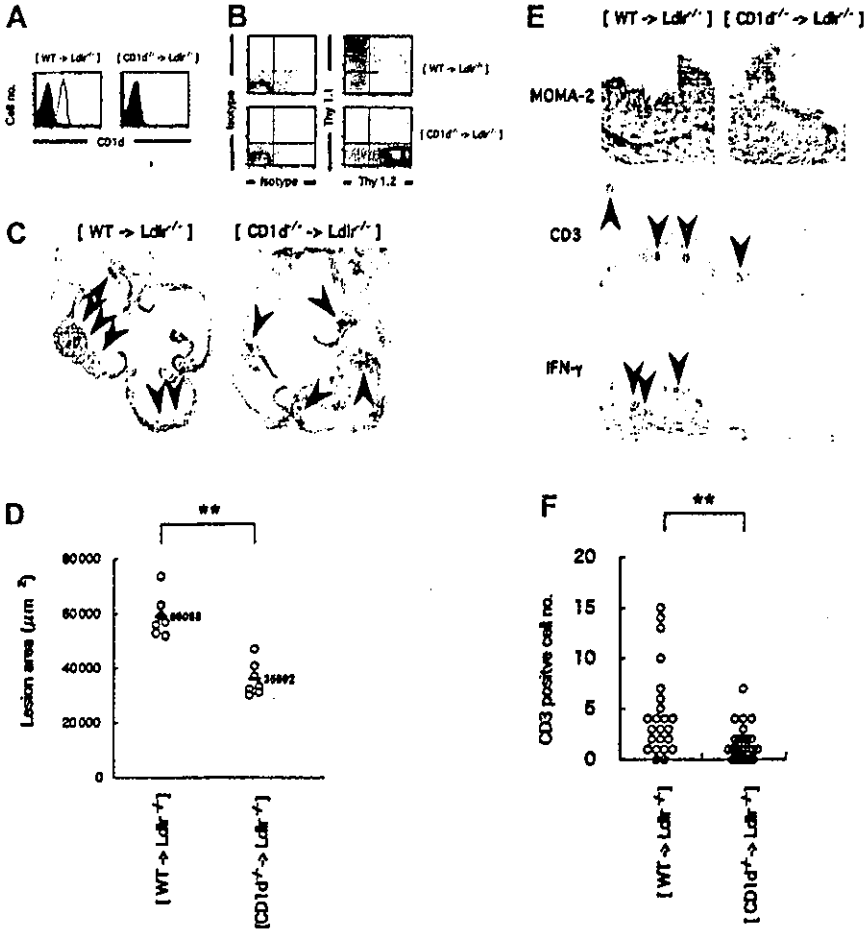

Figure 3. Atherosclerotic lesions in Ldlr$^{-/-}$ mice reconstituted with BM cells from CD1d$^{-/-}$ or WT mice. (A) Representative CD1d expression pattern on thymocytes from [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice. Red lines and filled histograms indicate CD1d staining and isotype control, respectively. (B) Thy1.1 and Thy1.2 expression on thymocytes from [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] chimeras. Representative results of Thy1.1 and Thy1.2 stainings are shown in the right panels with their respective isotype controls (left panels). (C) Representative histologic sections of [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice stained with oil red O (original magnification, × 40). Arrowheads represent oil red O–positive lesions. (D) Lesion area in [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice. Each symbol represents the lesion area of an individual mouse. Horizontal bars and numbers represent the mean of all mice within each group. $^{**}P < .01$ (E) Representative immunohistochemical section of [WT→Ldlr$^{-/-}$] and [CD1d$^{-/-}$→Ldlr$^{-/-}$] mice. Sections were stained with anti-MOMA-2, anti-CD3, and anti-IFN-γ mAb (original magnification, × 200). Arrowheads represent respective mAb-positive cells. (F) Numbers of CD3$^+$ cells per cross-section of lesion area. $^{**}P < .01$.

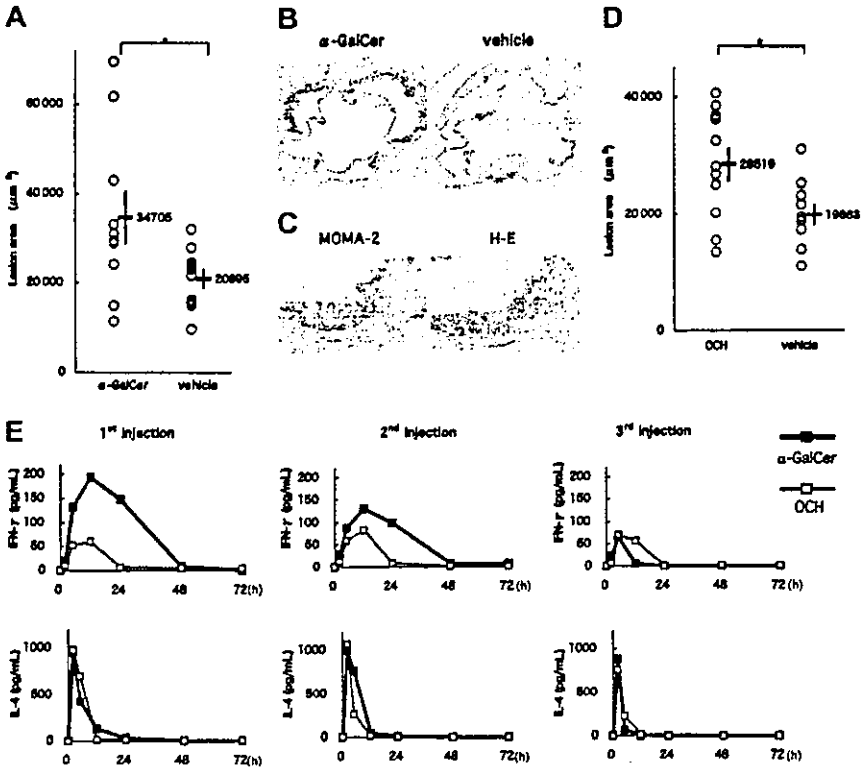

Figure 4. Effects of α-GalCer and OCH on the early phase of atherosclerosis. (A) ApoE$^{-/-}$ mice were intraperitoneally injected 3 times with α-GalCer or the vehicle alone, as described in "Materials and methods." Five weeks later, mice were examined for the development of atherosclerosis. Each symbol represents the lesion area of an individual mouse. Horizontal bars and numbers represent the mean of all mice within each group, and vertical bars represent SEM. (B) Representative histologic sections of the α-GalCer group and its control group stained with oil red O (original magnification, × 40). (C) Representative immunohistochemical section of the α-GalCer group stained with MOMA-2 and a serial section stained with hematoxylin and eosin (original magnification, × 200). (D) ApoE$^{-/-}$ mice were injected with OCH or vehicle. Mean lesion areas (OCH vs vehicle) are indicated as in Figure 4A. (E) Serum concentration of cytokines after administration of α-GalCer or OCH. Mean concentrations (n = 3) of IFN-γ (top) and IL-4 (bottom) in α-GalCer (■) and OCH (□) groups are shown after the first injection (left), the second injection (middle) and the third injection (right). Statistical analyses were performed using the Mann-Whitney *U* test. $^{*}P < .05$.

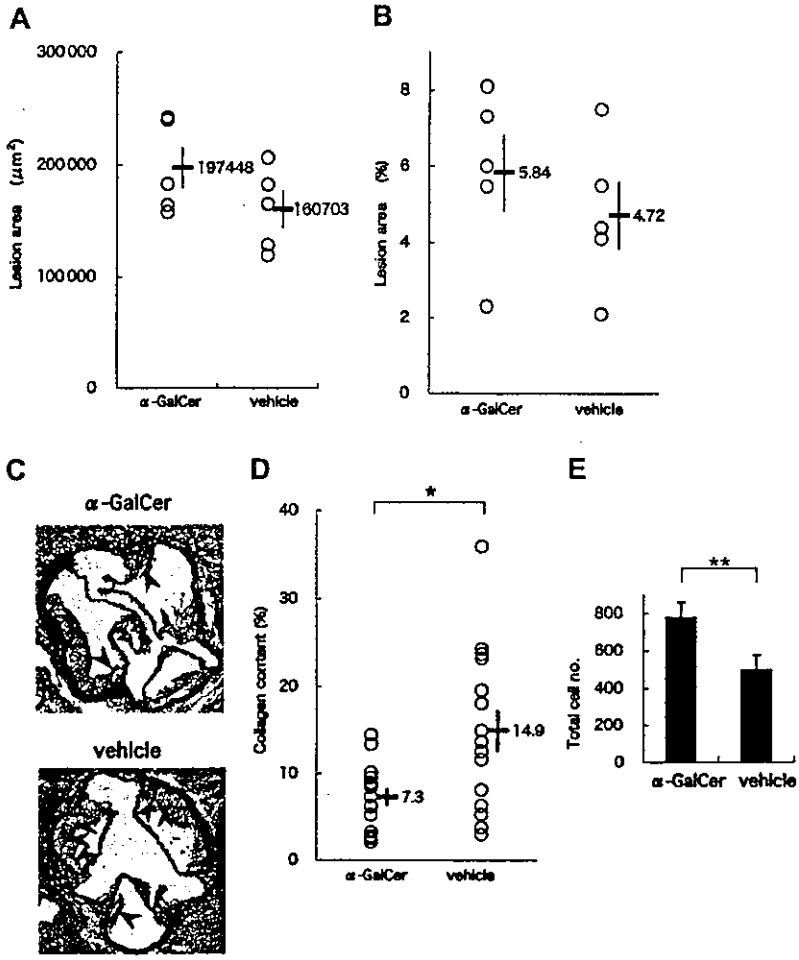

Figure 5. Effect of intensive α-GalCer administration on the late phase of atherosclerosis. ApoE$^{-/-}$ mice were injected weekly with α-GalCer or vehicle for a period of 11 weeks and examined for atherosclerosis at 19 weeks of age. (A) Mean lesion areas of each group are indicated as in Figure 4A. (B) Proportions of the oil red O–positive area to the whole lumen of the entire aorta were assessed by the en face method. (C) Representative histology of aortic sections from the α-GalCer group (top) or the control group (bottom) (Elastica-Masson staining). The collagen content is stained as blue in the lesion. Note that the blue region (arrowheads) in the α-GalCer–treated mouse is smaller than that in the control mouse. Original magnification, × 40. (D) Morphometric analysis of collagen contents of the atherosclerotic lesion. Mean lesion areas staining blue were quantitated with 3 aortic cross-sections per animal from a total of 10 animals. Statistical analyses were performed with the Mann-Whitney *U* test. $^{*}P < .05$. (E) Total cell numbers per cross-section of lesion area. Values are mean ± SE. $^{**}P < .01$.

addition, assessing lesion areas by the en face method showed slightly enlarged lesion areas in the α-GalCer group (5.8% ± 1.0%) compared with the control group (4.7% ± 0.9%) (Figure 5B). These results suggest that activating NKT cells exerts only slight influences on the development of advanced atherosclerotic lesions in apoE$^{-/-}$ mice. These findings are consistent with a previous report suggesting that lymphocytes are mostly involved in the early phase of atherogenesis.[6]

Figure 5C shows representative histologic analyses of the atherosclerotic lesions of α-GalCer– and vehicle-treated animals. Of note, the collagen content stained with Elastica-Masson was smaller in the α-GalCer–treated group than in the control group. When mean collagen content was compared between these 2 groups, the collagen content in the α-GalCer–treated group was significantly smaller than in the control group (7.3% ± 1.1% and 14.9% ± 2.4%, respectively; $P = .040$) (Figure 5D). However, the total cell number within the lesion per slice was significantly larger in the α-GalCer–treated group than in the control group (Figure 5E; $P = .009$). These findings suggest that NKT cell activation in the late phase of atherosclerosis alters the quality of the lesion from one rich in collagen to one characterized by high cellularity.

