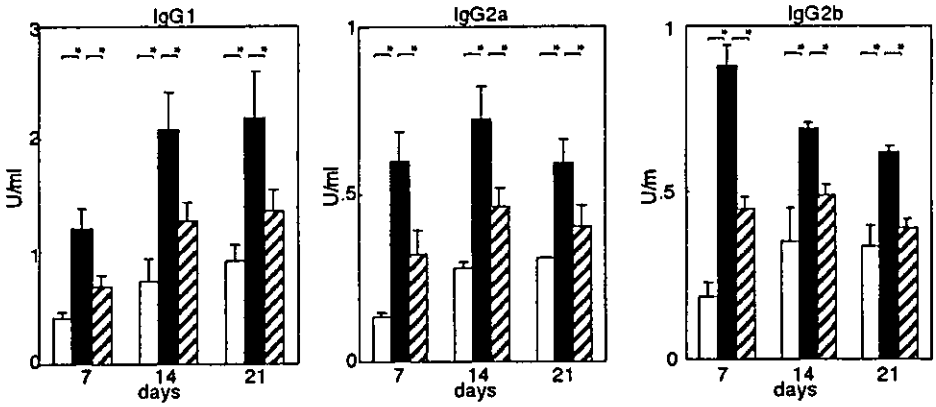

FIGURE 7. Effect of anti-B7h mAb treatment on serum anti-CII IgG titers. Serum levels of anti-CII IgG1, IgG2a, and IgG2b in unboosted immunized mice (□) and the CIA mice treated with control IgG (■) or anti-B7h mAb (▨) were measured by ELISA on days 7, 14, and 21. Data are shown as the mean ± SEM of 10 mice in each group from two experiments. *, Statistically different ($p < 0.05$).

is reasonable that the blockade of ICOS was less effective for preventing the priming of T cells. In contrast, ICOS is expressed on activated T cells and thus the ICOS-B7h interaction may play its primary role in costimulation of already Ag-primed T cells. Consistent with this notion, the expansion of $ICOS^+$ T cells in the draining LN upon second immunization was markedly inhibited by the anti-B7h mAb treatment. In addition, ICOS may also play a costimulatory role in the activation of $ICOS^+$ effector T cells infiltrating in the target tissues as discussed above.

The amelioration of CIA by the anti-B7h mAb treatment appeared to be correlated with the reduction of CII-specific T and B cell responses. T cell proliferative responses to CII and both IFN-γ and IL-10 production were significantly inhibited by the treatment. In addition, a marked reduction in all IgG1, IgG2a, and IgG2b subclasses of serum anti-CII Abs was observed. These results suggested that both Th1- and Th2-mediated immune responses against CII were comparably inhibited by the anti-B7h mAb treatment. It has been reported that the blockade of the ICOS pathway by ICOS-Ig fusion protein or a neutralizing anti-ICOS mAb exhibited prominent inhibitory effects in the effector phase of the Th2-mediated immune responses (10, 27, 45). ICOS is preferentially expressed on Th2 cells at higher levels than on Th1 cells (10, 18, 27) and the blockade of ICOS in vitro preferentially reduced the production of Th2 cytokines such as IL-4 and IL-10 (18). Several reports demonstrated the failure to inhibit Th1-mediated immune responses by the ICOS blockade (10, 45, 46). On the other hand, a successful inhibition of Th1-mediated immune responses such as acute allograft rejection (22) and EAE (23, 26) has been also demonstrated. Our present results add a new example in which the blockade of the ICOS-B7h interaction resulted in inhibition of both Th1- and Th2-mediated immune responses.

In addition to the CII-specific immune responses mediated by T and B cells, locally produced proinflammatory cytokines also play a critical role in the development of arthritis. Among such cytokines, TNF-α, IL-1β, and IL-6 are crucial and the intervention of their actions could be a potential strategy for the treatment of arthritis (29, 47–50). We here showed that the treatment with anti-B7h mAb efficiently reduced the expression of these proinflammatory cytokines in the inflamed joints. It has been reported that TNF-α induced B7h expression on fibroblasts and nonlymphoid cells (11, 12) and that ICOS costimulation strongly enhanced TNF-α production by T cells (51). Therefore, the reciprocal induction of TNF-α and B7h through the ICOS pathway may amplify and perpetuate the local inflammation. In the acute allograft rejection model, the ICOS blockade suppressed the production of chemokines as well as IFN-γ and IL-10 (22). Thus, the ICOS-B7h interaction may be involved in the regulation of multiple cytokines and chemokines that control local inflammatory responses.

