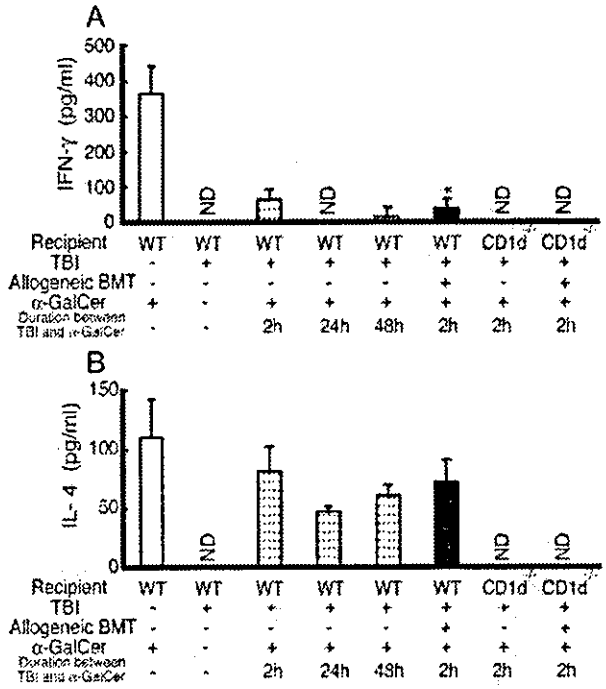

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen cells (5×10^6) from WT BALB/c donors immediately after TBI, followed by injection of α-GalCer 2 h after TBI. Six hours after the administration of α-GalCer, serum samples were collected, and levels of IFN-γ (*A*) and IL-4 (*B*) were measured. α-GalCer-treated control mice without TBI (□), recipients of TBI plus α-GalCer (▨), and recipients of TBI, allogeneic BMT, and α-GalCer (■) are shown ($n = 3$ per group). Results represent one of three similar experiments and are shown as mean ± SD. *, $p < 0.05$ vs nonirradiated controls. ND, Not detected.

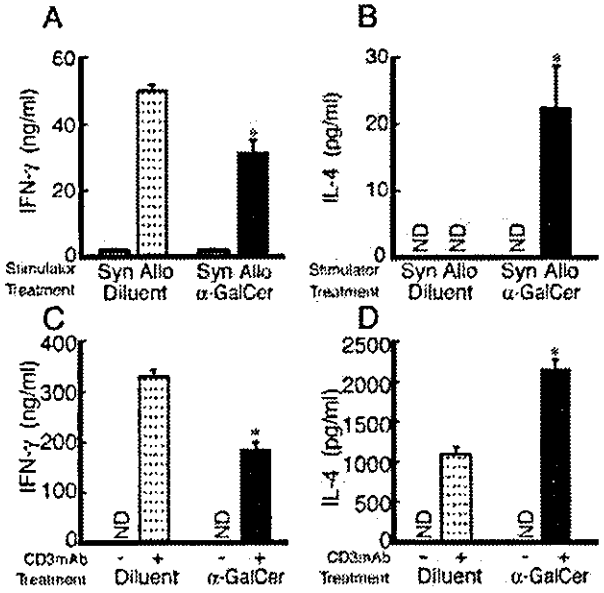

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control diluent. Mesenteric LN cells obtained from diluent-treated recipients (▨) and α-GalCer-treated recipients (■) 6 days after BMT were standardized for numbers of CD4$^+$ T cells as 5×10^4/well and were stimulated with 1×10^5/well of allogeneic or syngeneic peritoneal cells (*A* and *B*) or with CD3 (*C* and *D*). After 48 h, cytokine levels in the supernatant were measured by ELISA. Results shown are mean ± SD. *, $p < 0.05$ vs diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

either syngeneic (B6) or allogeneic (BALB/c) donors. Immediately after BMT, B6 recipients were injected i.p. with either α-GalCer or diluent. Six days after BMT, T cells isolated from mesenteric LN of recipient mice were cultured with irradiated B6 peritoneal cells or with anti-CD3ε mAbs and anti-CD28 mAbs for 48 h, and cytokine levels in the supernatant were determined. Flow cytometric analysis showed that >97% of LN T cells from both control recipients and α-GalCer-treated recipients were donor derived, as assessed by H-2^d vs H-2^b expression. T cells from α-GalCer-treated mice secreted significantly less IFN-γ, but more IL-4, in response to host alloantigens (Fig. 2, *A* and *B*) or to CD3 stimulation (Fig. 2, *C* and *D*) compared with those from controls. Similar results were obtained when T cells isolated from spleens were stimulated by anti-CD3ε and anti-CD28 mAbs. T cells from α-GalCer-treated mice secreted significantly less IFN-γ (18 ± 2 vs 164 ± 6 ng/ml), but more IL-4 (1022 ± 114 vs 356 ± 243 pg/ml), compared with controls. These results demonstrate that a single injection of α-GalCer to BMT recipients polarizes donor T cells toward Th2 responses after allogeneic BMT.

In α-GalCer-treated mice, serum levels of IFN-γ were dramatically reduced on day 6 compared with controls (Fig. 3*A*), and IL-4, which is usually hardly detectable in serum in this model, failed to be detected in the serum of mice of either group (data not shown). This impaired Th1 response of donor T cells was associated with a marked reduction of TNF-α levels in α-GalCer-treated mice (Fig. 3*B*).

Administration of α-GalCer or OCH to BMT recipients modulates acute GVHD

We next examined whether immune deviation mediated by administration of glycolipids can modulate acute GVHD. BMT was performed as above and α-GalCer was injected immediately after BMT on day 0. GVHD was severe in allogeneic controls, with 27% survival at day 50. A single injection of α-GalCer significantly improved survival to 86% ($p < 0.05$) (Fig. 4*A*). Allogeneic control mice developed significantly more severe clinical GVHD compared with syngeneic controls, as assessed by clinical GVHD scores (Fig. 4*B*). Clinical GVHD scores were significantly reduced in α-GalCer-treated recipients compared with allogeneic controls, but were greater than in syngeneic controls. Histological analysis showed that administration of α-GalCer significantly suppressed GVHD pathological scores in the intestine ($p < 0.05$). Analysis of donor cell engraftment at day 60 after BMT in spleens showed complete donor engraftment in α-GalCer-treated recipients (>99% H-2K^{d+}/H-2K^{b-} donor chimerism), ruling out rejection or mixed chimerism as a potential cause of GVHD suppression.

Similar protective effects against GVHD were observed in mice treated with OCH, further confirming the protective effects of NKT ligands (Fig. 4*C*). We performed BMT from B6 donors to BALB/c recipients to rule out strain artifacts. Again, a single injection of α-GalCer to BALB/c recipients reduced GVHD and significantly improved survival of animals (Fig. 4*D*).

Host NKT cells and host production of IL-4 are required for suppression of GVHD by α-GalCer

We examined the requirement of host NKT cells in this protective effect of α-GalCer, using NKT cell-deficient CD1d$^{-/-}$ mice as

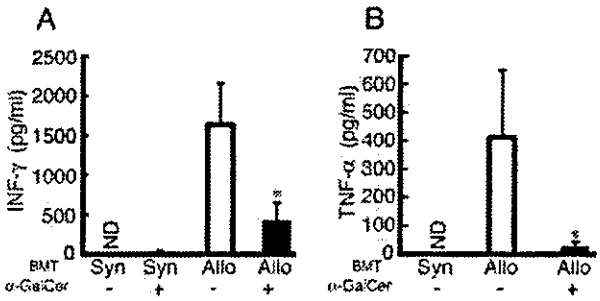

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of IFN-γ (*A*) and TNF-α (*B*) were determined. Results from three similar experiments are combined and shown as mean ± SD. *, $p < 0.05$ vs allogeneic, diluent-treated group. ND, Not detected; Syn, syngeneic; Allo, allogeneic.

BMT recipients. Lethally irradiated CD1d$^{-/-}$ mice were transplanted with BM cells and spleen cells from WT BALB/c donors, followed by administration of α-GalCer immediately after BMT on day 0. Protective effects of α-GalCer administration were not observed when CD1d$^{-/-}$ B6 mice were used as recipients, confirming the requirement for host NKT cells (Fig. 5*A*). We next examined the requirement of IL-4 production by host cells in this

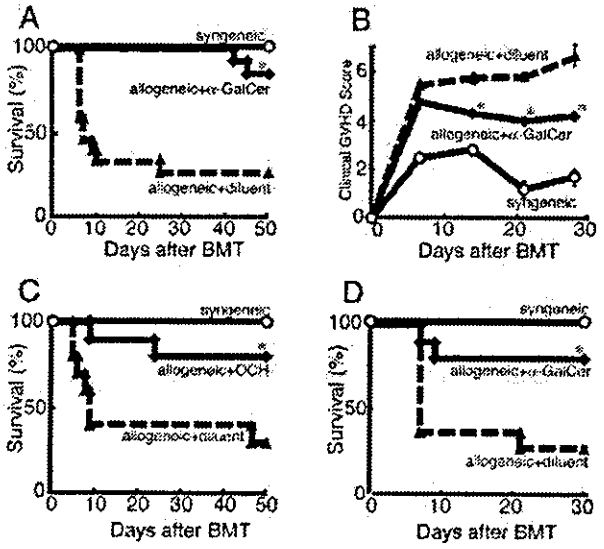

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined. *B*, Clinical scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE. *C*, Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, OCH-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *D*, Lethally irradiated (9 Gy) BALB/c mice were transplanted from B6 donors. Survival curves of the syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *, $p < 0.05$ vs diluent-treated group.

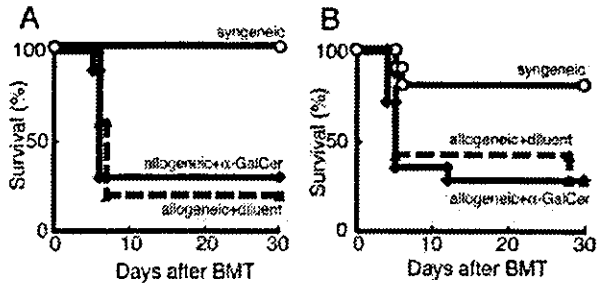

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line; n = 10); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 10) are shown. Data from two similar experiments were combined. *B*, Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 11); allogeneic, diluent-treated recipients (▲, dotted line; n = 14); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 14) are shown. Data from three similar experiments were combined.

protective effect. Lethally irradiated IL-4$^{-/-}$ B6 mice were transplanted from WT BALB/c donors and administered α-GalCer as above. α-GalCer did not confer protection against GVHD in IL-4$^{-/-}$ recipients (Fig. 5*B*). Taken together, these results indicate that protective effects of α-GalCer are dependent upon host NKT cells and host production of IL-4.

STAT6 signaling in donor T cells is required for modulation of GVHD by α-GalCer

To determine whether IL-4-induced signaling in donor T cells is critical for the protective effect of glycolipids on GVHD, we used donor spleen cells that lack STAT6 and have impaired IL-4 responses (34, 35). Spleen cells from STAT6$^{-/-}$ BALB/c mice and TCD BM cells from WT BALB/c mice were transplanted after lethal TBI, followed by a single injection of α-GalCer. α-GalCer treatment failed to reduce morbidity and mortality of acute GVHD when STAT6$^{-/-}$ BALB/c donors were used (Fig. 6), demonstrating that STAT6 signaling in donor cells is critical for the protective effect of α-GalCer against GVHD.

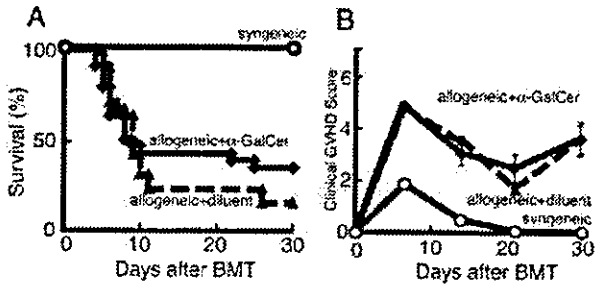

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic control group (○, solid line; n = 15); allogeneic, diluent-treated recipients (▲, dotted line; n = 25); and allogeneic, α-GalCer-treated recipients (◆, solid line; n = 25) are shown. Data from five similar experiments were combined. *B*, Clinical GVHD scores of syngeneic control group (○, solid line); allogeneic, diluent-treated recipients (▲, dotted line); and allogeneic, α-GalCer-treated recipients (◆, solid line) are shown as the mean ± SE.

Discussion

NKT cells are critically involved in the development and suppression of various autoimmune diseases. In experimental models, their regulatory mechanisms mostly depend on IL-4 production and subsequent inhibition of Th1 differentiation of autoreactive $CD4^+$ T cells (18). Previous studies have demonstrated that donor NKT cells regulate acute GVHD in an IL-4-dependent manner when administered together with donor inoculum (36). Considering these immunomodulating functions of NKT cells, we evaluated whether stimulation of host NKT cells could modulate GVHD in a mouse model of this disease.

Administration of α-GalCer stimulates NKT cells to produce both IFN-γ and IL-4 in naive mice, which can promote Th1 and Th2 immunity, respectively (18). We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. Surprisingly, irradiation of mice dramatically reduced IFN-γ production in response to α-GalCer, while preserving IL-4 production. This result may account for Th2, but not Th1, polarization of donor T cells by α-GalCer, even in conditions such as allogeneic BMT, which preferentially promotes Th1 polarization. Although mechanisms of selective suppression of IFN-γ production induced by irradiation need to be elucidated, irradiation may modulate the cytokine production profile of NKT cells or neighboring NK cells. Although OCH stimulates NKT cells to predominantly produce IL-4 compared with α-GalCer, resulting in potent Th2 responses (27, 31), both OCH and α-GalCer equally stimulate IL-4 production in irradiated mice and exert equivalent protection against acute GVHD.

Stimulation of host NKT cells by injecting α-GalCer or OCH polarized donor T cells toward Th2 cytokine secretion, resulting in marked reduction of serum IFN-γ levels after BMT. Th2 cytokine responses subsequently inhibited inflammatory cytokine cascades and reduced morbidity and mortality of acute GVHD, as previously described (10–12). Inflammatory cytokines have been shown to be important effector molecules of acute GVHD (37). α-GalCer treatment failed to confer protection against acute GVHD when $STAT6^{-/-}$ BALB/c donors were used, demonstrating that Th2 polarization via STAT6 signaling is critical for this protective effect of α-GalCer, although STAT6-independent Th2 induction has been reported (38, 39).