Vα14Jα18 TCR-α mRNA expression in atherosclerotic lesions of apoE$^{-/-}$ and AD-fed WT mice

Next, we examined atherosclerotic lesions by nested RT-PCR for detection of the invariant Vα14Jα18 TCR-α rearrangement characteristic of classic NKT cells. We were able to amplify the Vα14Jα18 rearrangement in the atherosclerotic tissues of apoE$^{-/-}$ mice and of WT mice on the AD (Figure 6), but we were unable to detect this rearrangement in the aortae of WT mice on the chow diet. Although we were unable to quantify numbers of NKT cells in the lesion, our results clearly demonstrated that the presence of Vα14Jα18-positive cells was restricted to the atherosclerotic lesions.

CD1d expression and IFN-γ production by WT peritoneal macrophages treated with LDL or OxLDL

Classic NKT cells recognize glycolipid antigens in the context of CD1d.[19,21,24-26,32,33] To investigate the mechanism by which NKT cells are activated and promote atherogenesis, peritoneal exudate

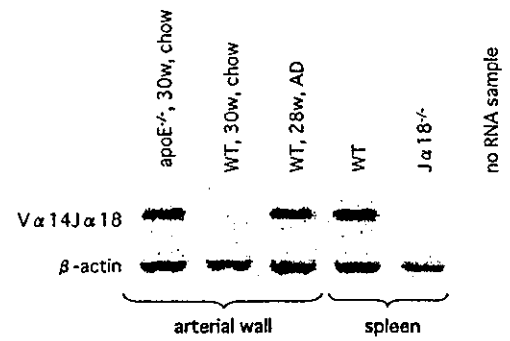

Figure 6. Vα14Jα18 mRNA in the atherosclerotic lesions. Expression of Vα14Jα18 mRNA in the atherosclerotic lesion was examined using RT-PCR. A sample from WT spleen was used as a positive control, and a sample from Jα18$^{-/-}$ spleen was used as a negative control. Note that Vα14Jα18 expression is detected only in the atherosclerotic tissues of apoE$^{-/-}$ mice (on the chow diet) and in WT mice on the AD. Representative result from 3 separate experiments is shown.

macrophages were harvested from WT mice, treated with LDL, OxLDL, or medium alone for 24 or 48 hours, and examined for expression of several surface molecules. The expression of CD1d on WT macrophages was enhanced by incubation with OxLDL for 24 hours, but not with LDL or medium alone (Figure 7A, top). No increase in the expression of MHC class 1 (H-2K^b) molecules was induced on macrophages by OxLDL (Figure 7A, bottom). In addition, OxLDL specifically enhanced CD1d expression in a dose-dependent manner (Figure 7B). No enhancement of H-2K^b, I-A^b or CD40 expression was seen by treatment with LDL or OxLDL. When cultured for a longer time (48 hours) with OxLDL, CD1d expression was further augmented (Figure 7B). Of note, at a high dose (50 μg/mL) and after a long incubation period (48 hours), LDL enhanced CD1d levels on macrophages (Figure 7B).

We then examined whether the enhanced expression of CD1d on OxLDL-treated macrophages related to their capacity to stimulate NKT cells. We mixed HMNCs isolated from WT mice (containing 15%-30% NKT cells) with irradiated peritoneal macrophages from WT or CD1d$^{-/-}$ mice treated with LDL or OxLDL for 48 hours. After culture for 24 hours, IFN-γ and IL-4 levels in the supernatants were quantitated. NKT cells produced significantly higher amounts of IFN-γ in the cultures with OxLDL (50 μg/mL)–treated peritoneal exudate cells from WT mice compared with control cultures (Figure 7C). CD1d$^{-/-}$ macrophages treated in the same manner induced no enhancement of IFN-γ production. No IL-4 was detected in the supernatant in our culture conditions (data not shown).

Discussion

In this study we demonstrate, using 3 atherosclerosis models (apoE$^{+/+}$ mice fed with AD, Ldlr$^{-/-}$ chimeras fed with AD, and apoE$^{-/-}$ mice fed with normal chow), that NKT cells play a significant role in the development of atherosclerosis. In addition, we show that NKT cell activation modulates the disease process. Atherosclerotic lesion areas in AD-fed CD1d$^{-/-}$ mice were significantly smaller than those in AD-fed WT mice (Figure 1B). Because the development of invariant NKT cells is markedly hampered in CD1d$^{-/-}$ mice,[30] our findings suggest that NKT cell deficiency is related to the amelioration of atherosclerosis. It has been reported that AD induces inflammatory cytokines in the liver because of its high concentration of cholesterol and cholic acid[42] and that it may alter physiologic conditions. In the present study, WT and CD1d$^{-/-}$ mice were subjected to AD feeding in an identical manner. These 2 groups of mice showed comparable degrees of liver steatosis and similar levels of serum alanine aminotransferase and total bilirubin. Thus, we conclude that the significant differences in the atherosclerotic lesions between WT and CD1d$^{-/-}$ mice are directly related to the presence and absence, respectively, of the intact CD1d-restricted T-cell population.

The prevalence of NKT cells (mainly the CD1d/α-GalCer tetramer$^+$ fraction) among HMNCs of WT mice substantially decreased through the AD. A slight reduction of NKT cells was also observed in splenocytes of the AD-fed WT mice (Figure 1C). One characteristic of NKT cells is the prominent production of cytokines, IFN-γ, and IL-4 shortly after stimulation of these cells through the TCR.[22,41] We found that, despite their decreased NKT cell numbers, on stimulation with α-GalCer, spleen cells from AD-fed WT mice produced levels of IFN-γ, IL-4, and IL-10 comparable to those from chow-fed WT mice. Of note, the cytokine production pattern of spleen cell cultures of AD-fed WT mice shifted toward a T_H1 profile, especially 2 hours after α-GalCer stimulation (Figure 2). This pattern of cytokine production would be expected to promote atherosclerosis.[10-14] However, the mechanism underlying this altered cytokine production pattern, with the concomitant decrease of NKT cells, remains elusive. One possibility is that the decrease of NKT cells in AD-fed WT mice is caused by a depletion of this population by activation-induced cell death (AICD). This may be mediated by the CD1d-restricted presentation of lipid antigens such as OxLDL, which accumulate during hyperlipidemia. An alternative explanation would be that chronic stimulation of the NKT cell population results in the continuous down-modulation of NK1.1 and TCR marker expression, resulting in the apparent loss of these cells.[43] Furthermore, the diminished population of NKT cells could be attributed to the migration of

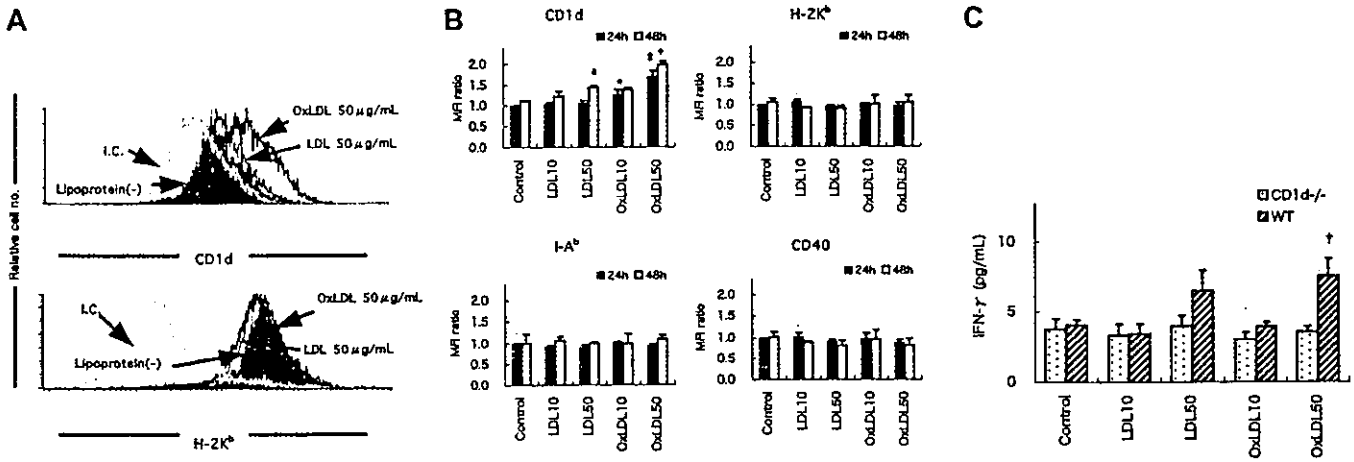

Figure 7. CD1d expression and IFN-γ production by WT peritoneal macrophages treated with LDL or OxLDL. WT peritoneal macrophages were treated with LDL or OxLDL or without additional lipoproteins (control) for 24 hours. (A) Representative histograms of CD1d or H-2K^b expression on the macrophages. I.C. indicates each isotype control for either anti-CD1d or anti-H-2K^b mAb. Cells with PI^{low} and Mac-1^{high} phenotypes were gated for analysis. (B) Mean fluorescence intensity (MFI) for CD1d, H-2K^b, I-A^b, or CD40 staining on WT peritoneal macrophages treated with LDL or OxLDL (10 or 50 μg/mL). Each column represents a ratio of MFI of a respective surface molecule to controls at either 24 hours (closed columns) or 48 hours (open columns). Values are mean ± SE of 3 independent experiments. *$P < .05$ vs control (24 hours). ‡$P < .01$ vs control, LDL10 or LDL50 (24 hours). §$P < .05$ vs control (48 hours). †$P < .01$ vs control, LDL10, LDL50, or OxLDL10 (48 hours). (C) Production of IFN-γ in the supernatant of the mixed culture of HMNCs with CD1d$^{-/-}$ or WT macrophages. HMNCs were cultured for 24 hours with peritoneal macrophages treated with LDL or OxLDL from CD1d$^{-/-}$ or WT mice. Then IFN-γ levels in the supernatant of the mixed culture were analyzed using ELISA. Values are mean ± SE of 3 independent experiments. †$P < .05$ vs control, LDL10, or OxLDL10 (WT). $P < .01$ vs OxLDL50 (CD1d$^{-/-}$).

these cells from liver or spleen to other peripheral tissues, such as the atherosclerotic lesion. In this context, we were able to detect mRNA corresponding to the invariant Vα14Jα18 TCR, which is characteristic of NKT cells and prerequisite for α-GalCer stimulation, within atherosclerotic lesions of AD-fed but not chow-fed WT mice by nested RT-PCR (Figure 6). The mechanisms that lead to NKT cell loss during AD feeding will be further addressed in future studies.