In conclusion, the blockade of ICOS costimulation by anti-B7h mAb ameliorated CIA through anti-inflammatory actions and suppression of both Th1- and Th2-mediated responses. Intervention of the ICOS costimulatory pathway may be a novel strategy for the treatment of human rheumatoid arthritis and possibly other chronic inflammatory diseases.

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Short Communication

Complete cDNA Coding Sequence of the HLA-DRB1*1405 Allele

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Cloning report of complete coding region of HLA-DRB1*1405 has never appeared in the literature. We have isolated and sequenced it from a B cell line with a DRB1*1405/1502 genotype. The sequence information of this gene and previously published DRB1 genes for the other DR serotypes facilitated us to find that the cytoplasmic domain sequences of DRB1 genes fall into three groups. It is known that antigen presenting cells are differentially activated when products of different MHC class II genes are triggered. Functional analyses would discern if these allelic differences in the cytoplasmic domain are of functional significance.

Keywords: cDNA; Coding sequence; DRB1*1405; HLA; Nucleotide sequence

Database Accession No: AB062112

Current DNA databases contain complete coding region sequence of a number of HLA genes. One of the exceptions was an DRB1*1405 allelic gene. The DRB1*1405 gene was first identified during survey of Japanese population with HLA-DR14 serotype, and accounts for roughly a quarter of all DR14 serotype-related alleles among Japanese (Obata *et al.*, 1990). We have found that this molecule is involved in T cell response to a mycobacterial bacillus Calmette–Guerin a (BCGa) peptide fragment (Matsushita *et al.*, 1996; 1997). Since this peptide is a major epitope of BCGa protein, immune reactions mediated by the DRB1*1405 allele should be involved in defense against *M. tuberculosis* infection. Nevertheless, its partial nucleic acid sequence published by the original report has never been stretched to the complete coding region.

In order to reveal full coding region sequence of the DRB1*1405 allele, peripheral B cells were isolated from a Japanese donor whose DRB1 genotype was DRB1*1405/1502. This genotype was defined with conventional sequence specific oligonucleotide hybridization technique that followed polymerase chain reaction to amplify DRB1 genes (Nishimura *et al.*, 1992). These peripheral B lymphocytes were immortalized with Epstein–Barr virus (EBV). The EBV-transformed B lymphocyte cell line, EBSM presented a relevant peptide to DRB1*1405-restricted BCGa-reactive T cell clones (Matsushita *et al.*, 1997). The EBSM cells were stimulated with 50 ng/ml of recombinant γ-interferon (Peprotech EC, London, UK) for 24 h. The total RNA were then extracted and converted to the first strand cDNA. The cDNA were PCR-amplified with a pair of oligonucleotide primers. The sense primer (DRB1405s: ATA TAA GCT TGC CAC CAT GGT GTG TCT GAG GCT CCC TGG) consisted of a Hind III restriction enzyme recognition site (underlined) and a oligonucleotide stretch that specified initial 23 nucleic acids of a signal sequence of previously reported HLA DRB1 genes (Gustafsson *et al.*, 1984). The anti-sense primer (DRB1405c: 5′-TAC CAC TAG TGC TCA GGA ATC CTG TTG GCT GAA) consisted of a Spe I restriction enzyme recognition site (underlined) and a oligo-nucleotide stretch that specified the last 23 consensus nucleic acids of previously reported DRB1 coding sequences (Marsh and Bodmer, 1992). Thirty-five

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cycles of the PCR-amplification reaction with ExTaq polymerase (Takara, Shiga, Japan) successfully generated products, which were subcloned into pGEM-T vectors (Promega, Madison, WI) for nucleic acid sequence determination. The sequences determined were identical to a HLA-DRB1*1502 gene or contained the previously published partial DRB1*1405 sequence.