α-GalCer did not confer protection against GVHD in $CD1d^{-/-}$ or $IL\text{-}4^{-/-}$ recipients. Therefore, the protective effect of α-GalCer against GVHD is dependent upon host NKT cells and host production of IL-4. Sublethal total lymphoid irradiation enriches NKT cells in host lymphoid tissues, and these NKT cells induce Th2 polarization of conventional T cells by IL-4 production, resulting in reduced GVHD (40–42). These findings are consistent with our observation that IL-4 production is critical for the protective effects of NKT cells against acute GVHD. It should be noted, however, that systemic administration of IL-4 is either ineffective or toxic (6). Because the cytokine environment during the initial interaction between naive T cells and APCs is critically important for induction of Th1 or Th2 differentiation (14), local IL-4 production in the secondary lymphoid organs where donor T cells encounter host APCs might be necessary to cause effective Th1→Th2 immune deviation after allogeneic HSCT (43).

Current strategies for prophylaxis and treatment of GVHD primarily target depletion or suppression of donor T cells. These interventions suppress donor T cell activation and are associated with increased risk of infection and relapses of malignant diseases. Th1→Th2 deviation of donor T cells represents a promising strategy to reduce acute GVHD while preserving cytolytic cellular effector functions against tumors and infectious agents (33, 44–47). To achieve Th1→Th2 immune deviation of donor T cells, cytokines have been administered to either donors or recipients in animal models of GVHD. Donor treatment with cytokines such as IL-18 and G-CSF, and recipient treatment with IL-11, induces Th2 polarization of donor T cells and reduces acute GVHD (33, 44, 48). The present study reveals an alternative strategy to induce Th2 polarization of donor T cells by injecting NKT ligands into recipients to activate recipient NKT cells.

Prior studies (36, 40–42, 49) and the current study suggest that both donor and host NKT cells can regulate acute GVHD through their unique properties to secrete large amounts of cytokines and subsequent modulation of adaptive immunity. These studies reveal that there are several ways by which the NKT cell system can be exploited to suppress GVHD. First, administration of donor NKT cells expanded in vitro by repeated stimulation with glycolipid (50) can suppress GVHD (36). Second, total lymphoid irradiation enriches host NKT cells in lymphoid organs and thereby skews donor T cells toward Th2 cytokine production (40–42). Third, as shown here, administration of glycolipid to recipients stimulates host NKT cells to suppress GVHD. A recent phase I trial for patients with various solid tumors demonstrated that administration of α-GalCer was well tolerated with minimal side effects, which included temporal fever, headache, vomiting, chills, and malaise (51). Therefore, α-GalCer treatment may provide an effective and relatively safe option for preventing GVHD.

Cells belonging to the innate arm of the immune system, such as monocytes/macrophages, NKT cells, and NK cells, can produce large amounts of cytokines quickly upon stimulation. Innate immunity can thereby augment donor T cell responses to alloantigens in allogeneic HSCT (3). Our findings reveal a novel role for host NKT cells in regulating GVHD and indicate that stimulation of host innate immunity may serve as an effective adjunct to clinical regimens of GVHD prophylaxis.

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Modulation of CD1d-restricted NKT cell responses by using *N*-acyl variants of α-galactosylceramides

Karl O. A. Yu*†, Jin S. Im*†, Alberto Molano*†, Yves Dutronc*‡, Petr A. Illarionov§, Claire Forestier*, Nagatoshi Fujiwara*¶, Isa Arias*, Sachiko Miyake‖, Takashi Yamamura‖, Young-Tae Chang**, Gurdyal S. Besra§, and Steven A. Porcelli*††

*Department of Microbiology and Immunology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461; §School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom; ‖Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan; and **Department of Chemistry, New York University, 29 Washington Place, New York, NY 10003

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A form of α-galactosylceramide, KRN7000, activates CD1d-restricted Vα14-invariant (Vα14i) natural killer (NK) T cells and initiates multiple downstream immune reactions. We report that substituting the C26:0 *N*-acyl chain of KRN7000 with shorter, unsaturated fatty acids modifies the outcome of Vα14i NKT cell activation. One analogue containing a diunsaturated C20 fatty acid (C20:2) potently induced a T helper type 2-biased cytokine response, with diminished IFN-γ production and reduced Vα14i NKT cell expansion. C20:2 also exhibited less stringent requirements for loading onto CD1d than KRN7000, suggesting a mechanism for the immunomodulatory properties of this lipid. The differential cellular response elicited by this class of Vα14i NKT cell agonists may prove to be useful in immunotherapeutic applications.

cytokines | inflammation | autoimmunity | immunoregulation

Natural killer (NK) T cells were defined originally as lymphocytes coexpressing T cell receptors (TCRs) and C-type lectin receptors characteristic of NK cells. A major subset of NKT cells recognizes the MHC class I-like molecule CD1d by using TCRs composed of an invariant TCR-α chain (mouse Vα14-Jα18, human Vα24-Jα18) paired with TCR-β chains with markedly skewed Vβ usage (1). These CD1d-restricted Vα14-invariant (Vα14i) NKT cells are highly conserved in phenotype and function between mice and humans (2). Vα14i NKT cells influence various immune responses and play an important role in regulating autoimmunity (3, 4). One example is the nonobese diabetic mouse. When compared with normal mice, nonobese diabetic mice have fewer Vα14i NKT cells, which are defective in their capacity to produce antiinflammatory cytokines like IL-4 (5, 6). Deficiencies in NKT cells have also been observed in humans with various autoimmune diseases (7, 8).

Vα14i NKT cells have been manipulated to prevent or treat autoimmune disease, mostly through the use of KRN7000, a synthetic α-galactosylceramide (α-GalCer, Fig. 1*A*) that binds to the hydrophobic groove of CD1d and then activates Vα14i NKT cells by means of TCR recognition (9). KRN7000 treatment of nonobese diabetic mice blocks development of T helper (T_H) type 1-mediated autoimmune destruction of pancreatic islet β-cells, thus delaying or preventing disease (10–12). There has been considerable interest in methods that would allow a more selective activation of these cells. In particular, the ability to trigger IL-4 production without eliciting strong IFN-γ or other proinflammatory cytokines may reinforce the immunoregulatory functions of Vα14i NKT cells. This effect is detected after Vα14i NKT cell activation with a glycolipid designated OCH, which is an α-GalCer analogue that is structurally distinct from KRN7000 in having a substantially shorter sphingosine chain and functionally by its preferential induction of IL-4 secretion (13, 14).

In this study, we investigated responses to α-GalCer analogues produced by alteration of the length and extent of unsaturation of their *N*-acyl substituents. Such modifications altered the outcome of Vα14i NKT cell activation and, in some cases, led to a T_H2-biased and potentially antiinflammatory cytokine response. This change in the NKT cell response was likely the result of an alteration of downstream steps in the cascade of events triggered by Vα14i NKT cell activation, including the reduction of secondary activation of IFN-γ-producing NK cells. These findings point to a class of Vα14i NKT cell agonists that may have superior properties for the treatment of autoimmune and inflammatory diseases.

Materials and Methods

Mice and Cell Lines. C57BL/6 mice (8- to 15-wk-old females) were obtained either from The Jackson Laboratory or Taconic Farms. $CD1d^{-/-}$ mice were provided by M. Exley and S. Balk (Beth Israel–Deaconess Medical Center, Harvard Medical School, Boston) (15). Vα14i NKT cell-deficient $Jα18^{-/-}$ mice were a gift from M. Taniguchi and T. Nakayama (Chiba University, Chiba, Japan) (16). Both knockout mice were in the C57BL/6 background. Animals were kept in specific pathogen-free housing. The protocols that we used were in accordance with approved institutional guidelines.

Mouse CD1d-transfected RMA-S cells (RMA-S.mCD1d) were provided by S. Behar (Brigham and Women's Hospital, Harvard Medical School) (17). WT or cytoplasmic tail-deleted CD1d-transfected A20 cells and the Vα14i NKT hybridoma DN3A4–1.2 were provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA) (18, 19). Hybridoma DN32D3 was a gift from A. Bendelac (University of Chicago, Chicago) (1). Cells were cultured in RPMI medium 1640 (GIBCO) supplemented with 10% heat-inactivated FCS (Gemini Biological Products, Calabasas, CA)/10 mM Hepes/2 mM L-glutamine/0.1 mM nonessential amino acids/55 μM 2-mercaptoethanol/100 units/ml penicillin/100 μg/ml streptomycin (GIBCO) in a 37°C humidified incubator with 5% CO_2.

Glycolipids. BF1508-84 was synthesized by Biomira (Edmonton, Canada). OCH [(2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-tetracosanoyl-2 amino-1,3,4-nonanetriol] was synthesized as described (13). An overview of the methods for synthesis of KRN7000 [(2*S*, 3*S*, 4*R*)-1-*O*-(α-D-galactopyranosyl)-*N*-hexaco-

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Abbreviations: Vα14i, Vα14 invariant; NK, natural killer; α-GalCer, α-galactosylceramide; T_H, T helper; TCR, T cell receptor; RMA-S.mCD1d, mouse CD1d-transfected RMA-S cells.

†K.O.A.Y., J.S.I., and A.M. contributed equally to this work.

‡Present address: Department of Dermatology, Bocage Hospital, BP 77908, 21079 Dijon Cedex, France.

¶Present address: Department of Host Defense, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan.

††To whom correspondence should be addressed. E-mail: porcelli@aecom.yu.edu.

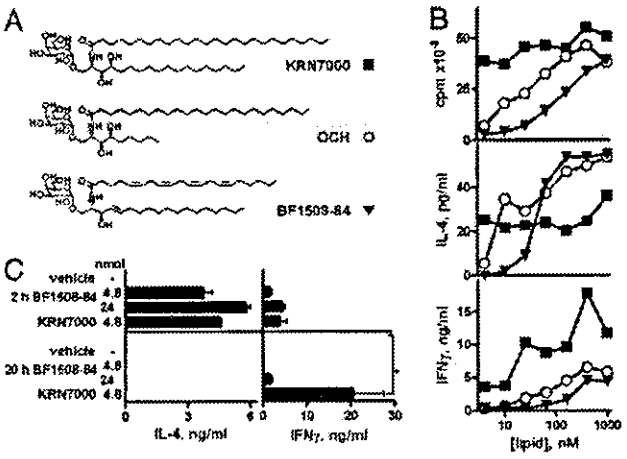

Fig. 1. Induction of a T_H2-polarized cytokine response by an unsaturated analogue of α-GalCer. (*A*) Glycolipid structures. (*B*) [^{3}H]thymidine incorporation and supernatant IL-4 and IFN-γ levels in 72-h splenocyte cultures with graded amounts of glycolipid. Means from triplicate cultures are shown; SEMs were typically <10% of the mean. (*C*) Serum IL-4 and IFN-γ levels (at 2 and 20 h) of C57BL/6 mice injected i.p. with 4.8 or 24 nmol of glycolipid. KRN7000 was the only glycolipid that induced significant IFN-γ levels at 20 h (*, $P < 0.05$, Kruskall–Wallis test, Dunn's posttest). Means ± SD of two or three mice per group are shown.

sanoyl-2-amino-1,3,4-octadecanetriol] and other *N*-acyl analogues used in this study is shown in Fig. 7, which is published as supporting information on the PNAS web site. Lipids were dissolved in chloroform/methanol (2:1 ratio) and stored at −20°C. Aliquots from this stock were dried and reconstituted to either 100 μM in DMSO for *in vitro* work or to 500 μM in 0.5% Tween-20 in PBS for *in vivo* studies.

***In Vitro* Stimulations.** Bulk splenocytes were plated at 300,000 cells per well in 96-well flat-bottom tissue culture plates with glycolipid diluted in 200 μl of medium. After 48 or 72 h at 37°C, 150 μl of supernatant was removed for cytokine measurements, and 0.5 μCi (1 Ci = 37 GBq) [^{3}H]thymidine per well (specific activity 2 Ci/mmol; PerkinElmer) was added for an 18-h pulse. Proliferation was estimated by harvesting cells onto 96-well filter mats and counting β-scintillations with a 1450 Microbeta Trilux (Wallac, Gaithersburg, MD; PerkinElmer).

Supernatant levels of IL-2, IL-4, IL-12p70, and IFN-γ were measured by ELISA using capture and biotinylated detection antibody pairs (BD PharMingen) and streptavidin–horseradish peroxidase (Zymed) with TMB-Turbo substrate (Pierce) or streptavidin–alkaline phosphatase (Zymed) with 4-nitrophenyl phosphate substrate (Sigma). IL-2 standard was obtained from R & D Systems; IL-4, IL-12p70 and IFN-γ were obtained from PeproTech (Rocky Hill, NJ).

Hybridoma Stimulations. CD1d$^+$ RMA-S or A20 cells (50,000 cells in 100 μl per well) were pulsed with graded doses of glycolipid for 6 h at 37°C. After three washes in PBS, Vα14i NKT hybridoma cells (50,000 cells in 100 μl) were added for 12 h. Supernatant IL-2 was assayed by ELISA. Alternatively, CD1d-transfected cells (RMA-S.mCD1d) were lightly fixed either before or after exposure to antigen (20). Cells were washed twice in PBS and then fixed in 0.05% glutaraldehyde (grade I, Sigma) in PBS for 30 s at room temperature. Fixative was quenched by addition of 0.2 M L-lysine (pH 7.4) for 2 min, followed by two washes with medium before addition of responders.

For cell-free presentation, recombinant mouse CD1d (1 μg/ml in PBS) purified from a baculovirus expression system (21) was adhered to tissue culture plates for 1 h at 37°C. After the washing off of unbound protein, glycolipids were then added at varying concentrations for 1 h at 37°C. Lipids were added in a 150 mM NaCl/10 mM sodium phosphate buffer (pH 7) with or without 0.025% Triton X-100. Wells were washed before addition of hybridoma cells.

***In Vivo* Studies.** Mice were given i.p. injections of 4.8 nmol of glycolipid in 0.2 ml of PBS plus 0.025% Tween-20 or vehicle alone. Sera were collected and tested for IL-4, IL-12p70, and IFN-γ, as described above. Alternatively, mice were killed at various times for FACS analysis.