To examine the role of NKT cells in a more advanced atherosclerosis model, we reconstituted lethally irradiated $Ldlr^{-/-}$ mice with BM cells from $CD1d^{-/-}$ or WT mice. It has been reported that lack of LDL receptors aggravates the development of atherosclerosis in AD-fed mice. Indeed, using [WT→$Ldlr^{-/-}$] chimeras, Boisvert et al[44] reported that AD-fed chimeras showed severe atherosclerotic lesions where donor-derived leukocytes were present. In the present study, we observed that significantly large atherosclerotic lesions developed in [WT→$Ldlr^{-/-}$] chimeras compared with [$CD1d^{-/-}$→$Ldlr^{-/-}$] chimeras (Figure 3). These findings demonstrate that NKT cell deficiency indeed ameliorates atherosclerosis in AD-fed animals. Immunohistochemistry studies in this BMT model demonstrated that the number of $CD3^+$ cells within the lesion was significantly larger in [WT→$Ldlr^{-/-}$] than that in [$CD1d^{-/-}$→$Ldlr^{-/-}$] mice, suggesting that these $CD3^+$ cells contain NKT cells. Furthermore, we demonstrated NKT cell (Vα14Jα18) messages in lesions of other animal models by RT-PCR (Figure 6). However, immunohistochemical identification of NKT cells in the lesion has thus far been unsuccessful and will be pursued in future studies.

Complementary to the above results, we showed that activation of NKT cells by α-GalCer or OCH in $apoE^{-/-}$ mice, before significant lesions had been formed (early-phase study), resulted in increased areas of atherosclerotic lesions (Figure 4). We did not expect the results with OCH because it was reported that OCH favors a T_H2 shift of NKT cells.[33] It has been suggested that a T_H2 bias suppresses atherogenesis.[10,11] Consistent with previous reports,[33] we found that a single injection of α-GalCer induced prominent production of IFN-γ and IL-4, whereas OCH induced little IFN-γ but similar levels of IL-4 (Figure 4E). However, after multiple administrations, IFN-γ induction in response to α-GalCer became reduced to levels similar to those for OCH. In contrast, repeated injection of these glycoplipids did not alter IL-4 induction. Although a number of studies support the idea that the T_H1 cytokine IFN-γ is proatherogenic, the precise role of the T_H2 cytokine IL-4 in atherogenesis remains elusive.[45] Our finding that both α-GalCer and OCH exacerbate atherosclerosis during the early stage of the disease process, but to a different degree (Figure 4A, D), may be attributed to differences in the amounts and kinetics of IFN-γ and IL-4 production.

We found that peritoneal exudate macrophages expressed augmented levels of CD1d after culture with OxLDL (either 10 μg/mL or 50 μg/mL) or LDL (50 μg/mL) (Figure 7A-B). Although intact LDL is not captured by macrophages, it is plausible that LDL is degraded by peroxidases released from macrophages during the incubation period and is involved in the enhancement of CD1d expression. Furthermore, these macrophages with high CD1d expression stimulated NKT cells to produce low but significant levels of IFN-γ in vitro (Figure 7C). Thus, the enhancement of CD1d expression on OxLDL-pulsed macrophages appeared to result in their augmented capacity to induce IFN-γ production by HMNCs. This finding may be of importance because physiologically degraded lipids are abundantly present in the atherosclerotic lesions and may provide a source of physiologic ligands for NKT cells.

In the late-phase study to evaluate the effects of α-GalCer on atherosclerosis in $apoE^{-/-}$ mice, α-GalCer administration failed to enlarge lesions but instead decreased collagen content (Figure 5C-D) and increased total cell numbers (Figure 5E) within the atherosclerotic lesions. It has been reported that IFN-γ decreases collagen synthesis[13] and plays a role in plaque stability. Accordingly, it is possible that IFN-γ, which is produced on α-GalCer stimulation, decreases collagen synthesis. Thus, NKT cell activation at the late phase may alter the lesion structure from a stable to an unstable state. Ostos et al[29] demonstrated that LPS-treated $apoE^{-/-}$ mice have larger atherosclerotic lesions than PBS-treated control $apoE^{-/-}$ mice. In atherosclerotic lesions of these LPS-treated mice, increased numbers of IL-4–producing $NK1.1^+$ cells were detected by immunohistochemistry. In our present study, the invariant Vα14Jα18 TCR was detected in aortic specimens with atherosclerotic lesions of either AD-fed WT or $apoE^{-/-}$ mice (Figure 6). These findings again favor the idea that NKT cells play a proatherogenic role in situ. However, it is also possible that NKT cells are activated in other tissues, such as the liver or spleen, and systemically affect the atherogenic process. Thus, the precise location where NKT cells are activated and demonstrate their effector functions during progression of atherosclerosis remains to be elucidated. Although T_H1 and T_H2 cytokines are probably important, other factors, such as chemokines and the capacity of NKT cells to exhibit cytotoxicity, should be considered in further investigations.[23,34] In summary, we have demonstrated that NKT cells accelerate atherogenesis in mouse models for this disease. In addition, we show that NKT cell activation in the early phase of the disease process exacerbates atherogenesis and that NKT cell activation in the late phase of the disease promotes plaque instability. Because NKT cells and CD1d molecules are highly conserved among different species,[46] our present results may be applicable to elucidation of the pathophysiology of atherosclerosis in humans, and they offer a novel approach for controlling the atherogenic process by intervening with certain NKT cell functions.

Acknowledgments

We thank the staffs at Kirin Brewery Company and Takeda Chemical Industries for providing α-GalCer and recombinant human IL-2, respectively. We also thank Keiko Kato and Mizuho Kasai for technical assistance.

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Journal of Autoimmunity 23 (2004) 293–300

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Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice

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Received 27 April 2004; revised 15 September 2004; accepted 29 September 2004

Abstract

Non-obese diabetic (NOD) mice develop diabetes mediated by pathogenic T-helper type 1 (Th1) cells. Vα14 Natural killer (NKT) cells are a unique lymphocyte subtype implicated in the regulation of autoimmunity and a good source of protective Th2 cytokines. We recently developed a Th2-skewing NKT cell ligand, OCH. OCH, a sphingosine truncated derivative of α-galactosylceramide (α-GC), stimulates NKT cells to selectively produce Th2 cytokines. Here we show that OCH prevented the development of diabetes and insulitis in NOD mice. The suppression of insulitis by OCH was more profound compared to α-GC. Infiltration of T cells, B cells and macrophages into islets is inhibited in OCH-treated NOD mice. OCH-mediated suppression of diabetes is associated with Th2 bias of anti-islet antigen response and increased IL-10 producing cells among islet-infiltrating leukocytes. Considering the non-polymorphic and well conserved features of the CD1d molecule in mice and humans, these findings not only support the proposed role of NKT cells in the regulation of self-tolerance but also highlight the potential use of OCH for therapeutic intervention in type I diabetes.

Keywords: NKT cells; Autoimmune disease; Type I diabetes; NOD mice; Glycolipid

1. Introduction

Non-obese diabetic (NOD) mice develop spontaneous autoimmune (type I) diabetes (T1D) very similar to the human disease. In female mice, insulitis usually begins at 3 to 5 weeks of age, eventually leading to β-cell destruction and overt diabetes by 4 to 6 months of age. Autoimmune destruction of β-cells is preceded by infiltration of pancreatic islets by macrophages, B cells and T cells. The capacity to transfer disease by islet specific T cells purified from diabetic NOD mice or T cell clones demonstrates the key role of T cells in the pathogenesis of diabetes. Th1 type CD4 cells, which preferentially secrete IFN-γ and TNF-α and CD8 T cells, have been implicated in the development of diabetes in NOD mice [1,2]. In parallel with these effector T cells, regulatory cells including $CD4^+CD25^+$ T 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 T1D [3]. Previous studies indicate that the β-cell-destructive immune response in NOD mice is biased toward Th1 and treatment with Th2 cytokines such as IL-4 or IL-10 have been shown to prevent the onset of spontaneous diabetes [4–8].

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doi:10.1016/j.jaut.2004.09.008

Natural killer T (NKT) cells are a unique subset of T cells that coexpress receptors of the NK lineage and α/β T cell receptor (TCR) [9–11]. NKT cells express an invariant TCRα chain (encoded by a Vα14-Jα281 rearrangement in mice and a homologous Vα24-Jα15 rearrangement in humans). Unlike conventional T cells that recognize peptides in association with MHC, NKT cells recognize glycolipid antigens such as α-galactosylceramide (α-GC) bound by the non-polymorphic MHC class I-like protein CD1d [12]. One striking feature of NKT cells is their capacity to secrete large amounts of cytokines including IL-4 and IFN-γ in response to TCR ligation. Although the precise function of NKT cells remains to be elucidated, evidence indicates that NKT cells play critical roles in the regulation of autoimmune responses [13–15]. Abnormalities in the number and function of 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. 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 thymocyte preparations [16] and by over-expression of NKT cells in Vα14-Jα281 transgenic NOD mice [17]. Moreover, several recent studies have investigated the effect of treating NOD mice with α-GC [18–21]. When started at around 3 or 4 weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes by inducing Th2 bias of autoreactive T cells. We have recently developed a synthetic glycolipid ligand, OCH, which stimulates NKT cells to selectively produce IL-4. OCH is a synthetic glycolipid, sphingosine truncation analogue of α-GC [22]. Administration of OCH inhibited experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) by inducing Th2 bias of autoreactive T cells [22,23]. These findings led us to examine the effect of OCH on the development of diabetes in NOD mice.

In the present study, we show that OCH can inhibit the development of insulitis and diabetes in NOD mice by inducing a Th2 bias of autoreactive T cells. These results imply that targeting NKT cells with OCH could be an attractive means for intervention in T1D.

2. Materials and methods

2.1. Mice

C57BL/6(B6) mice were purchased from CLEA Laboratory Animal Corp. (Tokyo, Japan). NOD/Shi mice were obtained from CLEA Japan (Tokyo, Japan). The animals were kept under specific pathogen-free conditions. We followed the guidelines for the use and care of laboratory animals of National Institute of Neuroscience, NCNP.

2.2. In vitro responses of NKT cells to α-GC or OCH

Splenocytes of naïve B6 mice were cultured with α-GC or OCH in RPM I1640 medium supplemented with 5×10^{-5} M 2-ME, 2 mM L-glutamine, 100 U/mg/ml penicillin/streptomycin, and 1% syngenic mouse serum for 72 h. Incorporation of [^{3}H]-thymidine (1 μCi/well) for the final 16 h of the culture was determined with a β-1205 counter (Pharmacia, Uppsala, Sweden). The levels of IL-4, IL-10 and IFN-γ in culture supernatant were measured by a standard sandwich ELISA, using purified and biotinylated antibody pairs and standards from BD PharMingen (San Jose, CA).

2.3. In vivo responses of NKT cells to α-GC or OCH

NOD mice were injected with 100 μg/kg of OCH or α-GC intraperitoneally and serum were collected after 2 h, 6 h and 24 h after injection. Serum levels of IL-4, IL-10 and IFN-γ were measured by ELISA.