Since PCR was employed to isolate the DRB1*1405 gene, the sequence of DNA strands that annealed to the oligonucleotide primers should be validated. First, the EBSM cDNA were polyG-tailed for anchored PCR to amplify the 5′ cDNA. The anti-sense primer specific to DRB1*1405 (B1405Cc: GAA ACG TGG TCT GGT GTC C) together with the anchor primers, mixture of AN and ANC, was used (Kohsaka *et al.*, 1993). After 20 cycles of the reaction, a part of the products were PCR-amplified further for 20 cycles with the AN primer and a nested DRB1*1405-specific anti-sense primer (B1405Bc: ACT CGT CGA CTC AGC ACC ATC AGT GTC A, underlined is Sal I recognition sequence). The products were subcloned into pGEM-T vector. Sequence determination revealed 5′ untranslated region and also confirmed that the DRB1*1405 allele shares the signal sequence with the original DRB1 gene (Gustafsson *et al.*, 1984). The 3′ RACE System (Invitrogen, San Diego, CA) was used to isolate the 3′ end of the DRB1*1405 transcript. Briefly, the total RNA of EBSM cells were converted to cDNA with oligo(dT)-containing Adapter primer. The cDNA were PCR-amplified with an Adapter-specific primer and a DRB1*1405-specific sense primer (B1405Es: ATG CGT CGA CGG GCT GTT CAT CTA CTT C, underlined is Sal I recognition sequence), and the products were subcloned into pGEM-T vectors. The sequence analysis disclosed the 3′ untranslated region sequence and also verified that the 3′ terminal sequence of the coding region is identical to the consensus sequence.

Figure 1 shows the coding region sequence with flanking sequences. The previously published sequence covered less than half of the entire coding sequence. Based on the sequence information of the cytoplasmic domain of the DRB1*1405 allele, we compared the cytoplasmic domains of the DRB1 alleles for all different DR specificities. The nucleotide and amino acid sequences of DRB1 cytoplasmic domains are largely conserved, but fall into three groups (Fig. 2). Each DRB1 allele has either threonine or arginine at the position 233. Among those that have threonine at the amino acid position 233, only the DR10-related alleles have proline instead of glutamine at the position 231. Since the genes in the same group are encoded by haplotypes with the same genetic structure, these differences should be generated early in the evolution of the HLA genes. Indeed, alleles with arginine at the position 233 share the secondary DRB locus, i.e. DRB3 that encodes for DR52 molecules.