Flow Cytometry. Splenocytes or thymocytes were isolated and used without further purification. Nonspecific staining was blocked by using FACS buffer (0.1% BSA/0.05% NaN_3 in PBS) with 10 μg/ml rat anti-mouse CD16/32 (2.4G2; The American Type Culture Collection). Cells ($\leq 10^6$) were stained with phycoerythrin or allophycocyanin-conjugated glycolipid/mouse CD1d tetramers (21) for 30–90 min at room temperature and then with fluorescently labeled antibodies (from Caltag, South San Francisco, CA, or PharMingen) for 30 min at 4°C. Data were acquired on either a FACSCalibur or LSR-II flow cytometer (Becton Dickenson) and analyzed by using WINMDI 2.8 (Scripps Research Institute, La Jolla, CA). For some experiments, dead cells were excluded by using propidium iodide (Sigma) or 4′,6-diamidino-2-phenylindole (Roche).

FACS-based cytokine secretion assays (Miltenyi Biotec, Auburn, CA) were used to quantitatively detect single-cell production of IL-4 or IFN-γ. Splenocytes were aseptically collected from mice that were previously injected i.p. with glycolipid analogues and not subjected to further stimulation. When applicable, 10^6 cells were prestained with labeled tetramer for 30 min at room temperature and then washed in PBS plus 0.1% BSA. Cells were then stained with the cytokine catch reagent according to the manufacturer's instructions, followed by incubation with rotation in 2 ml of medium at 37°C for 45 min. Cells were then washed, stained with fluorescently labeled antibodies to cell-surface antigens, phycoerythrin-conjugated anti-IFN-γ or IL-4, and propidium iodide, as described above.

Results

T_H2-Skewing Properties of an α-GalCer Analogue. During screening of a panel of synthetic glycosyl ceramides, we identified a compound that showed T_H2-skewing of the cytokine profile generated by Vα14i NKT cell activation. Glycolipid BF1508-84 differed structurally from both OCH and KRN7000 by having a shortened, unsaturated fatty-acid chain (C20:4 arachidonate) and a double bond in place of the 4-hydroxy in the sphingosine base (Fig. 1*A*). Despite these modifications, BF1508-84 activated proliferation and cytokine secretion by mouse splenocytes (Fig. 1*B*). These responses were Vα14i NKT cell-dependent, as demonstrated by their absence in both CD1d$^{-/-}$ and Jα18$^{-/-}$ mice (data not shown). Maximal proliferation and IL-4 levels were comparable with those obtained with KRN7000 and OCH, although a higher concentration of BF1508-84 was required to reach similar responses. Interestingly, IFN-γ secretion stimulated by BF1508-84, even at higher tested concentrations, did not reach the levels seen with KRN7000. This profile of cytokine responses suggested that BF1508-84 can elicit a T_H2-biased Vα14i NKT cell-dependent cytokine production, similar to OCH (13).

We measured serum cytokine levels at various times after a single injection of either KRN7000 or BF1508-84 into C57BL/6 mice. Our studies confirm published reports that a single i.p. injection of KRN7000 leads to a rapid 2-h peak of serum IL-4 (Fig. 1*C* and data not shown). However, IFN-γ levels were relatively low at 2 h but rose to a plateau at 12–24 h (13, 22). With

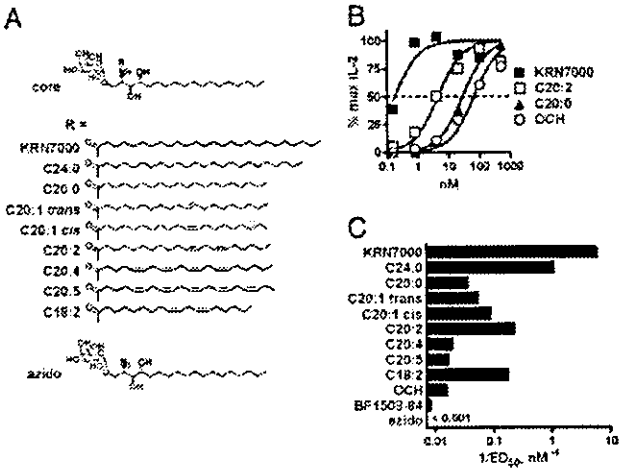

Fig. 2. Recognition of a panel of unsaturated analogues of KRN7000 by a canonical Vα14i NKT hybridoma. (*A*) Analogue structures. (*B*) Dose–response curves showing IL-2 production by hybridoma DN3A4–1.2 after stimulation with RMA-S.mCD1d cells pulsed with various doses of glycolipid. Maximal IL-2 concentrations in each assay were designated as 100%. Four-parameter logistic equation dose–response curves are shown; the dotted line denotes the half-maximal dose. (*C*) Relative potencies of the analogue panel in Vα14i NKT cell recognition, plotted as the reciprocal of the effective dose required to elicit a half-maximal response ($1/ED_{50}$). Similar results were obtained by using another Vα14i NKT hybridoma, DN32D3.

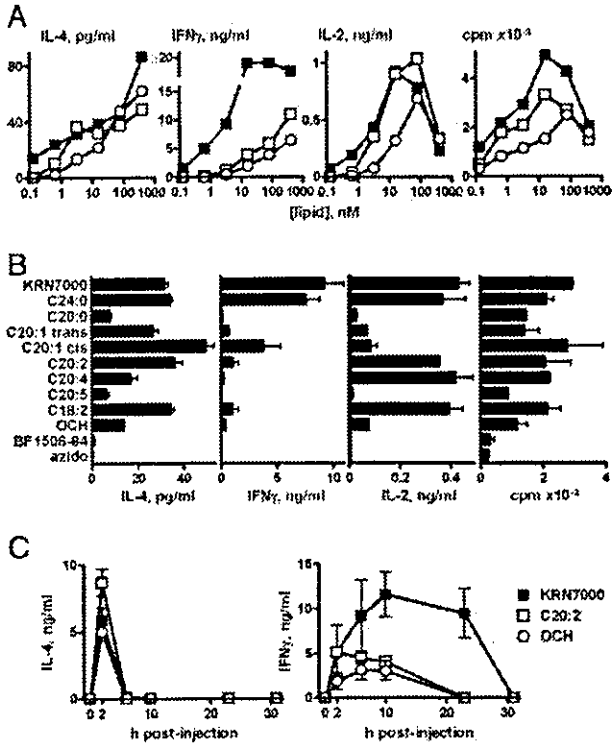

Fig. 3. T_H2-skewing of *in vitro* and *in vivo* cytokine responses to C20:2. (*A*) Dose–response curves reporting 48 h IL-4, IFN-γ, or IL-2 production, and cell proliferation of splenocytes in response to KRN7000, C20:2, and OCH. Means of duplicate cultures are shown; SEM were <10% of the means. (*B*) Cytokine and proliferation measurements on splenocytes exposed to a submaximal dose (3.2 nM) of the panel of α-GalCer analogues shown in Fig. 2. Mean ± SEM from duplicate cultures shown. (*C*) Serum IL-4 and IFN-γ levels in mice given 4.8 nmol of KRN7000, C20:2, or OCH. Mean ± SD of two or three mice are shown. Vehicle-treated mice had cytokine levels below limits of detection. The results shown are representative of two or more experiments.

BF1508-84, production of IL-4 at 2 h was preserved, whereas IFN-γ was barely detectable at 20 h (Fig. 1*C*). This pattern was identical to that reported for OCH (13, 22) and was not due to the lower potency of BF1508-84 because a 5-fold greater dose did not change the T_H2-biased cytokine profile (Fig. 1*C*).

Systematic Variation of Fatty-Acyl Unsaturation in α-GalCer. The cytokine response to BF1508-84 suggested that altering the fatty-acid length and unsaturation of α-GalCer could provide an effective strategy for creating Vα14i NKT cell activators with modified functional properties. We used a synthetic approach (Fig. 7, and G.S.B. and P.A.I., unpublished data) to generate lipids in which 20-carbon acyl chains with varying degrees of unsaturation were coupled onto the α-galactosylated sphingosine core structure (Fig. 2*A*). These compounds were first screened for the ability to activate a canonical Vα14-Jα18/Vβ8.2$^+$, CD1d-restricted NKT cell hybridoma cocultured with CD1d$^+$ antigen-presenting cells. Hybridoma DN3A4–1.2 recognized all C20 analogues of α-GalCer with various potencies when presented by CD1d-transfected RMA-S cells, and it failed to recognize an azido-substituted analogue lacking a fatty-acid chain (Fig. 2 *B* and *C*). As reported (9), mere shortening of the fatty-acid chain affected Vα14i NKT cell recognition, and reduction of saturated fatty-acid length from C26 to C20 was associated with a ≈2 log decrease in potency. However, insertion of double bonds into the C20 acyl chain augmented stimulatory activity. One lipid in particular, with unsaturations at carbons 11 and 14 (C20:2), was more potent than other analogues in the panel. This increase in potency seemed to be a direct result of the two double bonds, because an independently synthesized analogue with a slightly shorter diunsaturated acyl chain (C18:2) showed a potency similar to that of C20:2 (Fig. 2*C*).

We also studied *in vitro* splenocyte cytokine polarization resulting from Vα14i NKT cell stimulation by each lipid in the panel. Supernatant IL-4, IFN-γ, and IL-2 levels were measured over a wide range of glycolipid concentrations. All C20 variants induced IL-4 production comparable with that of KRN7000 (Fig. 3 *A* and *B*, and data not shown). However, IFN-γ levels for all but one C20 analogue (C20:1 *cis*) were markedly reduced to one-fourth of the maximal levels observed with KRN7000 and the closely related C24:0 analogue, or less. In addition, C20:1-*cis*, C20:2, and C18:2 were unique in this class of compounds in inducing strong IL-2 production and cellular proliferation similar to that seen with KRN7000 and C24:0 yet with much lower IFN-γ induction. This *in vitro* T_H2-bias was also evident *in vivo*. Mice given C20:2 and C20:4 showed systemic cytokine production that resembled stimulation by OCH or BF1508-84. Thus, a rapid burst of serum IL-4 was observed without the delayed and sustained production of IFN-γ typical of KRN7000 (Fig. 3*C* and data not shown). No significant difference between the glycolipids was seen in serum IL-12p70 levels at 6 h after treatment (data not shown).

Identification of Cytokine-Producing Cells *in Vivo*. Previous reports (23–25) established that Vα14i NKT cells are a predominant source of IL-4 and IFN-γ in the early (2 h) response to KRN7000 and that by 6 h after injection these cells become progressively undetectable because of receptor down-modulation, whereas secondarily activated NK cells begin to actively produce IFN-γ. Gating on either α-GalCer-loaded CD1d tetramer$^+$ or NK1.1$^+$ T cells, we observed similar strong cytokine secretion for both IL-4 (data not shown) and IFN-γ in Vα14i NKT cells at 2 h after injection of KRN7000 or C20:2 (Fig. 4 *A* and *B*). We concluded

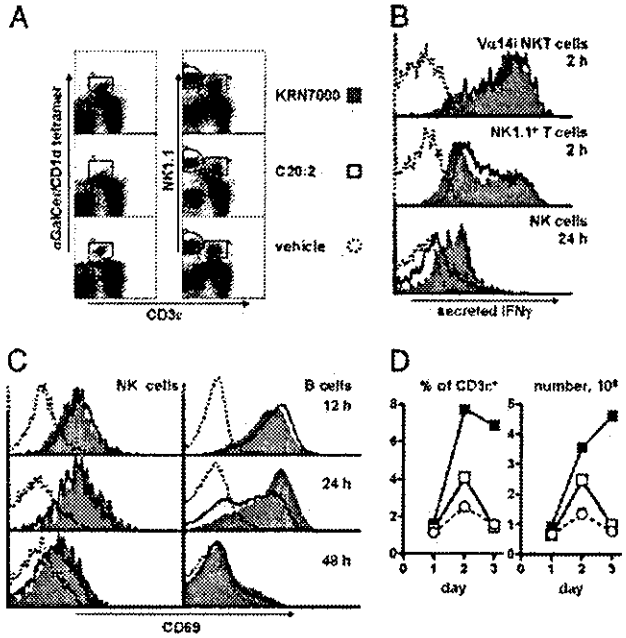

Fig. 4. Sequelae of KRN7000 and C20:2-induced Vα14i NKT cell activation. (*A*) Vα14i NKT cell (tetramer$^+$ CD3ε^{int}), NK cell (NK1.1$^+$ CD3ε^-), and NK1.1$^+$ T cell (NK1.1^{int} CD3ε^{int}) identification by FACS in splenocytes from mice given KRN7000, C20:2, or vehicle i.p. 2 h earlier. Lymphocytes gated as negative for B220 and propidium iodide are shown. (*B*) Histogram profiles for IFN-γ secretion of splenic Vα14i NKT, NK1.1$^+$ T, or NK cells from mice 2 or 24 h after treatment with glycolipid. IFN-γ-staining in C24:0-stimulated samples was identical to that of KRN7000-stimulated samples. (*C*) CD69 levels of splenic NK cells (gated as CD3ε^- NK1.1$^+$) or B cells (CD3ε^- NK1.1$^-$ B220$^+$) at 12, 24, or 48 h after injection of glycolipid. (*D*) Splenic Vα14i NKT cell (B220$^-$ CD3ε^{int} tetramer$^+$) frequency, measured as either percentages of T cells or as total NKT cell number, in mice 1, 2, or 3 days after glycolipid administration. The results shown are representative of three independent experiments.

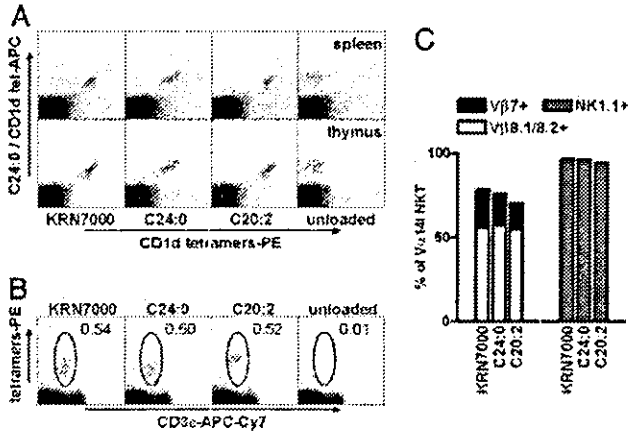

Fig. 5. Recognition of KRN7000, C24:0, and C20:2 by the same population of Vα14i NKT cells. (*A*) Costaining of C57BL/6 splenocytes or thymocytes with allophycocyanin-conjugated CD1d tetramers assembled with C24:0, and phycoerythrin-labeled CD1d tetramers assembled with various analogues. (*B*) Thymocytes were stained with C24:0, C20:2, KRN7000, or vehicle-loaded CD1d tetramers–phycoerythrin, and with antibodies to B220, CD3ε, Vβ7, Vβ8.1/8.2, or NK1.1. Dot plots show gating for tetramer$^+$ T cells, after exclusion of B lymphocytes, and dead cells. (*C*) TCR Vβ and NK1.1 phenotype of tetramer$^+$ CD3ε^{int} thymocytes. Analogous results were obtained with splenocytes. The results shown are representative of three or more experiments.

that cytokine polarization observed after C20:2 administration was not due to differences in the initial Vα14i NKT cell response but, rather, reflected altered downstream events such as the relatively late IFN-γ production by activated NK cells.