2.4. Assessment of diabetes and evaluation of insulitis

Diabetes was assessed by monitoring glucose levels every week in the blood using GLU-W (Fujifilm, Kanagawa, Japan). Mice with two consecutive positive blood glucose measurements greater than 250 mg/dl were considered diabetic. For histological evaluation of insulitis, mice were killed and pancreata were removed and fixed with 4% paraformaldehyde. Sections were stained with hematoxylin and eosin (HE). Multiple HE stained pancreatic sections were scored. Insulitis was graded as follows: grade 0, no inflammation; grade 1, peri-insulitis but no intra-insulitis; grade 2, mild intra-insulitis (cell infiltration in the area less than 25% of an islet); grade 3, moderate intra-insulitis (cell infiltration in the area more than 25% but less than 50% of an islet); and grade 4, severe intra-insulitis (cell infiltration in the area more than 50% of an islet).

2.5. Immunohistochemistry

Immunohistochemistry was performed on 15-mm-thick adjacent serial sections according to the avidin–biotin–peroxidase complex (ABC) method using ABC Elite (Vector) and 3,3′-diaminobenzidine tetrahydrochloride (DAB) as chromogen. Primary antibodies were used as follows; mouse CD4 (RM4-5, BD PharMingen), mouse CD8a (53-6.7, BD PharMingen), mouse B220 (RA3-6B2, BD PharMingen), mouse F4/80 antigen (A3-1, Serotec), mouse CD45 (sc-1121, Santa Cruz

Biotechnology Inc.), mouse interleukin-4 (11B11, ATCC), mouse interleukin-10 (JES-2A5), and mouse interferon-γ (XMG1.2, BD PharMingen). The biotinylated goat anti-mouse antibody or the biotinylated rabbit anti-goat antibody (Vector, Burlingame, CA, USA) was used as a secondary antibody. Cell infiltration was graded as follows: no infiltration, peri-infiltration, cell infiltration in peri-islet area but no intra-islet; mild infiltration, cell infiltration in less than 50% of the area of an islet; severe infiltration, cell infiltration in more than 50% of the area of an islet. For the quantification of cytokine staining, more than 100 CD45 positive cells per mice (five animals in each group) were evaluated for the IL-4 or IL-10 staining.

2.6. In vivo glycolipids treatment

α-GC and OCH were synthesized as described previously [24]. Synthetic glycolipids were used to treat NOD mice. Starting from 5 weeks of age, mice were injected intraperitoneally twice per week with either OCH or α-GC at a dose of 100 μg/kg. The control mice were injected with vehicle alone (10% DMSO in PBS).

2.7. Measurement of autoantigen specific IgG1 and IgG2a

Anti-glutamic acid decarboxylase (GAD) was measured by ELISA as described previously [19,25]. GAD (Sigma) (1 mg/ml) was coated onto ELISA plates (Sumitomo Bakelite, Co., Ltd, Tokyo, Japan) at 4 °C overnight. After blocking with 1% bovine serum albumin in PBS, serially diluted serum samples were added onto GAD-coated wells. For detection of anti-GAD antibodies, the plates were incubated with biotin-labeled anti-IgG1 and anti-IgG2a (Southern Biotechnology Associates, Inc., Brimingham, AL) or anti-IgG antibody (CN/Cappel, Aurora, OH) for 1 h and then incubated with streptavidin-peroxidase. After adding a substrate, the reaction was evaluated and antibody titers were calculated on the basis of dilution/absorbance curves. To control the experiments, dilutions of anti-GAD-positive serum from diabetic NOD mice were used as the control samples. Based on the standard values of the control samples, the relative value for each test sample was displayed.

3. Results

3.1. OCH induces selective production of Th2 cytokines in NOD mice

NOD mice have been reported to exhibit the defect in the number and the function of NKT cells [26]. We first examined whether OCH stimulates proliferation and selective IL-4 production in NOD mice. Spleen cells from NOD mice proliferated in response to in vitro stimulation with OCH and produced significant amounts of IL-4 and IL-10, although OCH was less active in inducing cell proliferation and the cytokine production compared with α-GC (Fig. 1A). In contrast, IFN-γ was barely detectable in response to OCH stimulation, whereas α-GC induces massive IFN-γ production (Fig. 1A). We next examined whether OCH treatment in vivo also induces the selective Th2 cytokines. We injected OCH or α-GC intraperitoneally in NOD mice and measured the serum level of IL-4, IL-10 and IFN-γ by ELISA. Consistent with in vitro data, OCH injection induced a rise in IL-4 and IL-10 (Fig. 1B, left and right panels) along with much less increase in the levels of IFN-γ (Fig. 1B, middle panel). In contrast, injection of α-GC induced the production of IL-4, IL-10 and IFN-γ. These results suggest that OCH preferentially induces Th2 cytokines in NOD mice similar to B6 mice.

3.2. OCH treatment prevents diabetes and insulitis in NOD mice

IL-4 and IL-10 have been reported as important protective cytokines for diabetes in NOD mice [4–8,16]. Thus, we tested whether multiple injections of OCH to NOD mice can modulate the development of diabetes. As shown in Fig. 2A, treatment of OCH starting at 5 weeks of age significantly delayed the onset and reduced the incidence of diabetes from 75% to 27% in female NOD mice at 30 weeks of age. α-GC inhibited the development of diabetes as well as previously described [18–21].

Next we examined HE-stained sections from pancreata of mice treated with either glycolipid ligands or vehicle twice per week from the age of 5 weeks. As shown in Fig. 2B, significantly greater percentages of islets were free of insulitis from OCH-treated mice than those from the control mice. Fifty percent of islets were free of infiltrating cells in OCH-treated mice, at which time less than 20% of islets were from vehicle-treated mice. The percentage of islets affected by severe insulitis (grade 4) was significantly lower ($p = 0.023$, Dunnett's multiple comparison tests) and the frequency of intact islet (grade 0) was significantly higher ($p = 0.0038$, Dunnett's multiple comparison tests) in OCH-treated mice than in α-GC treated mice. The mean score of insulitis of α-GC- or OCH-treated mice was significantly lower than vehicle-treated mice. Typical histological appearance was shown in Fig. 2C. These results indicate that the inhibitory effect of insulitis by OCH is stronger than α-GC even though OCH was about less active in inducing NKT cell proliferation (Fig. 1A). We further examined whether a particular subset of cells are preferentially affected by OCH treatment at the age of

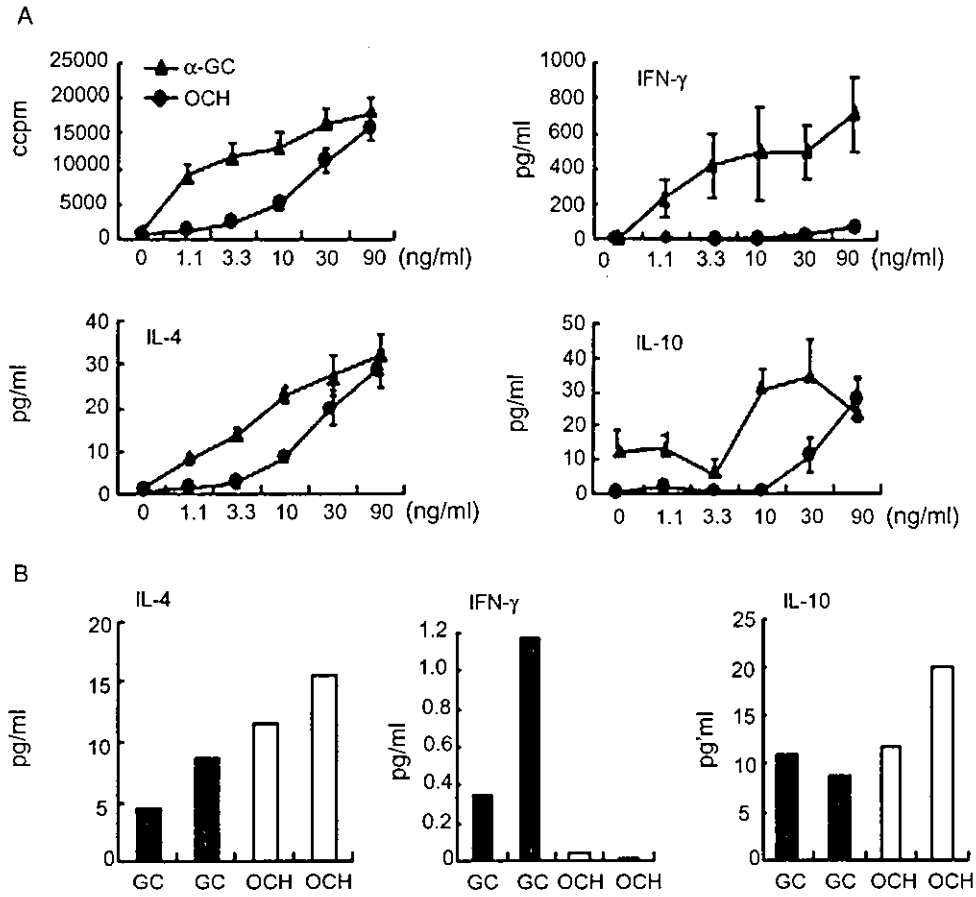

Fig. 1. Effects of OCH on cytokine production in NOD mice in vitro and in vivo. (A) Spleen cells were stimulated with various concentrations of glycolipid for 72 h. Incorporation of [^{3}H]-thymidine (1 μCi/well) for the final 16 h of the culture was examined (left top) and IFN-γ (right top), IL-4 (left bottom) or IL-10 (right bottom) contents in the supernatants were measured by ELISA. (B) Serum levels of IL-4, IFN-γ, IL-10 in NOD mice after intraperitoneal injection of OCH or α-GC were measured by ELISA. Data represent the individual mice and the peak level of each cytokine (2 h for IL-4, 24 h for IFN-γ and 6 h for IL-10) was displayed. Black bar: α-GC treated mice; white bar: OCH-treated mice.

16 weeks (Fig. 3). Massive infiltration of CD4 T cells, CD8 T cells, B cells into islets was observed in vehicle-treated NOD mice. Macrophages were also observed in vehicle-treated NOD mice. In contrast, OCH treatment inhibited infiltration of both T cells and B cells, and macrophages were barely detectable in OCH-treated mice. These results indicated that administration of OCH inhibited the development of diabetes and insulitis in NOD mice and the suppressive effect of insulitis is stronger than α-GC.

3.3. Administration of OCH promotes a Th2 response against autoantigens

To evaluate effects on immune responses against autoantigen, we measured GAD-specific IgG1 and IgG2a in the serum at the age of 24 weeks (Fig. 4, left and middle panels). The level of IgG2a was reduced both in OCH- and α-GC-treated mice compared to vehicle-treated mice. IgG1/IgG2a ratio significantly increases in OCH-treated mice. In α-GC-treated mice, IgG1/IgG2a ratio also tended to increase, even though the difference did not reach statistical significance. These results suggest that anti-GAD response in OCH-treated NOD mice shifted to Th2 response, and Th2 bias of autoantigen was more evident in OCH-treated mice compared to α-GC-treated mice.