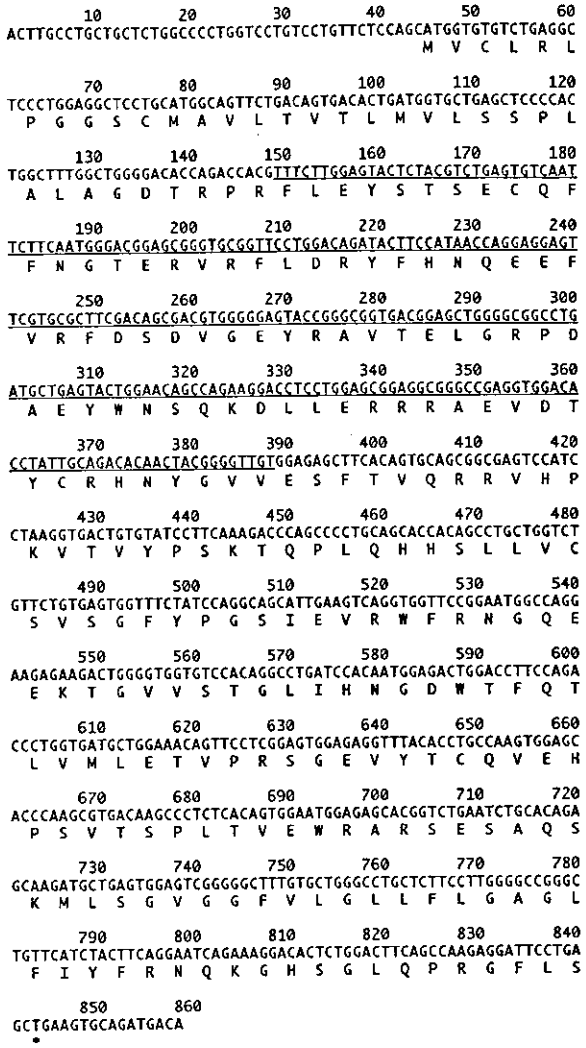

FIGURE 1 Nucleotide sequences of coding and flanking untranslated regions of the HLA-DRB1*1405 allele. The DRB1*1405 cDNA fragments were PCR-amplified from EBSM cDNA, and subcloned into pGEM-T plasmids. DH5α *E. Coli* were transformed with these plasmids, and screened with labeled internal oligonucleotide probes. The plasmids from positive clones were purified and subjected to sequencing reactions with Thermo Sequenase core sequencing kit with 7-deaza-dGTP (Amersham Life Science, England and Molecular Dynamics, CA). Texas Red-labeled M13 forward and reverse oligonucleotide primers (Hitachi Electronics Engineering, Tokyo, Japan) were employed for sequence determination on both strands. The products were fractionated with a Hitachi SQ-5500 sequencer (Hitachi Electronics Engineering), and analyzed with the associated software. The inferred amino acid sequence is shown in a single-letter format. Underlined were nucleic acids determined by the previous report (Obata *et al.*, 1990).

It is known that antigen presenting cells are activated via HLA molecules when they present antigens to T lymphocytes (Tabata *et al.*, 2000; Matsuoka *et al.*, 2001). They utilize surface HLA molecules as signaling receptors. Structural

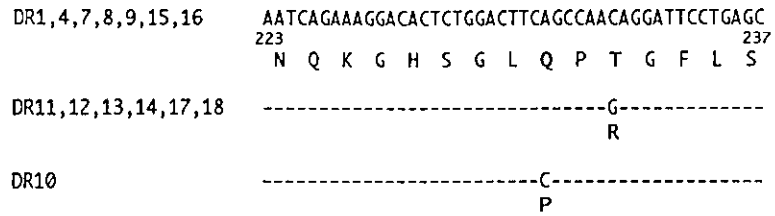

FIGURE 2 Cytoplasmic domain of the DRB1 gene alleles. Nucleotide and amino acid sequences (amino acid position 223–237) of the DRB1 alleles that define different DR serological specificities were shown. DRB1 alleles with the same DR specificity have identical cytoplasmic domain sequences. Hyphenated are nucleotides identical to those for the DR1 serotype. Amino acids are shown in a single letter format and variations are shown in bold letters.

difference of the cytoplasmic domains of the HLA molecules appeared to be important because triggering DR, DQ and DP molecules, each of which associates with a distinct isoform of protein kinases C in the cytoplasm, resulted in differential activation of antigen-presenting cells (Matsuoka *et al.*, 2001, and unpublished observation). Although the allelic variation does not necessarily have the same impact on the structure of the cytoplasmic domains, the future studies should address the question whether these differences induce distinct signals in the antigen presenting cells.

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Stimulation of Host NKT Cells by Synthetic Glycolipid Regulates Acute Graft-versus-Host Disease by Inducing Th2 Polarization of Donor T Cells[1]

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NKT cells are a unique immunoregulatory T cell population that produces large amounts of cytokines. We have investigated whether stimulation of host NKT cells could modulate acute graft-vs-host disease (GVHD) in mice. Injection of the synthetic NKT cell ligand α-galactosylceramide (α-GalCer) to recipient mice on day 0 following allogeneic bone marrow transplantation promoted Th2 polarization of donor T cells and a dramatic reduction of serum TNF-α, a critical mediator of GVHD. A single injection of α-GalCer to recipient mice significantly reduced morbidity and mortality of GVHD. However, the same treatment was unable to confer protection against GVHD in NKT cell-deficient CD1d knockout ($CD1d^{-/-}$) or $IL\text{-}4^{-/-}$ recipient mice or when $STAT6^{-/-}$ mice were used as donors, indicating the critical role of host NKT cells, host production of IL-4, and Th2 cytokine responses mediated by donor T cells on the protective effects of α-GalCer against GVHD. Thus, stimulation of host NKT cells through administration of NKT ligand can regulate acute GVHD by inducing Th2 polarization of donor T cells via STAT6-dependent mechanisms and might represent a novel strategy for prevention of acute GVHD. *The Journal of Immunology*, 2005, 174: 551–556.