Secreted cytokine staining confirmed that in both KRN7000- and C20:2-treated mice, NK cells were IFN-γ$^+$ at 6–12 h after treatment (26, 27). However, whereas splenic NK cells from mice that received either KRN7000 or the closely related C24:0 analogue strongly produced IFN-γ as late as 24 h after initial activation, NK cells from C20:2-treated mice showed substantially reduced staining (Fig. 4*B*). Together, these results pointed to a less sustained secondary IFN-γ production by NK cells (rather than a change in the initial cytokine response of Vα14i NKT cells) as the major factor responsible for the T_H2 bias of the systemic cytokine response to C20:2.

Sequelae of Vα14i NKT Cell Activation by C20:2. Secondary activation of bystander B and NK cells after KRN7000 administration has been studied by using expression of the activation marker CD69 (26, 28–30). We followed CD69 expression of splenic NK and B cell populations for several hours after KRN7000 or C20:2 administration. Both populations began to up-regulate CD69 at 4–6 h after injection (data not shown). Paradoxically, C20:2 induced slightly higher CD69 levels on both cell populations up until 12 h, although this trend was reversed from 24 h onwards, suggesting an earlier up-regulation yet faster subsequent down-regulation of the marker (Fig. 4*C*). NK cell forward scatter likewise remained higher in KRN7000-treated mice at days 1–3 compared with C20:2-treated mice (data not shown).

It is established that Vα14i NKT cells expand beyond homeostatic levels 2 or 3 days after KRN7000 stimulation (24, 25). In our study, a 3- to 5-fold expansion in splenic Vα14i NKT cell number occurred in KRN7000-treated mice at day 3 after injection. Interestingly, after *in vivo* administration of C20:2, only a minimal transient expansion was observed on day 2, with no expansion of the Vα14i NKT cell population thereafter, even as late as day 5 (Fig. 4*D* and data not shown). Together, our findings indicated pronounced alterations in the late sequelae of Vα14i NKT cell activation with the C20:2 analogue compared with KRN7000.

Recognition of KRN7000 and C20:2 by Identical Cell Populations. CD1d complexes containing the α-GalCer analogue OCH have been shown to have significantly reduced avidity for TCRs of Vα14i NKT cells compared with binding of KRN7000-loaded complexes (31). This finding suggests the possibility that the T_H2-biased response of C20:2 could be a result of preferential stimulation of Vα14i NKT cell subsets with TCRs of higher affinity for lipid-loaded CD1d. In fact, phenotypically defined subsets of murine and human NKT cells have been described that show a bias toward increased production of IL-4 relative to IFN-γ upon stimulation (32–36). However, by costaining of splenic and thymic Vα14i NKT cells by using CD1d tetramers loaded with different lipids, we demonstrated that identical populations recognized C24:0, C20:2, and KRN7000 (Fig. 5*A*). Single staining with these reagents revealed no difference in Vβ usage or NK1.1 status of cells reactive with the different analogue tetramers (Fig. 5 *B* and *C*). Interestingly, C20:2-loaded tetramers stained NKT cells more strongly than tetramers loaded with KRN7000, reflecting a slightly higher affinity of the C20:2–CD1d complex to the Vα14i TCR (J.S.I. and S.A.P., unpublished results). Together, these findings demonstrated that the altered cytokine response to C20:2 cannot be the result of preferential activation of a subset of Vα14i NKT cells.

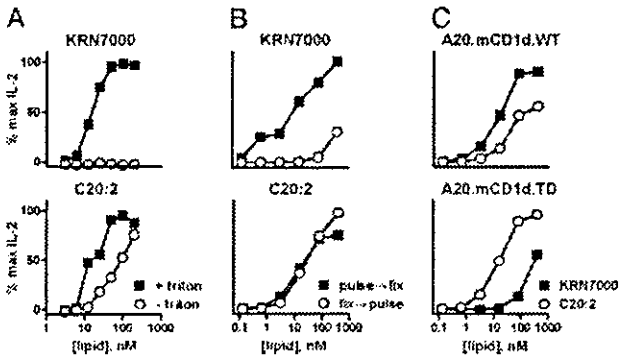

Fig. 6. Differential requirements for CD1d loading with KRN7000 and C20:2. IL-2 response of hybridoma DN3A4-1.2 to glycolipid presentation in three *in vitro* CD1d presentation systems: platebound CD1d loaded with varying amounts of KRN7000 or C20:2 in the presence or absence of the detergent Triton X-100 (*A*), RMA-S.CD1d cells pulsed with glycolipid before or after glutaraldehyde fixation (*B*), or WT or cytoplasmic tail-deleted (TD) CD1d-transfected A20 cells, loaded with either KRN7000 or C20:2 (*C*).

Loading Requirements of α-GalCer Analogues onto CD1d. To find an alternative explanation for the T_H2-biased response to C20:2, we studied requirements for handling of different forms of α-GalCer by antigen-presenting cells. We employed a cell-free system in which platebound mouse CD1d was loaded with doses of KRN7000 or C20:2 in the presence or absence of the detergent Triton X-100 (37). By using IL-2 production by DN3A4-1.2 as a readout for glycolipid loading of CD1d, we observed a marked dependence on detergent for loading of KRN7000 but not for C20:2 (Fig. 6*A*). This result suggested a significant difference in requirement for cofactors, such as acidic pH or lipid transfer proteins, that facilitate lipid loading onto CD1d in endosomes (38–41). We assessed this hypothesis further by using glutaraldehyde fixation of $CD1d^+$ antigen-presenting cells, which blocks antigen uptake and recycling of CD1d between endosomes and the plasma membrane. Vα14i NKT cell recognition of KRN7000 was markedly reduced if lipid loading was done after fixation of RMA-S.mCD1d cells, whereas recognition of C20:2 was unimpaired (Fig. 6*B*).

Similar conclusions were drawn from experiments by using A20 cells transfected with either WT or cytoplasmic tail-deleted CD1d (Fig. 6*C*). The tail-deleted CD1d mutant lacks the intracellular tyrosine-based sorting motif required for internalization and endosomal localization of CD1d (19). As was the case with RMA-S.mCD1d, WT CD1d-transfected A20 cells presented KRN7000 more potently than C20:2. However, the tail-deleted mutant presented C20:2 with at least 20-fold greater efficiency than KRN7000. Together, these results point to the conclusion that the T_H2-skewing C20:2 analogue had substantially less dependence on endosomal loading for presentation by CD1d when compared with compounds that produced a more mixed response with strong IFN-γ production, such as KRN7000.

Discussion

This study details *in vitro* and *in vivo* consequences of activation of Vα14i NKT cells with C20:2, a diunsaturated *N*-acyl substituted analogue of the prototypical α-GalCer, KRN7000. The T_H2 cytokine bias observed with C20:2 is not unique: OCH and other shortened fully saturated lipids have been shown to have this effect (13, 42). C20:2 differs from these other compounds in two potentially important respects. First, the *in vitro* potency of C20:2 for stimulation of certain Vα14i NKT cell functions (e.g., proliferation and secretion of IL-4 and IL-2) approaches that of KRN7000, whereas OCH appears to be a much weaker Vα14i NKT cell agonist. Second, staining with C20:2-loaded CD1d tetramers, as opposed to OCH, is undiminished compared with KRN7000. This finding would suggest that, as a therapeutic agent, C20:2 will be recognized by the identical global Vα14i NKT cell population (as KRN7000 is) and not limited to higher-affinity NKT cell subsets, as suggested for OCH (31).

A recent study showed that one mechanism by which OCH may induce a T_H2-biased cytokine response involves changes in IFN-γ production by Vα14i NKT cells themselves. Oki *et al.* (43) reported that the transcription factor gene *c-Rel*, a member of the NF-κB family of transcriptional regulators that is a crucial component of IFN-γ production, is inducibly transcribed in KRN7000-stimulated but not OCH-stimulated Vα14i NKT cells. Although we have not assessed *c-Rel* induction or other factors involved in IFN-γ production in response to C20:2, our findings did not suggest that early IFN-γ production by Vα14i NKT cells was different after activation with C20:2 versus KRN7000. Both lipids induced identical single-cell IFN-γ staining in Vα14i NKT cells and serum IFN-γ levels at 2 h after injection. However, in contrast to the apparent similarity in Vα14i NKT cells, NK cell IFN-γ production was significantly reduced and less sustained after *in vivo* administration of C20:2 compared with KRN7000. Hence, failure of C20:2 to fully activate downstream events leading to optimal NK cell secondary stimulation by activated Vα14i NKT cells appears to be the most likely mechanism by which C20:2 induces reduced IFN-γ and an apparent T_H2-biased systemic response.

C20:2 administration resulted also in a more rapid but less sustained CD69 up-regulation in NK and B cells, as well as a lack of a substantial Vα14i NKT cell expansion. These findings were surprising, given that TCR down-modulation observed on Vα14i NKT cells within the first few hours after C20:2 stimulation was similar to or greater than that induced by KRN7000 (Fig. 4*A* and data not shown), indicating strong TCR signaling in response to the analogue. These features of the response to C20:2 may be a further reflection of the failure of C20:2 to induce a full range of downstream events after Vα14i NKT cell activation, including the production of cytokines or other factors required to support the expansion of Vα14i NKT cells.

What mechanism can then be invoked to account for the altered cytokine response to C20:2 and other *N*-acyl variants of KRN7000? One intriguing possibility is provided by our analysis of requirements for presentation of C20:2 compared with KRN7000, which revealed marked differences between these glycolipids in their need for endosomal loading onto CD1d. CD1d and other CD1 proteins undergo transport into the endocytic pathway, leading to intracellular loading with lipid antigens and subsequent recycling to the cell surface (39). The importance of endosomal loading for KRN7000 most likely reflects the impact of factors in these compartments that facilitate the insertion of lipids into the CD1d ligand-binding groove. These factors include the acidic pH of the endosomal environment, as well as lipid transport proteins, such as saposins and GM2 activator protein (38, 40, 41). Our findings indicate that C20:2 can efficiently load onto CD1d in the absence of these endosomal cofactors. Consequently, we speculate that C20:2 may be strongly presented by any cell type that expresses surface CD1d, regardless of its ability to efficiently endocytose lipids from the extracellular space. This more widespread presentation could lead to a more pronounced presentation of C20:2 by nonprofessional antigen-presenting cell types compared with KRN7000. Because many cell types express CD1d, including all hematopoietic lineages and various types of epithelia (44–48), presentation of C20:2 by nonprofessional antigen-presenting cells may explain the more rapid trans-activation of bystander cells observed with C20:2. An alternative hypothesis is that the endosomal loading requirements of KRN7000 result in its preferential localization into CD1d molecules contained in membrane lipid rafts, whereas the permissive loading properties of

C20:2 would result in a more uniform glycolipid distribution across the cell membrane. Evidence of lipid raft localization of CD1d and raft influence on the T_H-bias of MHC class II-restricted $CD4^+$ T cells lend support to this model (49, 50). Either scenario would be expected to result in decreased delivery of costimulatory signals associated with professional antigen-presenting cells (e.g., dendritic cells) and, thus, lead to quantitative and qualitative differences in the outcome of Vα14i NKT cell stimulation. Consistent with both models, Vα14i NKT cell activation with KRN7000 *in vitro* in the presence of costimulatory blockade (anti-CD86) can polarize cytokine production to a T_H2 profile (22).

We have shown that structurally modified forms of α-GalCer with alterations in their *N*-acyl substituents can be designed to generate potent immunomodulators that stimulate qualitatively altered responses from Vα14i NKT cells. Our results confirm and extend several basic observations and principles established from earlier studies on less potent agonists, such as OCH. Further study of these and similar analogues may yield compounds with clear advantages for treatment or prevention of specific immunologic disorders or for the stimulation of protective host immunity against particular pathogens.

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The clinical implication and molecular mechanism of preferential IL-4 production by modified glycolipid-stimulated NKT cells

Shinji Oki, Asako Chiba, Takashi Yamamura, and Sachiko Miyake

Department of Immunology, National Institute of Neuroscience, National Center for Neuroscience and Psychiatry, Tokyo, Japan.

OCH, a sphingosine-truncated analog of α-galactosylceramide (αGC), is a potential therapeutic reagent for a variety of Th1-mediated autoimmune diseases through its selective induction of Th2 cytokines from natural killer T (NKT) cells. We demonstrate here that the NKT cell production of IFN-γ is more susceptible to the sphingosine length of glycolipid ligand than that of IL-4 and that the length of the sphingosine chain determines the duration of NKT cell stimulation by CD1d-associated glycolipids. Furthermore, IFN-γ production by NKT cells requires longer T cell receptor stimulation than is required for IL-4 production by NKT cells stimulated either with immobilized mAb to CD3 or with immobilized "αGC-loaded" CD1d molecules. Interestingly, transcription of *IFN-γ* but not that of *IL-4* was sensitive to cycloheximide treatment, indicating the intrinsic involvement of de novo protein synthesis for IFN-γ production by NKT cells. Finally, we determined *c-Rel* was preferentially transcribed in αGC-stimulated but not in OCH-stimulated NKT cells and was essential for IFN-γ production by activated NKT cells. Given the dominant immune regulation by the remarkable cytokine production of ligand-stimulated NKT cells in vivo, in comparison with that of (antigen-specific) T cells or NK cells, the current study confirms OCH as a likely therapeutic reagent for use against Th1-mediated autoimmune diseases and provides a novel clue for the design of drugs targeting NKT cells.

Introduction

Natural killer T (NKT) cells are a unique subset of T lymphocytes that coexpress the α/β T cell receptor (TCR) along with markers of the NK lineage such as NK1.1, CD122, and various Ly49 molecules. Most NKT cells express an invariant TCRα chain composed of Vα14-Jα281 segments in mice and Vα24-JαQ segments in humans associated with a restricted set of Vβ genes (1, 2). Unlike conventional T cells, which recognize peptides presented by MHC molecules, NKT cells recognize glycolipid antigens such as α-galactosylceramide (αGC) in the context of a nonpolymorphic MHC class I-like molecule, CD1d (3–5). After being stimulated by a ligand, NKT cells rapidly affect the functions of neighboring cell populations such as T cells, NK cells, B cells, and dendritic cells (6, 7). The various functions of NKT cells are mediated mainly by a rapid release of large amounts of cytokines, including IL-4 and IFN-γ. Whereas IFN-γ provides help for the Th1 responses required for defending against various pathogens and tumors, IL-4 controls the initiation of Th2 responses and has been shown to inhibit Th1-mediated autoimmune responses involved in experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis (CIA), and type 1 diabetes in NOD mice.