3.4. OCH treatment increased IL-10 producing cells among islet-infiltrated leukocytes in NOD mice

Since anti-GAD response shifted to Th2 in OCH-treated mice, we next examined the numbers of IL-4 or IL-10 producing cells among the infiltrating leukocytes. IL-10 producing cells among CD45 positive leukocytes were significantly increased in OCH-treated mice compared to vehicle-treated NOD mice (Fig. 5). IL-4 producing cells were not significantly increased in OCH-treated mice. The frequency of either IL-4- or IL-10-producing cells among leukocytes in α-GC-treated mice were not significantly different from vehicle-treated mice. These results suggest that OCH-mediated

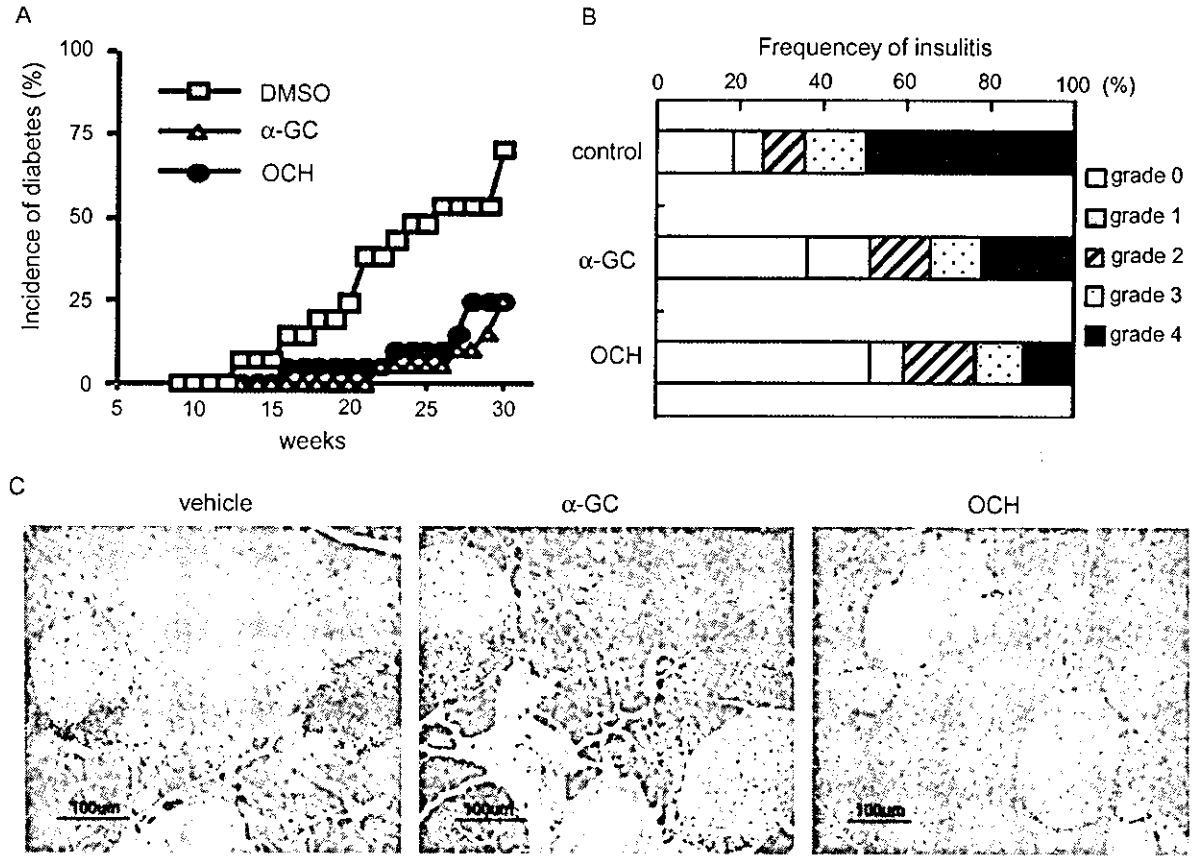

Fig. 2. Prevention of diabetes and insulitis in NOD mice by OCH treatment. (A) The effect of OCH on the incidence of diabetes. Female NOD mice were injected with OCH, α-GC or vehicle twice per week starting at 5 weeks of age ($n = 20$ per group). Diabetes was monitored by measurements of blood glucose levels. (B) Female NOD mice were treated with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. Pancreata were microscopically evaluated for the degree of insulitis at 16 weeks of age. Data shown represent the mean of six animals in each group and more than 20 islets per mice were scored for grading as described in Section 2. The mean score of insulitis of vehicle-, α-GC-, or OCH-treated mice was 1.56, 1.24, 2.7 ($p < 0.001$; vehicle vs α-GC, $p < 0.001$; vehicle vs OCH, $p < 0.0081$; α-GC vs OCH). (C) Representative histological appearance of hematoxylin and eosin-stained islets from non-diabetic female NOD mice treated with vehicle, α-GC, or OCH used in the experiments shown in (B).

inhibition of insulitis is associated with increase of IL-10 producing cells.

4. Discussion

In this study, we found that OCH treatment prevented spontaneous autoimmune diabetes in NOD mice, a model of human type I diabetes, by inducing Th2 bias. OCH treatment strongly inhibited insulitis in NOD mice. The proportion of IL-10 producing cells among infiltrated leukocytes was increased in OCH-treated mice. Although the correlation between a defect in NKT cells and the susceptibility of diabetes in NOD mice is still debated [13–15], the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by the prevention of diabetes following an infusion of NKT cell-enriched thymocytes [16], and by the increase of NKT cells in Vα14-Jα281 transgenic NOD mice [17]. In these mice, protection from diabetes by NKT cells is associated with the induction of a Th2 response to islet autoantigens. Furthermore, α-GC treatment has been demonstrated to delay the onset and reduce the incidence of diabetes in NOD mice [18–21]. And the mechanism of protection has been reported to be associated with Th2 shift of autoantigen response, which is similar to that observed by increasing the number of NKT cells in NOD mice. The mechanism of OCH-mediated inhibition of insulitis was also associated with Th2 polarization of the autoantigen response and increase of IL-10 producing cells. The stronger suppression of insulitis by OCH treatment than α-GC treatment may not be surprising because α-GC stimulates NKT cells to produce IFN-γ as well as Th2 cytokines. IFN-γ enhances the expression of major histocompatibility complex class I and II molecules as well as several other proteins involved in antigen processing and presentation, and supports the homing of activated T cells into islets in NOD mice [27–29].

Stimulation of NKT cells by injection of OCH inhibited EAE and CIA [22,23]. On the other hand,

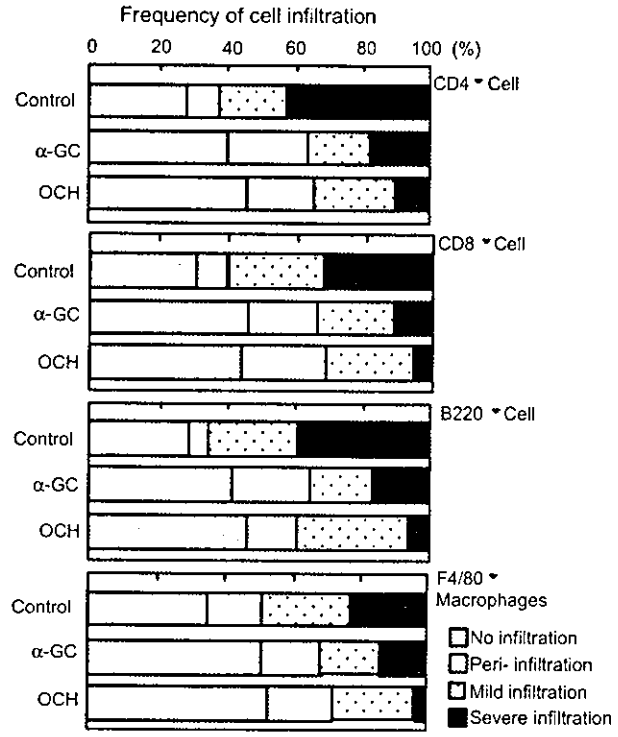

Fig. 3. Immunohistochemical analysis of cell composition of infiltrated cells into islets in NOD mice treated with OCH. Female NOD mice were treated with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. At 16 weeks of age, pancreata were microscopically evaluated for the degree of infiltrated cells positive for CD4, CD8, B220 or F4/80 antigen as described in Section 2. Data shown represent the mean of six animals in each group and more than 20 islets per animal.

α-GC ameliorates or exacerbates EAE, depending on the strain of mouse and stage of disease tested [30–32] and to have only a marginal effect on CIA [23], probably because α-GC induces both Th1 and Th2 cytokines whereas OCH predominantly elicits a Th2 response. In this situation, treatment with OCH might be preferable to α-GC for Th1-mediated diseases. Although the insulitis was severe in α-GC-treated mice compared to OCH-treated mice, we confirmed that multiple injection of α-GC inhibited diabetes in NOD mice as well as OCH

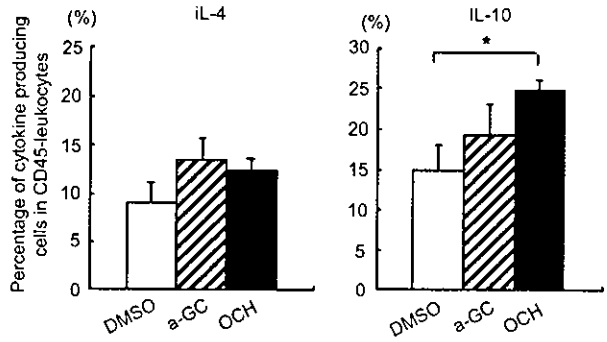

Fig. 5. Immunohistochemical analysis of IL-4 or IL-10 producing cells infiltrated into islets in NOD mice treated with OCH. Female NOD mice were injected with OCH, α-GC or vehicle twice per week starting at 5 weeks of age. Immunohistochemical examination was performed at 16 weeks of age. Data shown represent the mean of six animals in each group and more than 100 $CD45^+$ leukocytes per mice were evaluated. IL-4 positive or IL-10 positive cells were expressed as percentage of $CD45^+$ total leukocytes. $^*p = 0.017$ vs control, by Dunnett's multiple comparison tests.

as previously reported [18–21]. There might be several reasons to explain that the effect of OCH was not different in the inhibition of overt diabetes. For example, it has been documented that no modification of the diabetes incidence occurs in both IFN-γ knockout and IFN-γ receptor knockout mice, as compared with littermate controls. Therefore α-GC induced IFN-γ has less effect to mask the protective effect of Th2 cytokines in NOD mice, which is different to other models such as EAE or CIA. The other possibility to explain that OCH and α-GC showed similar effect on the development of overt diabetes is that diabetes in NOD mice develops spontaneously and multiple injection of α-GC or OCH were continued for several months, which are different from other inducible autoimmune models. The adjuvant used for the induction of other models such as EAE and CIA might have some effect on the cytokine production by NKT cells. Considering that the bacterial infection has been shown to induce predominantly Th1 cytokines from NKT cells [33], injection of α-GC at the same time the injection of CFA including mycobacterium extract could enhance Th1 cytokine production from NKT cells

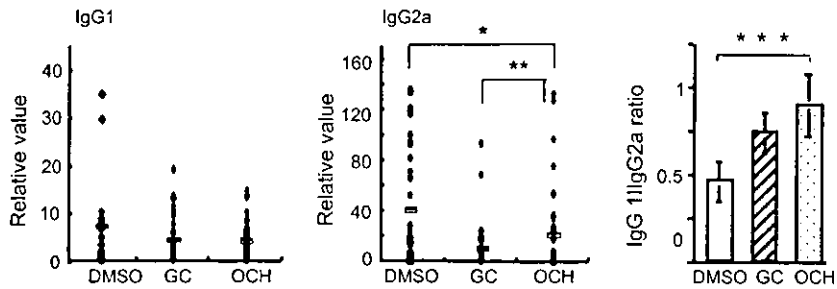

Fig. 4. GAD-specific antibody isotype levels in mice treated with OCH. Individual serum samples obtained from mice shown in Fig. 2 at 24 weeks were analyzed as in Section 2. IgG1:IgG2a ratios in OCH-, α-GC-, vehicle-treated mice are shown. Values are the mean and SEM ($n = 20$). $^*p = 0.0327$, $^{***}p = 0.0147$, OCH vs control, $^{**}p = 0.0002$, α-GC vs control by Mann–Whitney U test.

enough for the mask of the inhibitory effect of Th2 cytokines. In contrast, in NOD mice which spontaneously develop diabetes, Th1 cytokine production from NKT cells for the initial treatment might not be so harmful because NKT cells have been reported to have the tendency to predominantly produce Th2 cytokines after repeated injections [34] since the glycolipid treatment was continued for several months.

