigen specific switch in the immune response of NOD mice is unclear. We also observed the protective effect of OCH treatment in NOD diabetic mice in addition to α-GC treatment. The protective effect for insulitis by OCH was more profound compared to that by α-GC [56].

GLYCOLIPID THERAPY FOR MOUSE MODELS OF SYSTEMIC LUPUS ERYTHEMATOSUS

It has been reported that a selective reduction in $NK1.1^+$ T cells precedes the development of autoimmunity in MLR lpr/lpr mice. Mieza MA *et al.* also found a decrease in the expression of invariant Vα14 TCR mRNA of NKT cells before the onset of lymphocyte accumulation and autoimmune disease in MRL lpr/lpr mice, C3H gld/gld and NB/W F1 mice when compared to control mice [39]. Recently, Zeng D *et al.* demonstrated that treatment of NZB/W F1 mice with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a anti-double-stranded DNA (dsDNA) antibodies, and ameliorated lupus [40]. They also showed that multiple injection of α-GC induced an enhanced Th1-type response and exacerbated lupus associated with decreased serum levels of IgE and increased levels of IgG2a and IgG2a anti-ds DNA antibodies. This exacerbation of disease was associated with reduced IL-4 and tumor necrotic factor-α production and expansion of marginal zone B cells. These results suggested that activation of NKT cells augmented Th1-type responses and autoantibody production that contribute to lupus development in NZB/W F1 mice. In contrast, Yang JQ *et al.* reported that pristine-induced lupus nephritis was accelerated when induced in CD1d deficient mice [41]. They also demonstrated that repeated injection of α-GC resulted in the expansion of NKT cells and ameliorated dermatitis in MRL lpr/lpr mice [42]. Thus they postulated that NKT cells may play a protective role in lupus models. Since lupus models are not simply explained by only Th1-mediated or Th2-mediated pathology, the complexity of these models may explain the differences in results in these studies.