Given the exceptional ability of NKT cells to secrete regulatory cytokines in comparison with that of T cells or NK cells after primary stimulation, we have explored the possibility that ligand stimulation of NKT cells may lead to the suppression of Th1-mediated autoimmune diseases. We have previously demonstrated that OCH, a sphingosine-truncated analog of αGC, preferentially induces Th2 cytokines from NKT cells and that administration of OCH suppresses EAE and CIA by inducing a Th2 bias in autoantigen-reactive T cells (8, 9). However, the molecular mechanism accounting for the unique property of OCH to selectively induce IL-4 has not been clarified yet.

In this study, we used various stimuli, including the prototypic ligand αGC and its derivatives such as OCH, to investigate the molecular basis of the differential production of IL-4 and IFN-γ by NKT cells. We found that OCH, due to its truncated lipid chain, was less stable in binding the CD1d molecule than was αGC and exerted short-lived stimulation on NKT cells. IFN-γ production by NKT cells required longer TCR stimulation than was required for IL-4 production and de novo protein synthesis. *c-Rel* was preferentially transcribed in αGC-stimulated, but not in OCH-stimulated NKT cells and was shown to regulate IFN-γ production by NKT cells. Taken together, these results indicate that sustained TCR stimulation and concomitant c-Rel expression by αGC leads to the production of IFN-γ, whereas short-term activation and marginal *c-Rel* transcription by OCH results in preferential production of IL-4 by NKT cells.

Methods

Mice. C57BL/6 (B6) mice were purchased from CLEA Laboratory Animal Corp. (Tokyo, Japan). MHC class II–deficient I-$A^b\beta^{-/-}$ mice were purchased from Taconic (Germantown, New York, USA). All animals were kept under specific pathogen-free conditions and were used at 7–10 weeks of age. Animal care and use were in accordance with institutional guidelines.

Cell lines, antibodies, plasmids, and reagents. The NKT cell hybridoma (N38.2C12) (10) was a generous gift from K. Hayakawa (Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA) and NS0-derived

Nonstandard abbreviations used: altered glycolipid ligand (AGL); altered peptide ligand (APL); CD28 responsive element (CD28RE); collagen-induced arthritis (CIA); c-Rel lacking C-terminal transactivation domain (c-RelΔTA); cycloheximide (CHX); cyclosporin A (CsA); experimental autoimmune encephalomyelitis (EAE); α-galactosylceramide (αGC); natural killer T (NKT); nuclear factor of activated T cell (NF-AT); phycoerythrin (PE); T cell receptor (TCR).

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plasmacytoma cell lines expressing the Kb tail mutant of CD1d (11) were kindly provided by S. Joyce (Vanderbilt University, Nashville, Tennessee, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 2 mM sodium pyruvate, and 50 μM β-mercaptoethanol (complete medium). Phycoerythrin (PE)-labeled mAb to NK1.1 (PK136), peridinine chlorophyll protein/cyanine 5.5 -labeled mAb to CD3 (2C11), and recombinant soluble dimeric human CD1d:Ig fusion protein (DimerX I) were from BD PharMingen (San Diego, California, USA). For some experiments mAb's to NK1.1 (PK136) and CD3 (2C11) were conjugated with FITC. Polyclonal antibody to asialo GM_1 was purchased from WAKO Chemicals (Osaka, Japan). The pRc/CMV-c-Rel expression plasmid (12) was a generous gift from Grundström (Umeå University, Umeå, Sweden). The open reading frame of c-Rel cDNA was amplified by PCR and cloned into the retroviral pMIG(W) vector. The forward primer containing the *Xho*I recognition site was 5′-GACTCTCGAGATGGCCTCGAGTGGATATAA-3′ and the reverse primers used for wild-type c-Rel or the dominant negative mutant c-RelΔTA containing *Eco*RI recognition sites were 5′-GACTGAATTCTTATATTTTAAAAAAACCATATGTGAAGG-3′ and 5′-GACTGAATTCTTAACTCGAGATGGACCCGCATG-3′, respectively. The retroviral vector (pMIG) and packaging vector (pCL-Eco) were kindly provided by L. Van Parijs (Massachusetts Institute of Technology, Cambridge, Massachusetts, USA). Cyclosporin A (CsA) and cycloheximide (CHX) were from Sigma-Aldrich (St. Louis, Missouri, USA). All glycolipids were prepared as described in the Supplemental Methods (supplemental material available at http://www.jci.org/cgi/content/full/113/11/1631/DC1). The glycolipids were solubilized in DMSO (100 μg/ml) and were stored at –20°C until use.

Kinetic analysis of glycolipid stability on CD1d molecules. The kinetic analysis of glycolipid stability on CD1d molecules was performed as described previously with slight modifications (13). In brief, the NKT hybridoma was preincubated with 4 μM Fura red and 2 μM Fluo-4 (Molecular Probes, Eugene, Oregon, USA) at room temperature for 45 minutes, washed with RPMI 1640 medium containing 2% FCS (assay media), and resuspended in assay media. For determination of the optimal time for glycolipid loading onto $CD1d^+$ APCs, kinetic analysis was conducted using either αGC or OCH. According to the data obtained in Figure 2C, $CD1d^+$ APCs were pulsed with glycolipids (100 ng/ml) for 30 minutes. Then, cells were washed and resuspended in assay media. Glycolipid-pulsed APCs were harvested every 15 minutes after resuspension, mixed with NKT cells, and subjected to centrifugation in a table-top centrifuge (2,000 *g*) for 60 seconds. Cells were then resuspended briefly and analyzed for calcium influx into NKT hybridoma cells by flow cytometry (EPICS XL; Beckman Coulter, Tokyo, Japan). Activation was expressed as the percentage of Fura-red- and Fluo-4-stained cells in a high-FL1, low-FL4 gate.

In vivo glycolipid treatment and microarray analysis. Mice were injected intraperitoneally with 0.2 ml PBS containing 0.1 mg anti-asialo GM_1 Ab. Forty hours after injection, mice were injected intraperitoneally with αGC, OCH (100 μg/kg), or control vehicle in 0.2 ml PBS. After the indicated time point, liver mononuclear cells or spleen cells were harvested and NKT cells were purified with the AUTOMACS cell purification system using FITC-conjugated mAb to NK1.1 (PK136) and anti-FITC microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The purity of NKT cells in the untreated samples and in the samples treated for 1.5 hours was more than 90%. The purity of the liver-derived samples and spleen-derived samples treated for 12 hours was more than 80% and 74%, respectively. Total RNA isolation with the RNeasy Mini Kit (Qiagen, Chatsworth, California, USA) and whole-microarray procedures using U74Av2 arrays (GeneChip System; Affymetrix, Santa Clara, California, USA) were done according to the manufacturers' instructions. From data image files, gene transcript levels were determined using algorithms in the Gene Chip Analysis Suit software (Affymetrix). Each probe was assigned a "call" of present (expressed) or absent (not expressed) using the Affymetrix decision matrix. Genes were considered to be differentially expressed when (a) expression changed at least threefold in the case of liver NKT-derived samples or twofold in the case of spleen NKT-derived samples compared with the expression in the negative control and (b) increased gene expression included at least one "present call."

In vitro stimulation. Liver mononuclear cells were isolated from B6 mice by Percoll density gradient centrifugation and were stained with PE-NK1.1 and FITC-CD3 mAb's. The $CD3^+NK1.1^+$ cells and $CD3^+NK1.1^-$ cells were sorted with an EPICS ALTRA Cell Sorting System (Beckman Coulter). The purity of the sorted cells was more than 95%. Sorted cells were suspended in RPMI 1640 medium supplemented with 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 10% FCS and were stimulated with immobilized mAb to CD3. Incorporation of [^{3}H]thymidine (1 μCi/well) for the final 16 hours of the culture was analyzed with a β-1205 counter (Pharmacia, Uppsala, Sweden). We measured the content of cytokines in the culture supernatants by ELISA. For quantitative PCR analysis, we harvested the cells after stimulation with glycolipid to prepare total RNA. Glycolipid stimulation of spleen cells in vitro was done similarly except that 1% syngeneic mouse serum was used instead of FCS. In some experiments, plates were coated with DimerX I (1 μg in 50 μl PBS per well) for 16 hours. After plates were washed extensively with PBS, glycolipids (100–200 ng in 50 μl PBS per well) were added, followed by incubation for another 24 hours. Then, NKT cells were added and cytokine production was analyzed after 72 hours of incubation.

Real-time PCR to monitor gene expression. Real-time PCR was conducted using a Light Cycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's specifications using 4 mM $MgCl_2$ and 1 pM primers. Values for each gene were normalized to those of a housekeeping gene (*GAPDH*) before the "fold change" was calculated (using crossing point values) to adjust for variations between different samples. Primers used for the analysis of gene expression are described in Supplemental Methods.

ELISA. For evaluation of cytokine production by NKT cells, sorted liver $CD3^+NK1.1^+$ NKT cells were stimulated with immobilized mAb to CD3 in complete medium. The level of cytokine production in cell culture supernatants or in serum was determined by standard sandwich ELISA using purified and biotinylated mAb sets and standards from BD PharMingen. After the addition of a substrate, the reaction was evaluated using a Microplate reader (BioRad).

Retroviral infection of NKT cells. The 293T cells were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 2 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Liver mononuclear cells were purified and cultured in complete medium supplemented with IL-2 (200 U/ml) for 24–48 hours. Cells were infected with retrovirus prepared by cotransfection of pMIG retroviral vector and pCL-Eco packaging vector into 293T cells. Cells were cultured in complete medium containing IL-2 and IL-15 (50 ng/ml) continuously for 3 days, and

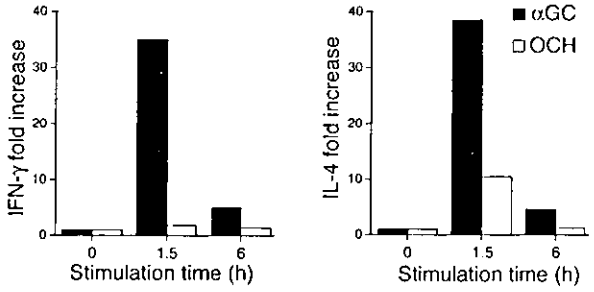

Figure 1
Transcriptional upregulation of cytokine genes by NKT cells stimulated with glycolipids in vivo. B6 mice were injected intraperitoneally with αGC or OCH (100 μg/kg), and liver NKT cells were isolated at the indicated time point. Total RNA was extracted and analyzed for cytokine mRNA by quantitative RT-PCR as described in Methods. Data are presented as "fold induction" of cytokine mRNA after glycolipid treatment. The amount of mRNA in NKT cells derived from untreated animals was defined as 1.

GFP-positive NKT cells were sorted and stimulated with immobilized mAb to CD3 for 48 hours. Culture supernatants were subjected to evaluation of cytokine production by ELISA.

Results

Preferential IL-4 production by OCH-stimulated NKT cells. The suppression of EAE by OCH was found to be associated with a Th2 bias of autoimmune T cells mediated by IL-4 produced by NKT cells (9). To confirm the primary involvement of NKT cells in the Th2 bias seen in the OCH treatment, we purified $CD3^+NK1.1^+$ NKT cells from B6 mice treated in vivo with αGC or OCH and measured the transcription of cytokine genes by quantitative RT-PCR. As shown in Figure 1, treatment with αGC greatly increased the expression of both IFN-γ and IL-4 at 1.5 hours after injection, whereas OCH induced a selective increase in IL-4 expression. When the IL-4/IFN-γ ratio was used for evaluating the Th1/Th2 balance, the NKT cells, isolated at 1.5 hours after injection of OCH were distinctly biased toward Th2 (Table 1). These results indicate that OCH is a selective inducer of rapid IL-4 production by NKT cells when administered in vivo.

Lipid chain length and cytokine production. Comparison of the structural difference between OCH and αGC (Figure 2A) raised the possibility that the lipid chain length of the glycolipid ligand may influence the cytokine profile of glycolipid-treated NKT cells. We compared αGC and OCH as well as newly synthesized analogs F-2/S-3 and F-2/S-7, which bear lipids of intermediate length (Figure 2A), for their ability to induce cytokine production by splenocytes. There was good correlation between the lipid tail length of each glycolipid and its ability to induce IFN-γ from the splenocytes, and a larger amount of IFN-γ was released into the supernatants after stimulation with the glycolipids with the longer sphingosine chain (Figure 2B, right). Regarding the ability to stimulate IL-4 production, the differences among OCH, F-2/S-3 and F-2/S-7 were less clear, as shown by IFN-γ induction. Similar results were obtained with liver mononuclear cells as responder cells (see Supplemental Figure 1). These results indicate that cytokine production by NKT cells, in particular IFN-γ production, is greatly influenced by lipid chain truncation of the glycolipid.

Differential half-life of NKT cell stimulation by CD1d-associated glycolipids. It is believed that the two lipid tails of the glycolipids (sphingosine base and fatty acyl chain) would be accommodated by the highly hydrophobic binding grooves of CD1d. To verify the hypothesis that the functional properties of each glycolipid may be determined by the stability of its binding to CD1d molecules, we evaluated the half-life of these glycolipids on CD1d molecules by estimating calcium influx into NKT hybridoma cells as described previously (13). To exclude the possible involvement of endosomal/lysosomal sorting in this assay, we used APCs expressing a CD1d mutant (Kb tail) that lacks the endosomal/lysosomal targeting signal (11). The cells express both β_2m and sCD1d1 fused to the transmembrane and cytosolic tail sequence of H-$2K^b$ at the carboxyl terminus and could bind to glycolipids such as αGC or OCH without their internalization and following endosomal/lysosomal sorting. Based on the kinetic analysis data for glycolipid loading efficiency shown in Figure 2C, we pulsed $CD1d^+$ APCs with glycolipids for 30 minutes.