It is still controversial whether the defects in NKT cells are causal for autoimmune disease or occur as a secondary consequence of the autoimmune process [13–15]. However, given the efficacy of glycolipid ligands such as OCH in the prevention of the development of diabetes in NOD mice, in addition to the suppression of EAE and CIA, stimulation of NKT cells with glycolipid seems to be an attractive strategy for the treatment of autoimmune diseases such as type I diabetes. The evolutionary conservation and the homogeneous ligand specificity of NKT cells allow us to apply a glycolipid ligand like OCH for the treatment of human disease without considering species barrier or genetic heterogeneity of humans.

Acknowledgements

This work was supported by the Pharmaceuticals and Medical Devices Agency (PMDA), Grant-in-Aid for Scientific Research (B) 14370169 from Japan Society for the Promotion of Science, Uehara Memorial Foundation, Kato Memorial Bioscience Foundation and Kanae Foundation for Life & Socio-Medical Science.

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NKT Cell-Stimulating Synthetic Glycolipids as Potential Therapeutics for Autoimmune Disease

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Abstract: Although T cells were previously believed to recognize only peptide antigen associated with the major histocompatibility complex (MHC), recent studies have shown that there are unique T cells specialized for recognition of lipid or glycolipid antigens bound to the MHC class I-like CD1 molecules (CD1a, b, c or d). Among these lipid-specific T cells, CD1d-restricted T cells, also referred to as natural killer (NK) T cells, are of special interest as a target of drug development, since their role in immunoregulation has been indicated in various physiological or disease conditions including autoimmunity. They are unique in their homogeneous ligand specificity for α-glycosylated sphingolipid and secrete large amounts of regulatory cytokines shortly after T cell receptor (TCR) engagement. The first glycolipid identified as an NKT cell ligand was α-galactosylceramide (α-GalCer) derived from marine sponges. α-GalCer exhibits significant immunomodulatory effects by stimulating NKT cells. However, we found that an altered analogue of α-GalCer with a shorter sphingosine chain (OCH), is more useful than α-GalCer for treatment of autoimmune disease models, because of its ability to selectively induce IL-4, a key cytokine for control of autoimmunity. As such, altered glycolipid ligands (AGL) of α-GalCer appear to be promising reagents for treatment of human autoimmune diseases.

INTRODUCTION

Autoimmune diseases such as multiple sclerosis (MS) and type I diabetes mellitus remain a major health problem in the 21st century that provide a fundamental challenge for drug development. The list of currently available drugs for MS includes interferon-β and copolymer 1 (Cop 1) for long-term management of the disease [1,2]. However, these drugs have only limited value at best, as they cannot halt the progression of neurological disability in a majority of the patients with MS.

Understanding the immunological mechanisms underlying MS has been greatly facilitated in the past two decades due to studies on an animal model for MS, experimental autoimmune encephalomyelitis (EAE) [3,4]. In addition, clinical trials of cytokines [5] and synthetic peptides [6] have given us deep insights into the pathogenesis of MS. A large body of evidence now supports the view that MS is an autoimmune disease, in which autoimmune T cells play a central role in mediating the inflammatory process within the central nervous system (CNS) [7,8]. The pathogenic autoimmune T cells involved in EAE/MS are known to produce interferon-γ (IFN-γ), IL-2 and tumor necrosis factor (TNF)-β when properly activated with antigen. This pattern of cytokine production would define the pathogenic autoimmune T cells as being T helper type 1 (Th1) cells. Of note is that Th1 cells and Th2 cells (the latter secreting IL-4, IL-5, IL-10 and IL-13) cross-regulate each other via secreting cytokines and that the cytokine milieu is critical for inducing Th1 or Th2 subsets from naive T cells. A previous observation that IFN-γ treatment induced exacerbation of MS [5] is now interpreted as a strong evidence for the role of Th1 cells in MS, given that IFN-γ would augment the activity of this T cell subset.

Regarding the target antigen for the Th1 cells mediating MS pathology, myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) have been explored as the prime candidates [9,10,11]. Immunization of susceptible laboratory animals with these antigens induces development of EAE, characterized by ascending limb paralysis with inflammatory demyelinating lesions in the CNS. In a recent clinical trial, a peptide analogue of the immunodominant sequence of MBP has caused clinical exacerbation in a proportion of the patients [6], although this peptide was expected to halt disease progression. This unsuccessful trial proved that T cells responding to the MBP mimic, that are most probably MBP-reactive autoimmune T cells, play an important role in these patients.

To control pathogenic Th1 cells by inducing Th2 bias, infusion of Th2 cytokines could be considered for clinical use. However, clinical trials of recombinant cytokines, except for IFN-β, have mostly failed because of accompanying side effects. Given a physiological role of each cytokine *in vivo*, cytokine therapy should work if an optimal amount of the cytokine is delivered selectively to the inflammatory lesions. To this end, non-pathogenic autoimmune T cells that can accumulate in the inflammatory lesions are being considered as a potential vehicle for cytokine delivery. Indeed, studies have documented that

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1568-0266/04 $45.00+.00

autoimmune T cells transfected with the genes encoding anti-inflammatory cytokines can suppress autoimmune inflammation [12,13]. However, this strategy seems to be impractical unless major technical advances in culturing autoimmune T cells *in vitro* take place.

REGULATORY CELLS AS TARGETS FOR DRUG DEVELOPMENT

Although self-reactive T cells are eliminated in the selection process in the thymus, it is now established that the negative selection for the potentially dangerous T cells (central tolerance) is not perfect; in fact autoimmune T cells represent a normal component of the T cell repertoire [14]. To maintain good health and to avoid development of autoimmune diseases, the autoimmune T cells exported from the thymus have to be properly controlled in the periphery by a mechanism that would protect against tissue injury mediated by autoimmune attack. Such control is referred to as peripheral tolerance. It is now widely accepted that regulatory cells play essential roles in peripheral tolerance. Natural killer (NK) T cells [15,16] on which we will focus in this review are within the up-dated list of regulatory cells, along with $CD25^+CD4^+$ T cells [17], and NK cells [18]. Because regulatory cells would control harmful auto-immunity in a highly sophisticated manner mainly via producing cytokines, it is an attractive therapeutic strategy to induce or strengthen the regulatory cells and let them produce cytokines in the relevant sites. Peptides have been exploited for inducing regulatory T cells in EAE and MS, which proves the feasibility but has also revealed the potential problems for clinical use [6, 19].

PROPERTIES AND ROLES OF NKT CELLS

NKT cells are a minor subset of lymphocytes that were classically defined as cells expressing both T cell receptor (TCR) and NK cell markers (such as NKRP1). Studies have revealed that the majority of the NKT cells are reactive to α-galactosylceramide (α-GalCer) bound to CD1d molecule. α-GalCer is a glycosylceramide containing an α-anomeric sugar with a longer fatty acyl chain (C_{26}) and sphingosine base (C_{18}) (Fig. (1)). The α-GalCer-reactive NKT cells have been most intensively studied in the past decade [reviewed in 15,16]. Here we focus on the glycolipid-specific NKT cells and the term "NKT cells" will be used for this cell type below.

As reflected by the name, NKT cells have unique properties that are intermediate between those of innate and acquired immunity. Here we point out just two of these: the

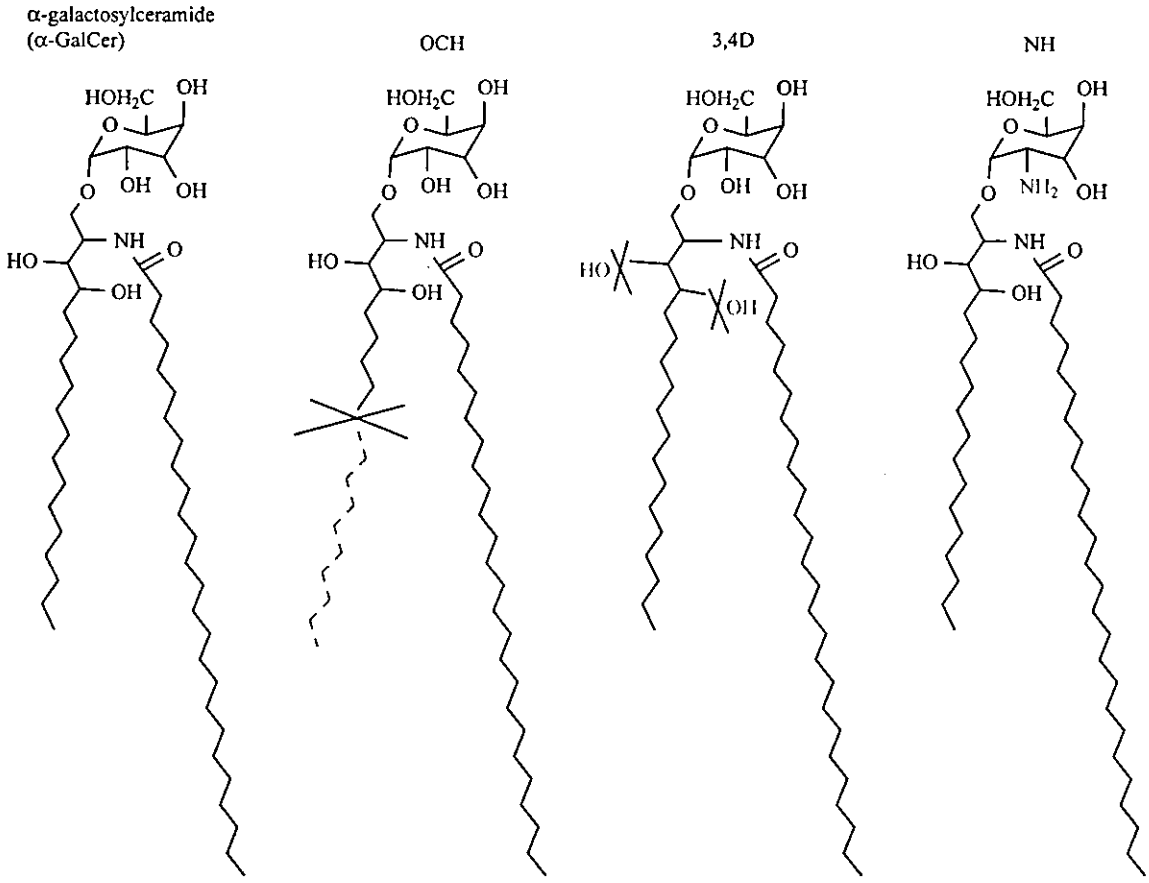

Fig. (1). Structure of α–galactosylceramide and its derivatives. Previous studies [23,24] showed that the 2-hydroxyl group of the galactose moiety and the 3,4-hydroxyl groups of the phytosphingosine are critical for NKT cell recognition of **α–GalCer**. The 2-hydroxyl group is replaced by NH_2 in the **NH** analogue, whereas the 3,4-hydroxyls are removed in the **3,4D** analogue. The **OCH** analogue has a shorter sphingosine chain compared with α-GalCer.