Allogeneic hemopoietic stem cell transplantation (HSCT)[3] cures various hematologic malignant tumors, bone marrow (BM) failures, and congenital metabolic disorders. Emerging evidence suggests that allogeneic HSCT is also useful for treatment of other diseases, including solid tumors and autoimmune diseases (1, 2). However, graft-vs-host disease (GVHD) is a major obstacle that precludes wider application of allogeneic HSCT. The pathophysiology of acute GVHD is complex, involving 1) donor T cell responses to the host alloantigens expressed by host APCs activated by conditioning regimens (i.e., irradiation and/or chemotherapy), and 2) dysregulation of inflammatory cytokine cascades, leading to further T cell expansion and induction of cytotoxic T cell responses (3).

$CD4^+$ helper T cells can be divided into two distinct subpopulations: Th1 and Th2 cells (4). Th1 cells produce IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5, and IL-13. Although the role of Th1 and Th2 cytokines in the pathophysiology of acute GVHD is complex and controversial (5–8), Th1 polarization of donor T cells predominantly plays a role in inducing the "cytokine storm" that is seen in several models of acute GVHD (3, 9), whereas Th2 polarization mostly suppresses inflammatory cascades and reduces acute GVHD (10–12). Many properties of dendritic cells (DCs), including the type of signal, the duration of activation, the ratio of DCs to T cells, and the DC subset that presents the Ag, influence the differentiation of naive $CD4^+$ T cells into Th1 or Th2 cells (13). The cytokines that are present during the initiation of the immune responses at the time when the TCR engages with MHC/peptide Ags are critically important for Th cell differentiation (14).

NKT cells are a distinct subset of lymphocytes characterized by expression of surface markers of NK cells together with a TCR. Although the NKT cell population exhibits considerable heterogeneity with regard to phenotypic characteristics and functions (15), the major subset of murine NKT cells expresses a semi-invariant TCR, Vα14-Jα18, in combination with a highly skewed set of Vβs, mainly Vβ8 (16). NKT cells can be activated via their TCR by glycolipid Ags presented by the nonpolymorphic MHC class I-like molecule CD1d expressed by APCs (17). Stimulation of NKT cells rapidly induces secretion of large amounts of IFN-γ and IL-4, thereby influencing the Th1/Th2 balance of conventional $CD4^+$ T cell responses (18). In particular, NKT cells are considered an important early source of IL-4 for the initiation of Th2 responses (19, 20), although these cells are not absolutely required for the induction of Th2 responses (21–23). NKT cells are absent in CD1d knockout ($CD1d^{-/-}$) mice because of defects in their thymic positive selection, which requires CD1d expression on hemopoietic cells, probably double-positive thymocytes (24, 25).

Considering the critical role of cytokines in the development of acute GVHD, we investigated the role of host NKT cells in an experimental model of GVHD, using synthetic NKT cell ligands,

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[3] Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; BM, bone marrow; GVHD, graft-vs-host disease; DC, dendritic cell; α-GalCer, α-galactosylceramide; BMT, bone marrow transplantation; TBI, total body irradiation; TCD, T cell depletion; LN, lymph node; WT, wild type.

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α-galactosylceramide (α-GalCer) (26), a glycolipid originally purified from a marine sponge, and its analog, OCH (27). Our findings indicate that stimulation of host NKT cells with NKT ligands can modulate acute GVHD.

Materials and Methods

Mice

Female C57BL/6 (B6, H-2^b) and BALB/c (H-2^d) mice were purchased from Charles River Japan. IL-$4^{-/-}$ B6 and STAT6$^{-/-}$ BALB/c mice were purchased from The Jackson Laboratory. CD1d$^{-/-}$ B6 mice were established by specific deletion of the CD1d1 gene segment (22). Mice, between 8 and 16 wk of age, were maintained in a specific pathogen-free environment and received normal chow and hyperchlorinated drinking water for the first 3 wk post-bone marrow transplantation (BMT). All experiments involving animals were performed under the auspices of the Institutional Animal Care and Research Advisory Committee at the Department of Animal Resources, Okayama University Advanced Science Research Center.