Figure 2D shows that OCH was rapidly released from the CD1d molecule. A 30% reduction in calcium influx was observed after 15 minutes of incubation and only 25% of the initial amount of glycolipid remained after 60 minutes of incubation. In contrast, αGC was not released from CD1d molecule in the first 15 minutes and more than 50% of the initial amount of glycolipid remained after 60 minutes of incubation. F-2/S-3 and F-2/S-7 showed intermediate levels of release from CD1d molecule. These results support the idea that a glycolipid with a shorter sphingosine chain has a shorter half-life for NKT cell stimulation because of less-stable association with the CD1d molecule.

Kinetic analysis of cytokine production by activated NKT cells. Previous in vivo studies demonstrated that injection of αGC into B6 mice can induce a rapid and transient elevation of the serum IL-4 level and a delayed and persistent rise in IFN-γ (9, 14), suggesting that there is an intrinsic difference in kinetics for the production of IL-4 and IFN-γ by NKT cells. To address this issue further, we sorted $CD3^+NK1.1^+$ NKT cells, and conventional $CD3^+NK1.1^-$ T cells as a control, from liver lymphocytes and stimulated the sorted cells with immobilized mAb to CD3 for various periods of time. The cells were then incubated at rest without further stimulation and culture supernatants were harvested at 72 hours after initiation of the TCR stimulation. We found that TCR stimulation of NKT cells for as little as 2 hours could induce detectable IL-4 in the supernatant (Figure 3A, center). The amount of IL-4 in the supernatant rapidly increased in proportion to the duration of TCR stimulation (Figure 3A, center). In contrast, production of IFN-γ by NKT cells required at least 3 hours of TCR stimulation and gradually increased corresponding to the duration of TCR stimulation (Figure 3A, right). Conventional T cells required longer TCR stimulation for efficient cytokine production. We repeatedly confirmed that IFN-γ production by NKT cells required initial stimulation that was 1–2 hours longer and showed a slower accumulation than that of IL-4 production in this experimen-

Table 1
Transcriptional upregulation of cytokine genes by NKT cells stimulated with glycolipids in vivo

Stimulus	Time	*IFN-γ*	*IL-4*	Ratio (*IL-4*/*IFN-γ*)
αGC	1.5 h	35.0	38.3	1.09
	6 h	5.0	4.6	0.92
OCH	1.5 h	1.8	10.3	5.58
	6 h	1.5	1.1	0.72

The relative amounts of transcripts of *IFN-γ* and *IL-4* obtained from the experiment shown in Figure 1 are presented as "fold induction" relative to that of NKT cell–derived samples from untreated animals.

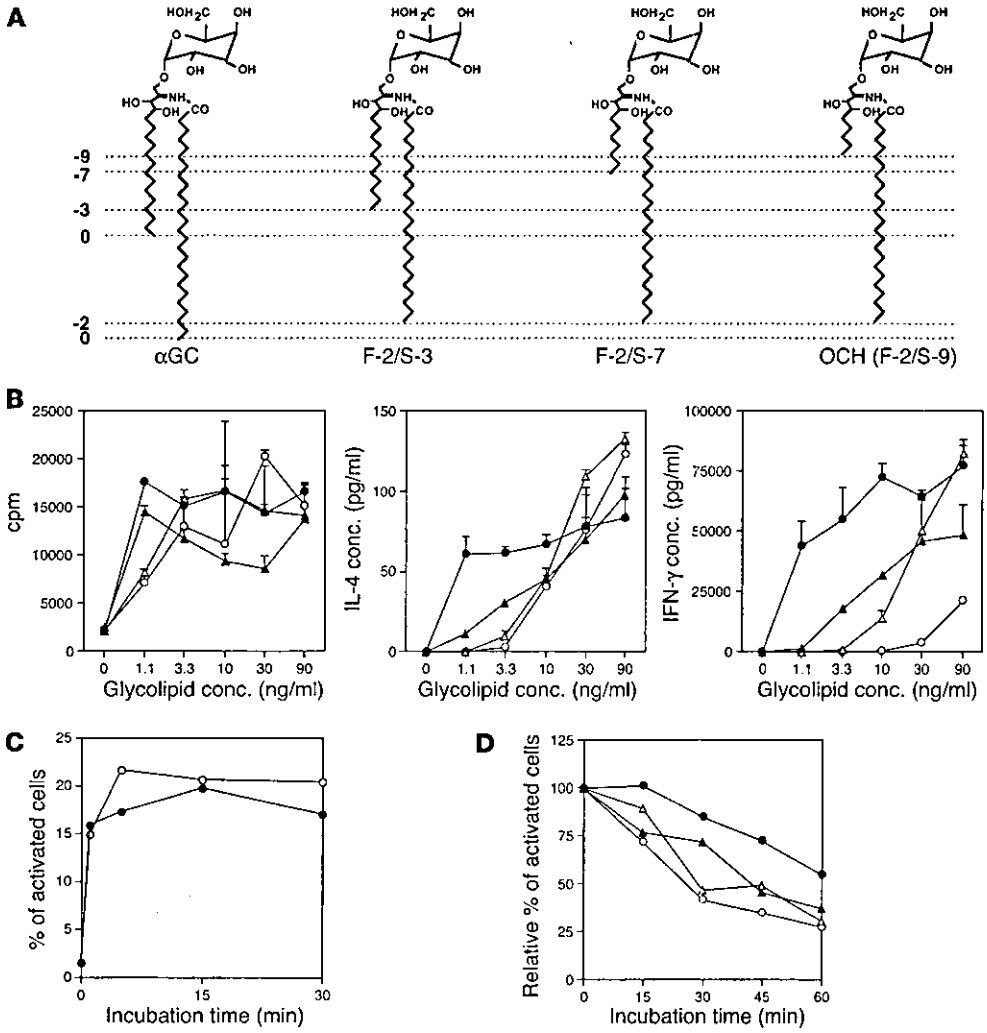

Figure 2

Differential properties of structurally distinct glycolipid derivatives. (**A**) Structures of αGC, OCH, and two other glycolipid ligands for NKT cells. F-2/S-3 has a truncation of two hydrocarbons in the fatty acyl chain (F) and of three hydrocarbons in the sphingosine chain (S) in comparison with αGC. OCH can be called F-2/S-9 accordingly. The numbers of truncated hydrocarbons in either lipid chain are shown along the left margin as negative integers. (**B**) Effect of αGC, OCH, and other glycolipids on proliferation and cytokine production of splenocytes. Splenocytes were stimulated with various concentrations (conc.) of αGC (filled circles), OCH (open circles), F-2/S-3 (filled triangles), or F-2/S-7 (open triangles) for 72 hours. Incorporation of [^{3}H]thymidine (1 μCi/well) during the final 16 hours of the culture was assessed (left), and IL-4 (center) or IFN-γ (right) in the supernatants was measured by ELISA. (**C**) Kinetic analysis of the loading of αGC (filled circles) or OCH (open circles) onto CD1d$^+$ APCs. See Methods for details. One experiment representative of two independent experiments with similar results is shown. (**D**) Calcium influx into NKT hybridoma cells after coculture with CD1d$^+$ APCs pulsed with αGC, OCH, F-2/S-3, or F-2/S-7. Data are presented as the activity remaining when the respective activity of glycolipid-loaded APCs for activation of the NKT cell hybridoma at time 0 was defined as 100%. Data are representative of three experiments with similar results.

tal setting. A similar kinetic difference was also observed when we used spleen-derived NKT cells (data not shown). These results indicate that NKT cells could produce IL-4 after a shorter period of TCR stimulation than is required for IFN-γ production.

To exclude the possibility that a qualitatively different CD1d complex with either αGC or OCH may bind with altered affinity to the TCR, we stimulated NKT cells with plate-bound αGC-CD1d complexes instead of mAb to CD3 for the periods of time indicated in Figure 3B. Consistent with the previous results obtained with anti-CD3 stimulation, the level of IL-4 in the culture supernatant was increased after shorter periods of incubation. In contrast, IFN-γ was efficiently produced after longer incubation, showing that the short pulse of NKT cells with plate-bound αGC-CD1d complexes could recapitulate the OCH phenotype. These results demonstrate that the timing of the CD1d-lipid interaction rather than the "shape" of the OCH-CD1d complex is the decisive factor in controlling polarization of cytokine production by NKT cells.

Differential transcriptional properties of cytokine genes. To clarify the molecular basis for different kinetics of cytokine production by activated NKT cells, we next examined the effects of CsA or CHX on the NKT cell responses. Without any inhibitors, IL-4 production was more rapid and had a higher rate than IFN-γ production (Figure 3C), confirming the kinetic difference required for induction of each cytokine shown in Figure 3A. Production of both IL-4

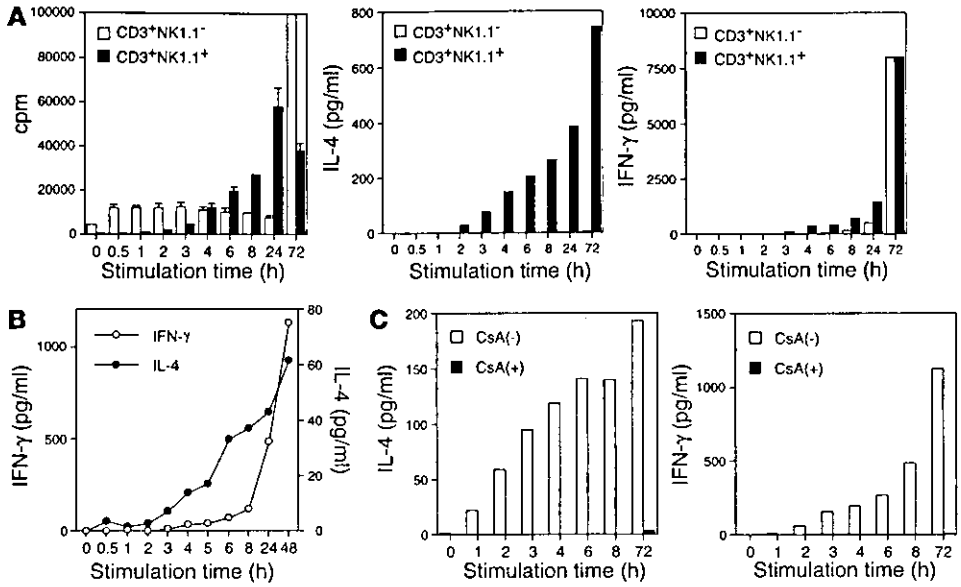

Figure 3

Kinetic analysis of NKT cell activation and cytokine production after glycolipid stimulation. (**A**) Differential production of IFN-γ and IL-4 by activated NKT cells. $CD3^+NK1.1^+$ NKT cells and conventional $CD3^+NK1.1^-$ T cells were purified from liver mononuclear cells by cell sorting. The sorted cells were stimulated with immobilized mAb to CD3 for the time indicated on the *x* axis and were then removed and recultured on a fresh culture plate without anti-CD3 stimulation for up to 72 hours from the start of the anti-CD3 stimulation. Incorporation of [^{3}H]thymidine (1 μCi/well) during the final 16 hours of the culture was assessed (left), and culture supernatants were analyzed for the production of IL-4 (center) and IFN-γ (right) by ELISA. One experiment representative of three independent experiments with similar results is shown. (**B**) NKT cells purified from liver mononuclear cells were stimulated with plates coated with DimerX I loaded with αGC and were analyzed as shown in **A**. (**C**) NKT cells purified from liver mononuclear cells were stimulated as shown in **A** in the presence [CsA(+)] or absence [CsA(–)] of CsA (1 μg/ml). Culture supernatants were analyzed for the production of IL-4 and IFN-γ by ELISA.

and IFN-γ after TCR stimulation, however, was almost completely inhibited by pretreatment of NKT cells with CsA.

Similarly, CsA abolished the transcriptional activation of *IL-4* and *IFN-γ* genes in activated NKT cells (Figure 4A), indicating that TCR signal–induced activation of nuclear factor of activated T cell (NF-AT) is indispensable for the production of both cytokines by NKT cells. Meanwhile, transcription of these cytokine genes showed different sensitivities to CHX treatment (Figure 4A). Although transcriptional activation of *IL-4* was barely affected by CHX treatment, transcription of *IFN-γ* gene was almost completely blocked after treatment with CHX. These results indicate that transcriptional activation of *IFN-γ*, but not that of *IL-4*, requires de novo protein synthesis.

Next, we analyzed the sensitivities of other cytokine genes to CsA and CHX treatment (15–17). As shown in Figure 4B, transcriptional activation of all cytokine genes tested was completely blocked by pretreatment of NKT cells with CsA. Interestingly, transcription of the *IL-2* gene and *GM-CSF* gene were blocked by CHX treatment. In contrast, transcriptional activation of *TNF-α* was resistant to CHX treatment. These results indicate that cytokines produced by NKT cells could be divided into two groups based on their dependence on de novo protein synthesis.

Selective c-Rel induction after stimulation with αGC. Although NKT cells secrete a large number of cytokines upon stimulation, the regulatory mechanisms for the expression of each cytokine are still poorly understood. The susceptibility of IFN-γ production to CHX indicates that some newly synthesized protein(s) would promote specific transcription of the *IFN-γ* gene in NKT cells. To identify the protein responsible for αGC-induced transcription of the *IFN-γ* gene, we purified NKT cells from glycolipid-administered I-$A^b\beta$–deficient mice, which have two- to threefold higher numbers of NKT cells in the liver and the spleen than do wild-type B6 mice (18), and assessed NKT cell–derived total RNA by microarray analysis. As shown in Table 2, a number of cytokines and chemokines were differentially expressed after in vivo treatment with either αGC or OCH. It is noteworthy, however, that significant induction of *IFN-γ* transcription was observed only in αGC-treated samples, not in OCH-treated samples. Overall, the data obtained correlated well with previous results showing that OCH is a selective inducer of IL-4 production from NKT cells (9). There was no transcriptional upregulation of cytokines genes such as the *IFN-γ* and *IL-4* genes 12 hours after treatment with either glycolipid, indicating that NKT cells have undergone quiescence at this time point in the context of transcriptional upregulation of cytokine genes, although some genes are still upregulated.

Through analyzing the microarray data, we identified the protooncogene *c-Rel*, a member of the NF-κB family of transcription factors, as a candidate molecule that may play a role in the *IFN-γ* transcription. As shown in Figure 5A, *c-Rel* was inducibly expressed in NKT cells 1.5 hours after stimulation with αGC. In contrast, OCH treatment did not induce *c-Rel* transcription (Figure 5A). The transcription of other NF-κB family genes such as *p65/RelA* and *RelB* was not upregulated after treatment with αGC or OCH. Real-time PCR analysis also confirmed the selective induction of *c-Rel* after αGC stimulation (Figure 5B). CsA treatment inhibited *c-Rel* transcription, but CHX did not (Figure 5C), indicating that the inducible transcription of c-Rel is directly controlled by TCR signal–mediated activation of the NF-AT (19).