semi-invariant TCR expression and the rapid production of large amounts of cytokines. The TCR of NKT cells is composed of the invariant α-chain [Vα14-Jα281 in mice; Vα24-JαQ in human] and the β-chain that is heterogeneous but uses a selective gene segment [Vβ8.2 or Vβ7 segment in mice; Vβ11 in humans]. This restricted TCR expression is consistent with their homogeneous specificity for glycosylceramide bound to the non-polymorphic CD1d molecule. Upon TCR engagement, the NKT cells would rapidly produce large amounts of IL-4 and IFN-γ. The immunological role of NKT cells has been evaluated intensively, making use of NKT cell-deficient mice (CD1d knockout or TCR Jα281 knockout) or NKT cell TCR transgenic mice. Nowadays, it is widely recognized that they play a critical role in tumor rejection, regulation of autoimmune diseases, protection against infection, and tolerance induction [15,16]. Of interest, the number of NKT cells are greatly reduced in human autoimmune diseases, such as type I diabetes and MS [20,21], suggesting that NKT cells can be a target for treatment of autoimmune diseases.

GLYCOLIPID LIGANDS FOR NKT CELLS

α-GalCer, the representative ligand for NKT cells, was first isolated from the marine sponge *Agelas Mauritanius* [22]. Synthetic α-GalCer [(2S, 3S, 4R)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol] and its derivatives were later used to study glycolipid recognition by NKT cells (Fig. 1). In the pioneering work by Masaru Taniguchi and his colleagues [23], it was shown first that ceramide itself or β-galactosylceramide (β–GalCer) do not induce proliferation of mouse NKT cells, indicating that α-anomeric conformation of the sugar moiety is essential for the glycolipid to act as an efficient ligand for NKT cells. It is of note that α–linked glycosphingolipids have not been found in mammalian cells and therefore, α–GalCer and its derivatives are not natural ligands for NKT cells. Among α-linked glycosphingolipids examined, α-GlcCer [(2S, 3S, 4R)-1-*O*-(α-D-glucopyranosyl)–*N*-hexacosanoyl-2-amino-1,3,4-octadecanetriol] was stimulatory for NKT cell proliferation, but α-ManCer [(2S, 3S, 4R)-2-amino-*N*-hexacosanoyl-1-*O*-(α-D-mannopyranosyl)-1,3,4-octadecanetriol] was not. This indicates that although the 4-hydroxyl configuration of the sugar may not be important, the 2-hydroxyl group is probably critical for contact with the TCR. Taniguchi *et. al.* also proved that 3,4-hydroxyl groups of the phytosphingosine are important. NKT cells also recognized diglycosylated ceramides such as Galα1-6Galα1-1'Cer. Of note, the α-anomeric configuration of the inner sugar was critical for NKT cell recognition, but that of the outer sugar was not.

Comparison of α-GalCer with its derivatives having a shorter hydrophobic chain demonstrated that truncation of the fatty acyl chain as well as that of the sphingosine base reduces the stimulatory activity of the sphingolipid [23]. In contrast, Brossay *et al.* reported that an α–GalCer derivative with a very short fatty acyl chain (C_2) was still efficient in stimulating mouse NKT cell hybridomas [24]. The discrepancy in the results can be explained by the differences in the properties of hybridoma cells used by Brossay *et. al.* [24] and the freshly isolated NKT cells from NKT transgenic mice used in the prior study [23]. Shortly after α-GalCer was identified as a ligand for mouse NKT cells, it was found that human Vα24 NKT cells would also recognize α-GalCer bound to CD1d [25,26]. This striking conservation of human and mouse NKT cells in recognition of the glycolipid/CD1d complex may indicate the essential role of NKT cells in mammalian species.

Besides α-GalCer and its derivatives, natural glycosylphosphatidylinositols (GPI) [27] and phospholipids [28] have been reported to stimulate NKT cells. It is interesting to postulate that GPI recognition by NKT cells may help trigger antibody production and contribute to eradicating parasite infection. However, there are still controversies regarding the NKT recognition of GPI or phospholipids. We need to explore these possibilities more intensively in the future.

TREATMENT OF EAE WITH α–GALCER

EAE is the prototype Th1 autoimmune disease model that helps evaluation of new therapeutic compounds designed for autoimmune disease. Given the property of NKT cells to produce IL-4, we speculated that α–GalCer might protect against development of EAE by inducing IL-4 production by NKT cells. To evaluate preventive effects of α-GalCer on EAE, we induced EAE in C57BL/6 (B6) mice by immunizing with MOG 35-55 peptide [29]. Although we tried protocols with varying doses of α-GalCer or different timing of injection, we did not observe any significant effect of the synthetic glycolipid on the clinical course of EAE. Of note, α-GalCer did strongly stimulate NKT cells and induced cell proliferation as well as IL-4 production. However, it also induced IFN-γ production by NKT cells (Fig. 2, middle). We postulated that α-GalCer could not prevent EAE because the therapeutic effect of IL-4 was neutralized by the IFN-γ simultaneously produced by NKT cells. We showed several lines of evidence supporting this idea [29]. First, we found that α-GalCer would inhibit EAE induced in IFN-γ knockout mice whose NKT cells are unable to produce IFN-γ but could produce IL-4. Secondly, α-GalCer was found to augment the clinical signs of EAE induced in IL-4 knockout mice, whose NKT cells would produce IFN-γ but not IL-4. Thirdly, we showed that stimulation of NKT cells with α-GalCer in the absence of CD28/B7.2 co-stimulation would lead to selective IL-4 production. Injection of α-GalCer-pulsed spleen cells whose B7.2 expression was blocked by antibody led to the suppression of EAE in wild-type mice. As such, EAE could be prevented when ligand stimulation would lead to selective production of IL-4 by NKT cells *in vivo* (Table 1).

ALTERED GLYCOLIPID LIGANDS AND THEIR IMMUNOMODULATORY EFFECTS

Conventional T cells are known to change their pattern of cytokine production, when they are triggered with a suitably altered ligand. Such a ligand, referred to as altered peptide ligand (APL), generally has an alternative residue at a critical site(s) responsible for TCR contact. Studies showed that APLs of MBP could change MBP-reactive Th1 T cells into harmless and disease-protective Th2 T cells [6,30]. Given this information, we hypothesized that there might

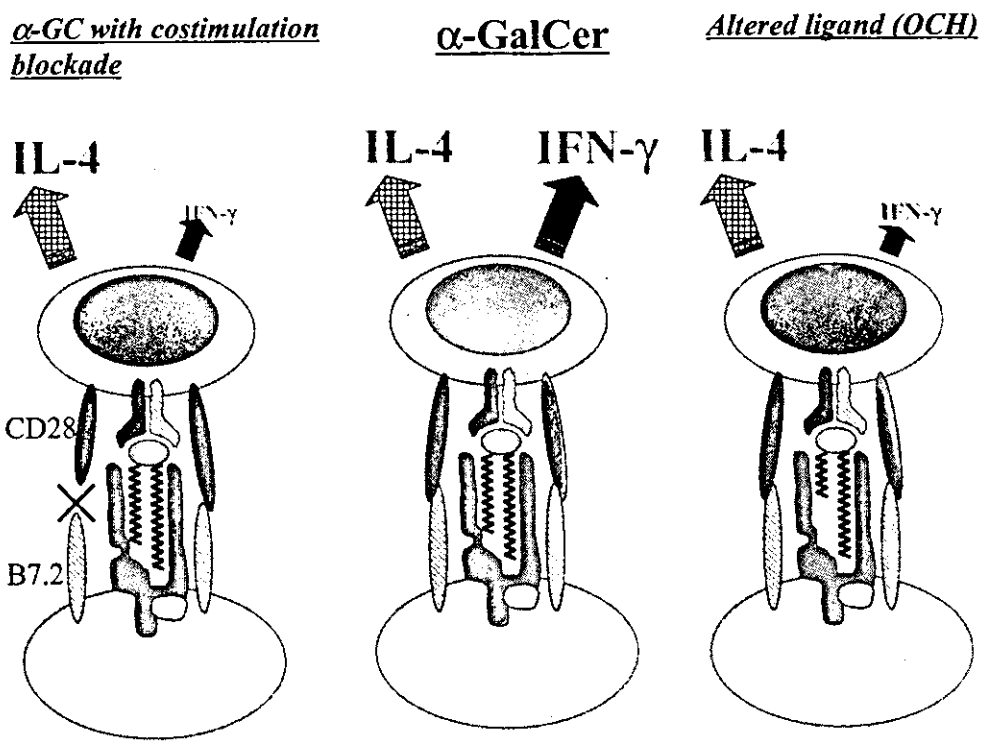

Fig. (2). Modulation of NKT cell cytokine production by co-stimulation blockade or with an altered ligand. α-GalCer induces production of both Th1 (IFN-γ) and Th2 (IL-4) cytokines, and therefore, is unable to control EAE (middle). We have found that NKT cells predominantly produce IL-4 when they are stimulated with α-GalCer in the absence of CD28/B7.2 co-stimulation [29] or if stimulated with an altered ligand OCH [32].

Table 1. Suppression of EAE with NKT Cell Stimulation by Glycolipid Ligands.

Mice	Glycolipid	NKT cell production of		EAE
		IL-4	IFN-γ	
Wild-type B6	α-GalCer	+	+	*no change*
IFN-γ $^{-/-}$	α-GalCer	+	-	*suppressed*
IL-4 $^{-/-}$	α-GalCer	-	+	*enhanced*
Wild-type B6	α-GalCer+anti-B7.1	+	+	*no change*
Wild-type B6	α-GalCer+anti-B7.2	+	-	*suppressed*
Wild-type B6	*OCH*	+	-	*suppressed*

This is the summary of the results obtained in our laboratory [29, 32].

exist an altered form of α-GalCer, or an altered glycolipid ligand (AGL), that would change the cytokine profile of NKT cells from Th0 type (producing both IFN-γ and IL-4) to Th2 type (predominantly producing IL-4). Such a ligand could be an ideal therapeutic for EAE.