Bone marrow transplantation

Mice were transplanted according to a standard protocol described previously (28). Briefly, B6 mice received lethal total body irradiation (TBI; x-ray), split into two doses separated by 6.5 h to minimize gastrointestinal toxicity. Recipient mice were injected with 5 × 10^6 BM cells plus 5 × 10^6 spleen cells from either syngeneic (B6) or allogeneic (BALB/c) donors. T cell depletion (TCD) of donor BM cells was performed using anti-CD90 MicroBeads and the AutoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. Donor cells were resuspended in 0.25 ml of HBSS (Invitrogen Life Technologies) and injected i.v. into recipients on day 0. Survival was monitored daily. The degree of systemic acute GVHD was assessed weekly by a scoring system incorporating five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity, as described (29).

Glycolipids

α-GalCer, (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (KRN7000), was synthesized and provided by Kirin Brewery Company (30). A homologue of α-GalCer, OCH, was selected from a panel of synthesized α-GalCer analogues by replacing the sugar moiety and/or truncating the aliphatic chains, because of its ability to stimulate enhanced IL-4 and reduced IFN-γ production by NKT cells, as previously described (27, 31). BMT recipient mice were injected i.p. with α-GalCer or OCH (100 μg/kg) immediately after BMT on day 0. Mice from the control groups received the diluent only.

Flow cytometric analysis

mAbs used were FITC- or PE-conjugated anti-mouse CD4, H-2K^b, and H-2K^d (BD Pharmingen). Cells were preincubated with 2.4G2 mAb (rat anti-mouse FcγR) for 10 min at 4°C to block nonspecific binding of labeled Abs, and then were incubated with the relevant mAbs for 15 min on ice. Finally, cells were washed twice with 0.2% BSA in PBS and fixed. After lysis of RBCs with FACS lysing solution (BD Pharmingen), cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). 7-Amino-actinomycin D (BD Pharmingen)-positive cells (i.e., dead cells) were excluded from the analysis. Fluorochrome-conjugated irrelevant IgG were used as negative controls. At least 5000 live events were acquired for analysis.

Cell cultures

Mesenteric lymph nodes (LNs) and spleens were removed from animals 6 days after BMT and four to six mesenteric LNs or spleens from each experimental group were combined. Numbers of cells were normalized for T cells and were cultured in complete DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.02 mM 2-ME, and 10 mM HEPES in wells of a 96-well flat-bottom plate, at a concentration of 5 × 10^4 T cells/well with 1 × 10^5 irradiated (20 Gy) peritoneal cells harvested from naive B6 (allogeneic) animals, or with 5 μg/ml plate-bound anti-CD3ε mAbs (BD Pharmingen) and 2 μg/ml anti-CD28 mAbs (BD Pharmingen). Forty-eight hours after the initiation of culture, supernatants were collected for the measurement of cytokine levels.

ELISA

ELISA was performed according to the manufacturer's protocols (R&D Systems) for measurement of IFN-γ, IL-4, and TNF-α levels, as described previously (32). Samples were obtained from culture supernatant and blood from retro-orbital plexus, diluted appropriately, and run in duplicate. Plates were read at 450 nm using a microplate reader (Bio-Rad). The sensitivity of the assays was 31.25 pg/ml for IFN-γ, 7.6 pg/ml for IL-4, and 23.4 pg/ml for TNF-α.

Histology

Formalin-preserved livers and small and large bowels were embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined in a blinded fashion by a pathologist (C. Liu). A semiquantitative scoring system was used to assess the following abnormalities known to be associated with GVHD, as previously described (33): 0, normal; 0.5, focal and rare; 1.0, focal and mild; 2.0, diffuse and mild; 3.0, diffuse and moderate; and 4.0, diffuse and severe. Scores were added to provide a total score for each specimen. After scoring, the codes were broken and data were compiled. Pathological GVHD scores of intestine are the sum of scores for small bowel and colon.