It is already known that c-Rel serves as a pivotal transcription factor for the Th1 response that would directly induce IFN-γ production in conventional T cells (20). However, very little is known about the function of this protooncogene in NKT cells during TCR-mediated activation. We therefore conducted time course analysis for transcriptional activation of *c-Rel* in parallel with *IL-4* and *IFN-γ*. We stimulated NKT cells with immobilized mAb to CD3 for 30–100 minutes and then cultured them without further stimulation for a total of 120 minutes. As shown in Figure 5D, *IFN-γ* expression was slightly downregulated in the first 90 minutes of TCR stimulation and was significantly upregulated when the cells were stimulated for 100 minutes. Interestingly, we found that the kinetics of *c-Rel* transcription were similar to those of *IFN-γ* transcription (Figure 5D, right). In contrast, transcrip-

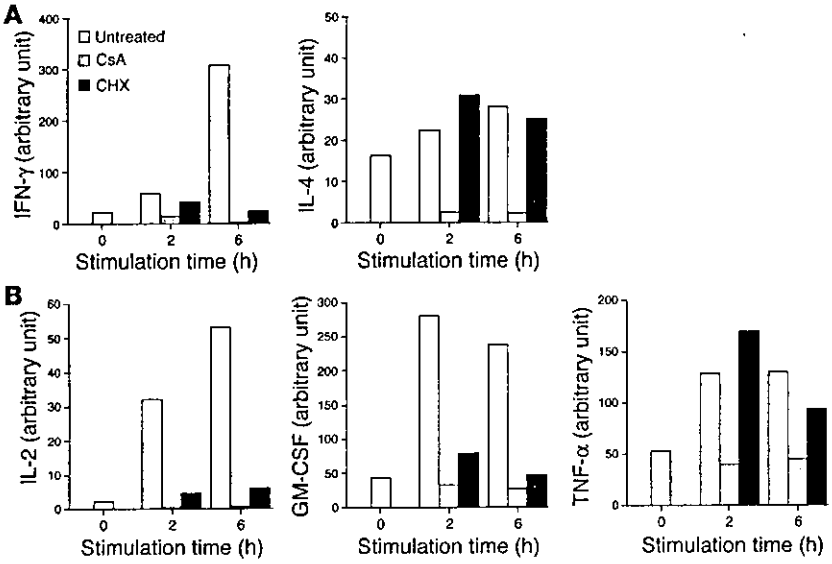

Figure 4
Differential sensitivity to CsA and CHX for transcriptional upregulation of *IFN-γ*, *IL-4*, and other cytokines. (**A**) Sorted NKT cells were pretreated with CsA (1 μg/ml) or with CHX (10 μg/ml) or without either reagent for 10 minutes and were then stimulated with immobilized mAb to CD3 for the indicated periods of time. Total RNA was extracted from each sample and analyzed for the relative amount of transcript of *IFN-γ* or *IL-4*. Data are presented as the amount of transcript in each sample relative to GAPDH. (**B**) Sorted NKT cells were pretreated with CsA (1 μg/ml) or with CHX (10 μg/ml) or without either reagent as shown in **A**. Total RNA was extracted from each sample and was analyzed for the relative amount of transcripts of *IL-2*, *GM-CSF*, or *TNF-α*. Data are presented as the relative amount of transcript in each sample.

tional activation of *IL-4* became evident 30 minutes after TCR stimulation and the transcript accumulated gradually in proportion to the duration of TCR stimulation. This result further confirmed that NKT cells require a longer TCR stimulus for IFN-γ expression.

Transcription of IFN-γ genes depends on c-Rel expression in NKT cells. To further investigate the functional involvement of c-Rel in the transcription of *IFN-γ* gene in NKT cells, we next examined whether forced expression of wild-type c-Rel or of its loss-of-function mutant could affect IFN-γ production by NKT cells. For this, we used bicistronic retroviral vectors expressing c-Rel along with GFP (pMIG/c-Rel) or a c-Rel dominant negative mutant that lacks the C-terminal transactivation domain but retains an intact Rel homology domain of c-Rel protein (pMIG/c-RelΔTA) (21) (Figure 6A). We infected liver-derived mononuclear cells with either retrovirus and stimulated sorted GFP-positive NKT cells with immobilized mAb to CD3 to analyze cytokine production. Retroviral transduction led to expression of GFP in approximately 10% of NKT cells (Figure 6B). Upon stimulation with mAb to CD3, GFP-positive cells from pMIG/c-Rel-infected cultures showed slightly augmented IFN-γ production compared with that of control pMIG-infected cells (Figure 6C). In contrast, GFP-positive cells from pMIG/c-RelΔTA-infected cultures secreted almost no IFN-γ after TCR stimulation (Figure 6C). These results demonstrate that inhibition of c-Rel function, via the introduction of a mutant form of c-Rel, abolishes IFN-γ production and that functional c-Rel is important for effective production of IFN-γ in activated NKT cells.

Discussion

In this study, we investigated the molecular mechanism for differential production of IFN-γ and IL-4 by activated NKT cells through a comparative analysis using the prototypic NKT cell ligands αGC and OCH. Treatment with αGC induced expression of both IFN-γ and IL-4 simultaneously, but OCH induced selective expression of IL-4 by NKT cells. Furthermore, we demonstrated that the CD1d-associated glycolipids with various lipid chain lengths showed different half-lives for NKT cell stimulation when applied in an endosome/lysosome-independent manner and induced the differential cytokine production by NKT cells in a lipid length–dependent manner. Accordingly, we demonstrated that IFN-γ production by NKT cells required longer TCR stimulation than did IL-4 production and depended on de novo protein synthesis. An NF-κB family transcription factor gene, the *c-Rel* gene, was inducibly transcribed in αGC-stimulated but not in OCH-stimulated NKT cells. Retroviral transduction of a loss-of-function mutant of c-Rel revealed the functional involvement of c-Rel in IFN-γ production by ligand-activated NKT cells. These results have provided a new interpretation of NKT cell activation — that the duration of TCR stimulation is critically influenced by the stability of each glycolipid ligand on CD1d molecules, which leads to the differential cytokine production by NKT cells.

We have previously demonstrated that administration of OCH consistently suppresses the development of EAE by inducing a Th2 bias in autoimmune T cells and that this Th2 shift is probably mediated by selective IL-4 production by NKT cells in vivo (9). Here we directly evaluated the cytokine profile of OCH-stimulated NKT cells using quantitative PCR analysis. Consistent with the previous assumption, NKT cells stimulated with OCH induced rapid production of IL-4 but led to only marginal induction of IFN-γ, confirming the presumed mechanism for the effect of OCH on EAE and CIA. As the "fold induction" of *IFN-γ* transcript after 1.5 hours of stimulation with αGC in microarray analysis was relatively low (fivefold for liver NKT cells and fourfold for spleen NKT cells) compared with the in vivo data, there are several possibilities to explain these results. First, quiescent transcripts of *IFN-γ* pre-existing in resting Vα14-invariant NKT cells (22) may raise the baseline of signal intensity in samples from untreated animals, resulting in a relative decrease in "fold induction" after glycolipid treatment. Second, detection of *IFN-γ* transcription in αGC-stimulated NKT cells might not be optimal, as injection of αGC induced a rapid elevation in IL-4 with the peak value at 2 hours and a delayed and prolonged elevation in IFN-γ in B6 mice (9). Third, αGC treatment significantly induces transcription of *CD154* (18.0-fold for αGC vs. 5.4-fold for OCH; data not shown), whose promoter has a functional NF-AT binding site and CD28 responsive element (CD28RE) (23, 24). Thus, augmented CD40/CD154 interaction may induce IL-12 expression by APCs, resulting in additional IFN-γ production (25). Finally, NKT cells are not necessarily the only source of IFN-γ after in vivo stimulation with αGC. The "serial" production of IFN-γ by NKT cells and NK cells has been demonstrated (6, 26). In particular, a C-glycoside analog of αGC has

Table 2
Differential gene expression patterns in αGC-treated and OCH-treated murine NKT cells

		Liver CD3⁺NK1.1⁺								Spleen CD3⁺NK1.1⁺							
		Untreated	αGC			OCH				Untreated	αGC			OCH			
Common name	GenBank		1.5 h		12 h		1.5 h		12 h		1.5 h		12 h		1.5 h		12 h
IFN-γ	K00083	1.0 P	5.0	P	0.3	P	1.2	P	0.1	P	1.0 P	4.0	P	2.3	P	0.7	P	1.0 P
IL-2	m16762	1.0 A	391.4	P	1.2	A	12.3	P	1.3	A	1.0 A	23.4	P	0.2	A	1.0	A	0.3 A
IL-2	K02292	1.0 A	129.6	P	0.6	A	32.8	A	1.1	A	1.0 A	16.1	A	0.7	A	10.7	A	1.5 A
GM-CSF	X03020	1.0 P	38.0	P	0.4	A	4.1	P	0.1	A	1.0 A	15.7	P	1.4	A	2.7	A	2.1 A
IL-4	X03532	1.0 P	276.8	P	2.5	P	47.3	P	0.2	A	1.0 A	364.9	P	35.1	P	38.8	P	4.7 P
IL-4	M25892	1.0 P	38.2	P	0.2	P	7.7	P	0.1	A	1.0 P	69.6	P	7.6	P	9.1	P	1.1 P
IL-4	X03532	1.0 A	34.8	P	3.9	A	9.4	A	1.9	A	1.0 A	2.2	A	4.2	A	1.1	A	0.7 A
IL-13	M23504	1.0 A	993.0	P	1.4	A	56.1	P	1.8	A	1.0 A	140.7	P	12.3	A	19.1	A	2.3 A
TNF-α	D84196	1.0 P	30.8	P	2.1	P	1.7	P	1.2	P	1.0 P	16.5	P	2.5	P	1.8	P	2.6 P
Lymphotoxin A	M16819	1.0 P	6.9	P	0.2	A	1.4	P	0.1	A	1.0 P	2.5	P	1.7	P	1.2	P	0.9 P
IL-1α	M14639	1.0 P	25.1	P	5.6	P	3.1	P	4.4	P	1.0 P	6.7	P	5.8	P	1.1	P	2.7 P
IL-1β	M15131	1.0 P	8.0	P	9.8	P	1.3	P	7.9	P	1.0 P	3.3	P	2.2	P	0.6	P	1.5 P
*IL-1*RA	L32838	1.0 P	10.9	P	15.2	P	1.1	A	11.3	P	1.0 P	5.3	P	28.0	P	0.9	P	23.4 P
IL-3	K01668	1.0 A	33.2	P	2.6	A	4.7	A	1.2	A	1.0 A	4.0	A	1.1	A	1.4	A	1.7 A
IL-6	X54542	1.0 A	34.8	P	16.5	P	8.8	P	10.7	P	1.0 A	19.1	P	17.8	P	1.8	A	12.2 A

Real-time PCR analyses were conducted for *IFN-γ* and *IL-4* as well as for other selected cytokine genes listed in Figure 4 (data not shown) to confirm the correlation with those obtained from microarray analysis. Each probe was assigned a "call" of present (P; expressed) or "absent" (A; not expressed) using the Affymetrix decision matrix. GenBank, GenBank accession number; *IL-1*RA, *IL-1* receptor antagonist.

recently been shown to induce Th1-type activity superior to that induced by αGC, and IL-12 is indispensable for the Th1-skewing effect of the analog (27), indicating the importance of IL-12 in augmenting IFN-γ production in vivo (14, 28). Interestingly, the C-glycoside analog induces production of IFN-γ and IL-4 by NKT cells less strongly than does αGC at 2 hours after in vivo administration. Given that αGC and C-glycoside analog have the same structure for their lipid tails, they might be expected to have comparable affinity for CD1d molecules, and the slightly "twirled" α-anomeric galactose moiety between C-glycoside and O-glycoside may modulate the agonistic effect of these glycolipids. Furthermore, the C-glycoside is more resistant to hydrolysis in vivo and may have an advantage for effective production of IL-12 by APCs. In fact, OCH induces marginal IL-12 production after in vivo administration (data not shown), which makes it unable to induce IFN-γ production by various cells. Therefore, the beneficial feature of OCH as an immunomodulator is that it does not trigger production of IFN-γ in vivo.

As described previously, NKT cells recognize glycolipid antigens in the context of the nonpolymorphic MHC class I-like molecule CD1d (4). Crystal structure analysis revealed that the mouse CD1d molecule has a narrow and deep binding groove with extremely hydrophobic pockets, A′ and F′ (29). Thus the two aliphatic hydrocarbon chains would be captured by this binding groove of CD1d and the more hydrophilic galactose moiety of αGC or OCH would be presented to TCR on NKT cells. As OCH is an analog of αGC with a truncated sphingosine chain, it could be predicted that truncation of the hydrocarbon chain would make it more unstable on CD1d, which might then affect the duration of TCR stimulation on NKT cells. We demonstrated in this study that OCH detached from the CD1d molecule more rapidly than did αGC after a short-term pulse in which the glycolipids were segregated from the endosomal/lysosomal pathway. Accordingly, we showed that the initiation of IFN-γ production by NKT cells required more prolonged TCR stimulation than was required for IL-4 production. Methods such as surface plasmon resonance were not appropriate for direct assessment of the interaction between glycolipids and CD1d, possibly because of unpredictable micelle formation and the poor solubility of glycolipids in aqueous solvents (30). The half-life of the interaction of glycolipids and CD1d was reported to be less than 1 minute by surface plasmon resonance (31), contradicting functional assays suggesting a much longer half-life. Therefore, we applied a biological assay to evaluate the stability of these glycolipids on CD1d molecules, as described previously (13).

The characteristics of OCH are somewhat analogous to those of an altered peptide ligand (APL) that has been shown to induce a subset of functional responses observed in intact peptide and, in some cases, induce production of selected cytokines by T cells (32–34). Thus, OCH and possibly other αGC derivatives could be called "altered glycolipid ligands" (AGLs). Although the biological effects of APLs and AGLs could mediate a series of similar molecular events in target cells, it should be noted that APLs and AGLs differ in their "conceptual features." That is, APLs are usually altered in their amino acid residues to modify their affinity for TCRs, whereas AGLs have truncation of their hydrocarbon chain responsible for CD1d anchoring. This paper has highlighted the duration of NKT cell stimulation by CD1d-associated glycolipids as being a critical factor for determining the nature of AGLs for selective induction of cytokine production by NKT cells.