One may postulate that a modification at the TCR contact site of α-GalCer is appropriate for obtaining such an analogue. Regarding the lipid antigen recognition by T cells, it is currently believed that the hydrophilic cap of the sugar moiety contacts the TCR of NKT cells and hydrophobic aliphatic chains bind to the CD1 molecule expressed by antigen presenting cells [31]. Previous studies have identified the critical parts of α-GalCer for inducing NKT cell proliferation [23,24]. We thus synthesized AGLs of α-GalCer with modification at the known critical sites or in the length of the CD1d-binding aliphatic chains (Fig. 1). We have so far examined three of these AGLs in depth for their abilities to stimulate NKT cells and to modulate clinical course of EAE.

We first noticed that the AGLs NH and 3,4D were unable to induce a proliferative response by NKT cells *in vitro*. In contrast, the third AGL, OCH, possessing a shorter

sphingosine chain could induce a significant proliferation of spleen NKT cells, although the response was about five- to tenfold lower than that induced with α-GalCer [32]. We also measured the amounts of IFN-γ and IL-4 in the culture supernatant. We could not detect these cytokines in the supernatant of spleen NKT cells stimulated with NH or 3,4D. Stimulation with α-GalCer and OCH were found to induce both IFN-γ and IL-4. Of interest, OCH induced less IFN-γbut more IL-4 *in vitro* compared with α-GalCer. In parallel, we measured the serum levels of the cytokines after intraperitoneal injection of the glycolipids into wild-type B6 mice (Fig. **3A**). Injection of α-GalCer induced a rapid elevation of IL-4 with the peak value at 2 h and a delayed and prolonged elevation of IFN-γ in the mice. While NH was non-stimulatory, injection of 3,4D induced a lower production of both IL-4 and IFN-γ than did α–GalCer injection. Most interestingly, OCH injection dissociated the production of IL-4 and IFN-γ : production of IL-4 was unaffected but IFN-γ was much lower (Fig. **3A**; right panel). Injection of OCH into mice deficient for NKT cells did not induce an elevation of serum cytokines, indicating that NKT cells mediated the robust cytokine responses in the wild-type mice.

Given that OCH would induce predominant IL-4 production by NKT cells, we postulated that this glycolipid might prevent development of EAE by inducing Th2 bias of NKT cells. In support of this postulate, intraperitoneal or oral administration of OCH on the day of sensitization with MOG 35-55 peptide was found to prevent development of EAE in wild-type mice in both clinical and pathological parameters (Fig. **3B**; left panel). We interpreted that the effect of OCH on EAE was mediated by IL-4 produced by NKT cells, because OCH was not effective in EAE induced in NKT cell-deficient or IL-4 knockout mice (Fig. **3B**; right) or when it was co-injected with neutralizing antibody against IL-4 (Fig. **3B**; middle). Furthermore, IgG1 antibody against MOG 35-55 peptide (the hallmark of Th2 response) was elevated in the mice treated with OCH, demonstrating the Th2 bias of autoimmune T cells by OCH treatment.

Since OCH induced a weaker proliferation of NKT cells than did α-GalCer, it was theoretically possible that α-GalCer, given at lower doses, might induce selective

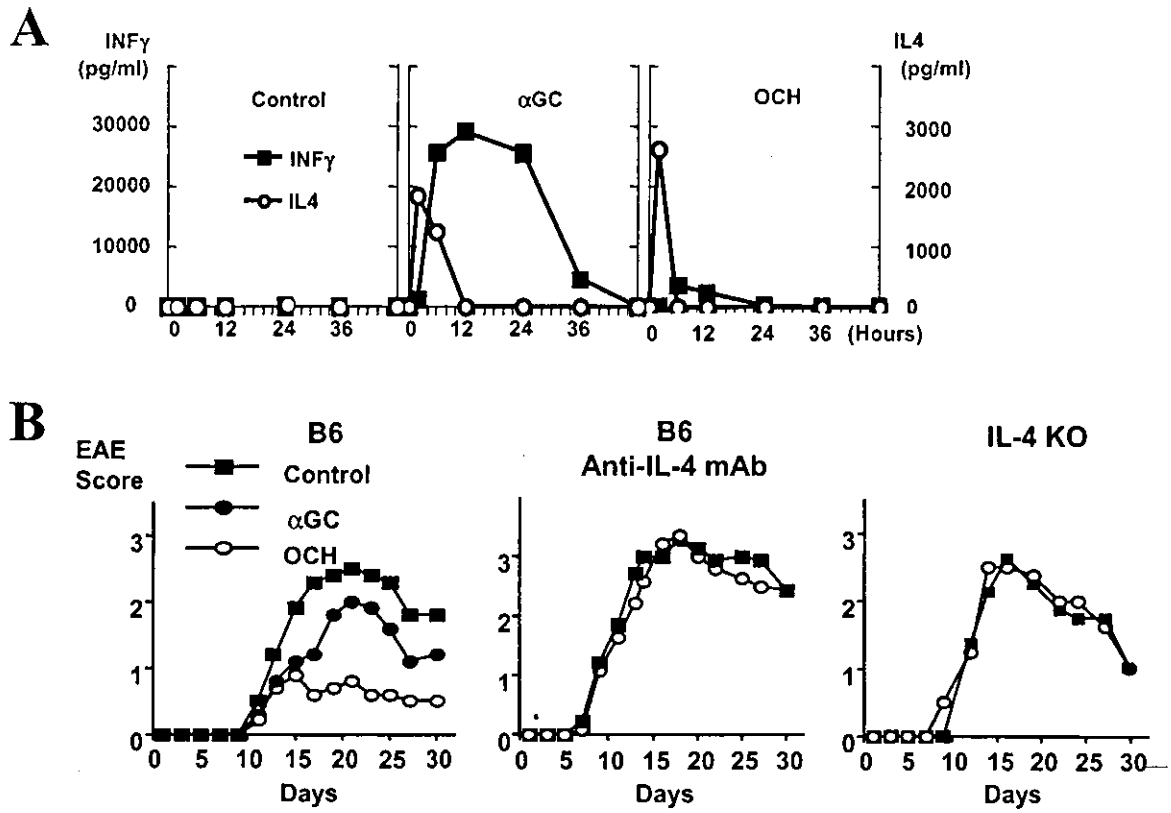

Fig. (3). Effect of glycolipid ligands *in vivo.* A: Changes in serum cytokine levels after injection of α–GalCer or OCH B6 mice were injected intraperitoneally with 100 μg/kg of α-GalCer or OCH, and serum samples were obtained at indicated times after injection. Serum levels of IL-4 and IFN-γ were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA). B: Preventive effects of OCH against EAE. Wild-type B6 or IL-4$^{-/-}$ mice were injected with an innoculum containing 100 μg of MOG35-55 peptide and 1 mg of *Mycobacterium tuberculosis* in incomplete Freunds adjuvant. Pertussis toxin (200 ng) was injected intravenously on day 0 and day 2 after immunization. Oral administration of OCH on the day of sensitization significantly suppressed the development of EAE (left). In contrast, OCH was not effective in IL-4 knockout mice (right), or when OCH was co-injected with anti-IL-4 mAb. The clinical signs were scored as follows: 0, no clinical signs, 1, partial loss of tail tonicity; 2, completely limp tail and abnormal gait; 3, partial hind-limb paralysis; 4, complete hind-limb paralysis; 5, fore- and hind-limb paralysis or a moribund state.

induction of IL-4 by NKT cells. However, we experimentally ruled out this possibility: lower doses of α-GalCer injected into wild-type mice induced elevation of both IL-4 and IFN-γ and did not alter the ratio of serum IFN-γ to IL-4 at their peak [32].

α-GALCER THERAPY FOR OTHER AUTOIMMUNE DISEASE MODELS

While we were studying the preventive effect of OCH on EAE, other laboratories were interested to know if α-GalCer might be preventive against development of autoimmune type 1 diabetes in NOD mice [33,34,35]. The results of these independent studies were in basic agreement that multiple injections of α-GalCer (twice/week) would induce Th2 bias of NKT cells and significantly inhibit diabetes development. We have confirmed that multiple injections of α-GalCer as well as of OCH would suppress diabetes in NOD mice (Miyake *et. al.* unpublished). These results demonstrate that spontaneous autoimmune disease such as NOD diabetes can be treated by repeated stimulation of NKT cells with glycolipid ligands. Given the unique property of OCH as inducing Th2 bias of NKT cells, it is of interest to know if OCH is more efficacious than α-GalCer in promoting disease protection. We would propose that the dynamic changes of NKT cells after repeated stimulation need to be further characterized, given that α-GalCer injection would induce a short-term depletion of NKT cells *in vivo*.

The preventive effect of α-GalCer on EAE has recently been reported with protocols different from ours [36,37]. In these studies, α-GalCer was injected prior to sensitization with peptide [36] or a mixture of α-GalCer with encephalitogenic peptide was co-immunized [37]. We have tried to reproduce these results, but to date have been unsuccessful for unknown reasons. In any event, it seems that a single injection of α-GalCer on the day of EAE induction or after EAE onset is not preventive against EAE not only in Japan but in laboratories in the USA as well.

CONCLUDING REMARKS

As we discussed in this review, ligand stimulation of regulatory cells is an attractive strategy for prevention or treatment of autoimmune diseases. Our results support the idea that NKT cell stimulation with glycolipid ligand is truly useful in control of autoimmune disease in model systems. Whereas both rodent and human NKT cells recognize α-GalCer in the context of CD1d, human NKT cells also recognize the *OCH* analogue (Araki *et al.* unpublished). Owing to the evolutionary conservation and the homogeneous ligand specificity of NKT cells, we could apply a glycolipid ligand like OCH for the treatment of human disease without considering species barrier or genetic heterogeneity of humans. Apart from considering the clinical point, our current energy should be focused on understanding how stimulation with different glycolipid ligands leads to differential effector functions of NKT cells. Screening of AGLs for inducing functional modulation of NKT cells is also important as this process would help us identify novel ligands of clinical value. It is also surely worth while to screen natural products in the same manner. Although microbial lipids have not been identified as NKT cell ligands, it can be speculated that they are probably an interesting natural source of useful ligands for CD1-restricted regulatory cells.

ABBREVIATIONS

α-GalCer	=	α-galactosylceramide
AGL	=	Altered glycolipid ligand
APL	=	Altered peptide ligand
B6	=	C57BL/6
CNS	=	Central nervous system
EAE	=	Experimental autoimmune encephalomyelitis
GPI	=	Glycosylphosphatidylinositol
IFN	=	Interferon
MBP	=	Myelin basic protein
MHC	=	Major histocompatibility complex
MOG	=	Myelin oligodendrocyte glycoprotein
MS	=	Multiple sclerosis
NK	=	Natural killer
PLP	=	Proteolipid protein
TCR	=	T cell receptor
Th1	=	T helper type 1
TNF	=	Tumor necrosis factor

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