Statistical analysis

Mann-Whitney *U* test was applied for the analysis of cytokine data and clinical scores. We used the Kaplan-Meier product limit method to obtain survival probability, and the log-rank test was applied for comparing survival curves. Differences in pathological scores between the α-GalCer-treated group and the diluent-treated group were examined by two-way ANOVA. We defined $p < 0.05$ as statistically significant.

Results

Administration of α-GalCer stimulates lethally irradiated mice to produce IFN-γ and IL-4

We first determined whether administration of synthetic NKT ligands such as α-GalCer and OCH can stimulate heavily irradiated mice to produce cytokines. B6 mice were given 13 Gy TBI and were injected i.p. with α-GalCer, OCH, or diluent 2 h after TBI. Six hours later, blood samples were obtained, and serum samples were prepared for measurement of IFN-γ and IL-4. TBI alone or BMT itself did not stimulate diluent-treated mice to produce IFN-γ or IL-4 (Fig. 1). Administration of α-GalCer increased serum levels of IFN-γ and IL-4, even in mice receiving TBI. However, serum levels of IFN-γ were much less in irradiated mice than in unirradiated mice. By contrast, the ability of irradiated mice to produce IL-4 to α-GalCer was maintained for 48 h after irradiation. Serum levels of IFN-γ and IL-4 in response to α-GalCer were not altered when irradiated wild-type (WT) mice were injected with 5 × 10^6 BM cells and 5 × 10^6 spleen cells isolated from allogeneic (BALB/c) donors. Furthermore, these cytokine responses were not observed when α-GalCer was injected into irradiated NKT cell-deficient CD1d$^{-/-}$ mice with or without BMT. These results suggest that host NKT cells that survive for at least 48 h after irradiation, rather than from infused donor cells, are critically involved in the production of these cytokines in response to glycolipids. Irradiation appears to impair the ability of mice to produce IFN-γ while preserving IL-4 production in response to α-GalCer. Similar cytokine profiles were observed when OCH was administered (data not shown).

Administration of α-GalCer to recipients polarizes donor T cells toward Th2 cytokine production after allogeneic BMT

Induction of GVHD fundamentally depends upon donor T cell responses to host alloantigens. We next evaluated the effect of glycolipid administration on donor T cell responses after allogeneic BMT in a well-characterized murine model of acute GVHD (BALB/c→ B6) directed against both MHC and multiple minor histocompatibility Ags. Lethally irradiated B6 mice were transplanted with 5 × 10^6 BM cells and 5 × 10^6 spleen cells from

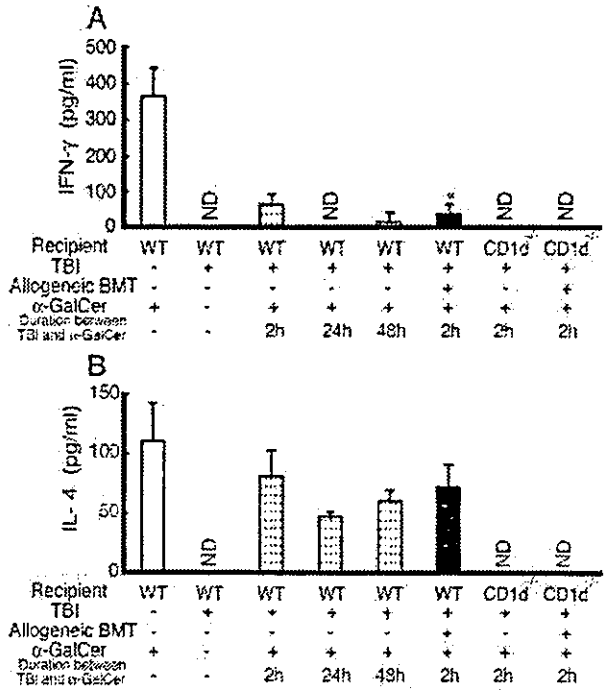

FIGURE 1. Cytokine responses to α-GalCer in lethally irradiated mice with or without BMT. WT and CD1d$^{-/-}$ B6 mice received 13 Gy TBI. Two, 24, or 48 h later, mice were injected i.p. with α-GalCer (100 μg/kg) or diluent. A cohort of animals were transplanted with allogeneic BM cells (5×10^6) and spleen 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.