Given that IL-4 secretion consistently precedes IFN-γ production by NKT cells after TCR ligation, we speculated there were critical differences in the upstream transcriptional requirements for the *IFN-γ* and *IL-4* genes in NKT cells. In support of this speculation, CHX treatment specifically inhibited the transcription of *IFN-γ* but not that of *IL-4*. In contrast, transcription of both cytokines was abolished by CsA treatment, indicating that TCR-mediated activation of NF-AT is essential for the production of both cytokines. TCR signal–induced NF-AT activation occurs promptly corresponding to calcium influx (35). Meanwhile, the protein expression of specific

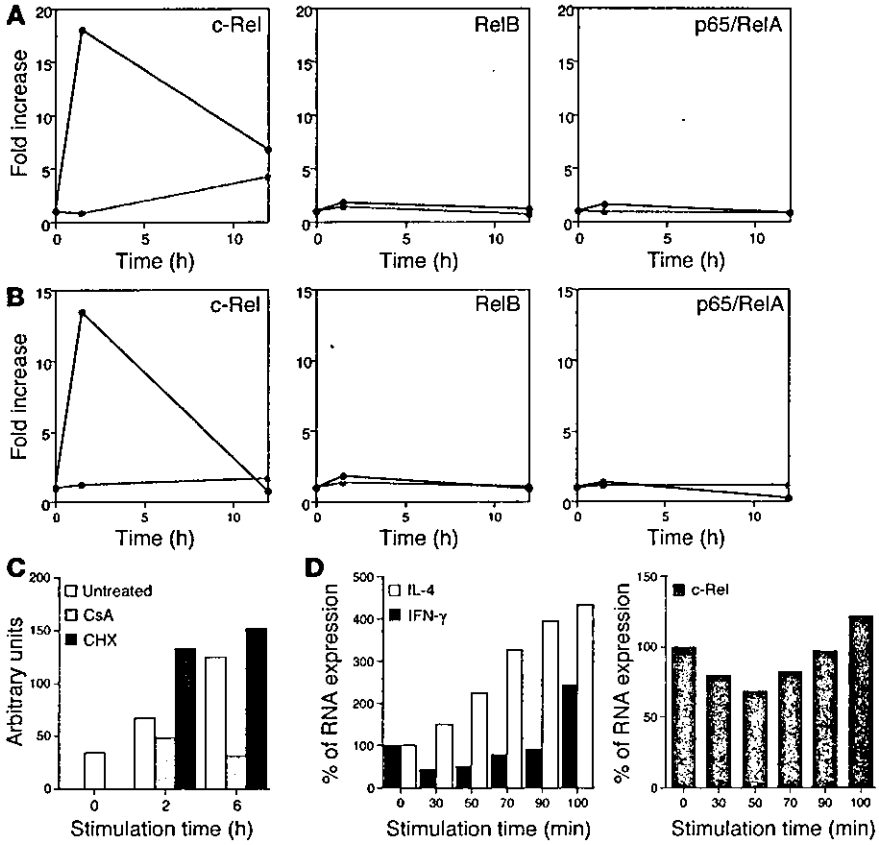

Figure 5
Induction of NF-κB family members in activated NKT cells. (**A**) Plotted values represent data of Affymetrix microarray analysis for the indicated genes.The αGC-stimulated (red lines) or OCH-stimulated (green lines) cells as well as unstimulated liver NKT cells were analyzed at the same time points and the data are presented as the relative value for stimulated NKT cells when the value in NKT cells derived from untreated animals was defined as 1. (**B**) Real-time PCR analysis for the same genes as in **A**. Data are presented as described in Figure 4. (**C**) Sorted liver NKT cells were pretreated with CsA or CHX and were stimulated with immobilized mAb to CD3, and comparative values of c-Rel transcripts relative to GAPDH were determined. (**D**) Sorted liver NKT cells were stimulated with immobilized mAb to CD3 for the indicated periods of time and then were cultured without stimulation for up to a total of 120 minutes after the initial stimulation. Total RNA was extracted from each sample and analyzed for relative amounts of transcripts of *IFN-γ* or *IL-4* (left), or *c-Rel* (right). The amount of RNA derived from unstimulated NKT cells was defined as 100%.

transcription factors takes more time to accomplish. The requirement for prolonged TCR stimulation for initiation of *IFN-γ* transcription may be due to its dependency on specific gene expression.

Recently, Matsuda et al. have shown using cytokine reporter mice that Vα14-invariant NKT cells express cytokine transcripts in the resting state, but express protein only after stimulation (22). We obtained a similar result with our microarray analysis, in that many cytokine transcripts including *IFN-γ* and *IL-4* were detectable in unstimulated NKT cells derived from liver or spleen, because most of them were assigned a "call" of "present" by the Affymetrix decision matrix, which means they were significantly expressed. The mechanism of translation of pre-existing cytokine transcripts after activation of NKT cells remains to be investigated.

Through microarray analysis and real-time PCR, we next identified a member of the NF-κB family of transcription factors, c-Rel, as being a protein rapidly expressed after αGC treatment and possibly responsible for the transcription of *IFN-γ*. Treatment with αGC selectively upregulated *c-Rel* transcription 1.5 hours after stimulation of NKT cells in vivo. OCH treatment, however, showed no induction of *c-Rel* transcription. Although *c-Rel* is transcriptionally upregulated after TCR stimulation of T cells (36), transcription of other NF-κB family members such as *p65/RelA*, *RelB*, *NF-κB1*, and *NF-κB2* was unchanged (data not shown). CsA treatment inhibited c-Rel transcription, but CHX did not, indicating that inducible transcription of c-Rel was directly controlled by TCR signal–mediated activation of NF-AT, which is consistent with a previous report (19). Although the pre-existing NF-κB proteins in general provide a means of rapidly altering cellular responses by inducing the destruction of IκB in order to enable NF-κB to be free for nuclear translocation and DNA binding, most of the nuclear c-Rel induced after T cell stimulation has been shown to be derived from newly translated c-Rel proteins. In contrast, pre-existing c-Rel scarcely translocates to the nucleus at all (36), indicating that the nuclear induction of c-Rel in T lymphocyte requires ongoing protein synthesis. The retrovirally transduced loss-of-function mutant c-Rel (c-RelΔTA) significantly inhibited transcription of *IFN-γ* genes, indicating the crucial role of c-Rel in their transcription after activation of NKT cells. Although it is possible that the Rel domain of the dominant negative mutant may affect a number of NF-κB dimers, it is unlikely, because IFN-γ production by stimulated NKT cells were CHX sensitive and other NK-κB members were not induced after stimulation in the microarray analysis. Retroviral transduction of wild-type c-Rel into NKT cells resulted in slightly augmented expression of IFN-γ after stimulation. Induction of endogenous c-Rel after in vitro stimulation might reduce the effect of retrovirally introduced c-Rel protein.

Whereas c-Rel has been associated with the functions of various cell types, its role in the immune system was first demonstrated in its involvement in *IL-2* transcription (37), in which it possibly induced chromatin remodeling of the promoter (38). Recently, the promoters for the genes encoding *IL-3*, *IL-5*, *IL-6*, *TNF-α*, *GM-CSF*, and *IFN-γ* were shown to contain κB sites or the κB-related CD28RE. Gene targeting of *c-Rel* in mice revealed that *c-Rel*–deficient T cells have a defect in the production of IL-2, IL-3, IL-5, GM-CSF, TNF-α, and IFN-γ, although expression of some of the cytokines was rescued by the addition of exogenous IL-2 (39, 40). Regarding the involvement of c-Rel in IFN-γ production, the c-Rel inhibitor pentoxyfylline (41) selectively suppresses Th1 cytokine production and EAE induction (42), and transgenic mice expressing the *trans*-dominant form of IκBα have a defect in IFN-γ production and the Th1 response (43). Recently, an elegant study using *c-Rel*–deficient mice revealed *c-Rel* has crucial roles in IFN-γ production by activated T cells and conse-

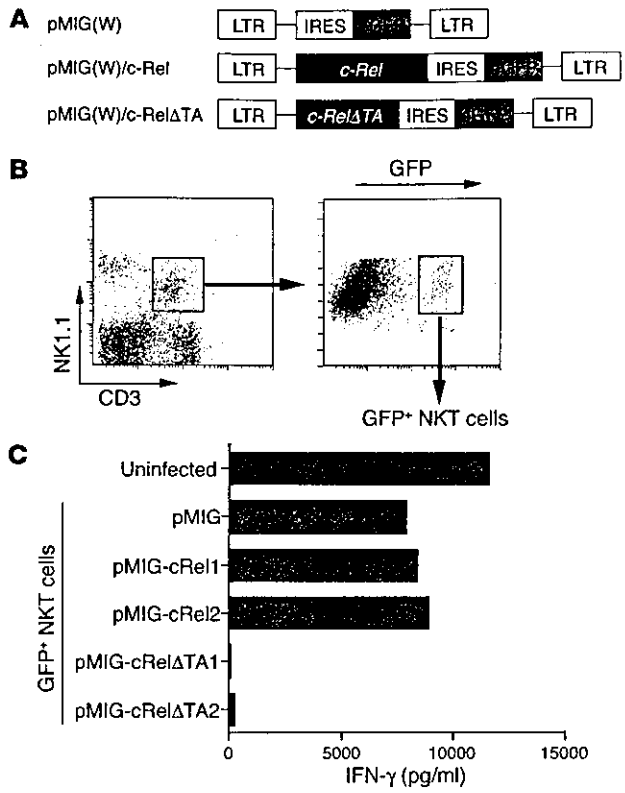

Figure 6
Cytokine production after retroviral transduction of c-Rel or c-RelΔTA into NKT cells. (**A**) DNA fragments encoding wild-type c-Rel or its mutant were cloned into the pMIG(W) bicistronic retrovirus vector. The mutant form of c-Rel (c-RelΔTA) lacks the transactivation domain of the c-Rel protein. LTR, long terminal repeat; IRES, internal ribosome entry site; eGFP, enhanced GFP. (**B**) Flow cytometric identification of cells transfected with the viral vector. Among the NK1.1+CD3+ liver NKT cells identified in the left panel, approximately 10% were GFP positive. The GFP-positive NKT cells were sorted for further analysis. (**C**) IFN-γ production by NKT cells transfected with c-Rel or its dominant negative mutant. The CD3+NK1.1+ NKT cells infected with the viruses were isolated based on their expression of GFP and were stimulated with immobilized mAb to CD3. For transduction of c-Rel or c-RelΔTA into NKT cells, two independent clones of each retroviral vector were used. The level of IFN-γ in the supernatants was measured by ELISA.

quent Th1 development by affecting the cellular functions of both T cells and APCs (20).Thus, the critical involvement of c-Rel for IFN-γ production in NKT cells is consistent with these findings.

Our results indicate that rapid calcium influx and subsequent NF-AT activation is essential for IFN-γ production by activated NKT cells and that c-Rel plays a crucial role in IFN-γ production as well. NF-AT shows quick and sensitive nucleocytoplasmic shuttling after TCR activation (35). Feske et al. demonstrated that the pattern of cytokine production by T cells was determined by the duration of nuclear residence of NF-AT (44) and that sustained NF-AT signaling promoted IFN-γ expression in CD4+ T cells (45). Considering the structural feature of αGC with longer lipid chain, sustained stimulation by αGC induces long-lasting calcium influx, resulting in sustained nuclear residence of NF-AT, and c-Rel protein synthesis, which enables NKT cells to produce IFN-γ. In contrast, the rather sporadic stimulation by OCH induces short-lived nuclear residence of NF-AT, followed by marginal c-Rel expression, which leaves NKT cells unable to produce IFN-γ (Figure 7). Thus, the kinetic and quantitative differences between αGC and OCH in the induction of transcription factors, such as NF-AT and c-Rel, determine the pattern of cytokine production by NKT cells. As CD1d molecules are nonpolymorphic and are remarkably well conserved among the species, the preferential induction of IL-4 production through NKT activa-

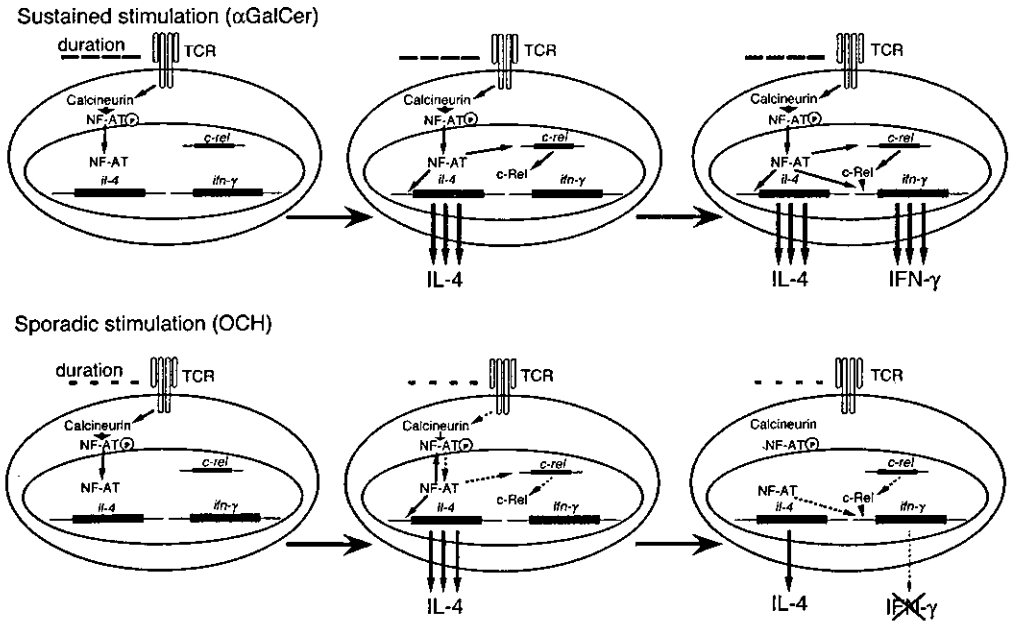

Figure 7
A model for the differential expression of IFN-γ and IL-4 after treatment of NKT cells with αGC or OCH. See text for details.

tion and subsequent Th2 polarization suggest that OCH may be an attractive therapeutic reagent to use for Th1-mediated autoimmune diseases such as multiple sclerosis and rheumatoid arthritis.

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Address correspondence to: Sachiko Miyake, Department of Immunology, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. Phone: 81-42-341-2711; Fax: 81-42-346-1753; E-mail: miyake@ncnp.go.jp.

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