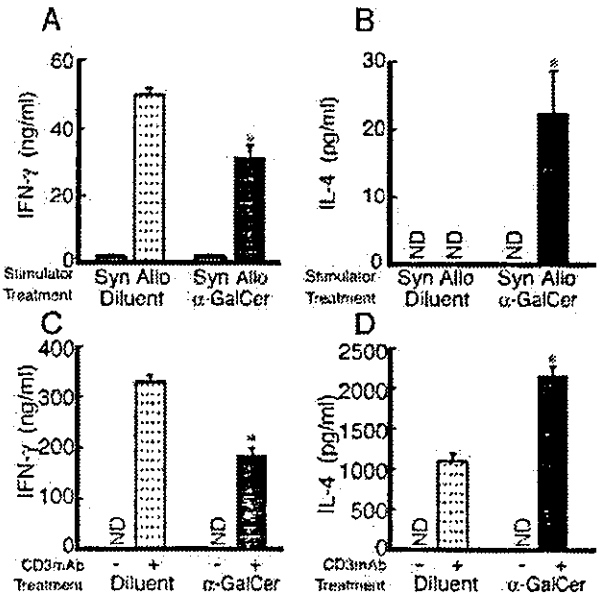

FIGURE 2. Administration of α-GalCer to recipients of allogeneic BMT polarizes donor T cells toward Th2 cytokine secretion. Lethally irradiated (13 Gy) B6 mice were transplanted with BM cells (5×10^6) and spleen cells (5×10^6) isolated from BALB/c mice, followed by injection of either α-GalCer or control 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

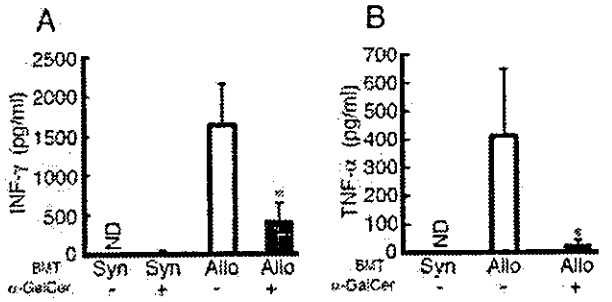

FIGURE 3. A single injection of α-GalCer to recipients of allogeneic BMT markedly reduces serum levels of IFN-γ and TNF-α. WT B6 mice were transplanted as in Fig. 2. Sera (n = 3–10/group) were obtained from diluent-treated (□) and α-GalCer-treated (■) recipients on day 6 after BMT, and serum levels of 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

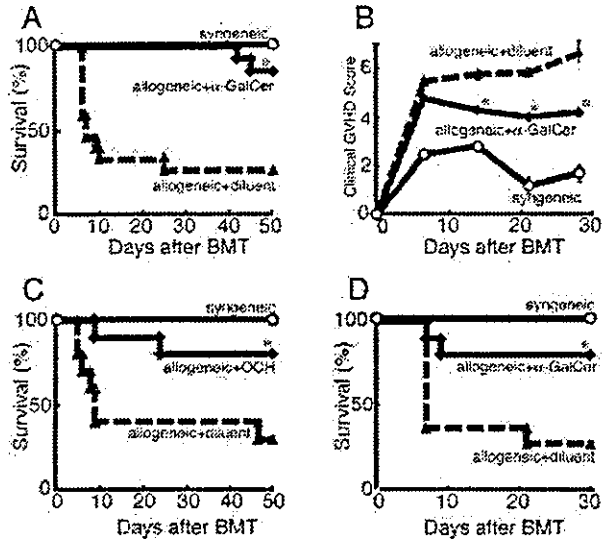

FIGURE 4. A single injection of NKT ligands to BMT recipients modulates acute GVHD. BMT was performed as in Fig. 2. *A*, Survival curves of syngeneic control group (○, solid line; n = 9); allogeneic, diluent-treated recipients (▲, dotted line; n = 15); and allogeneic, α-GalCer-treated recipients (◆, 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.

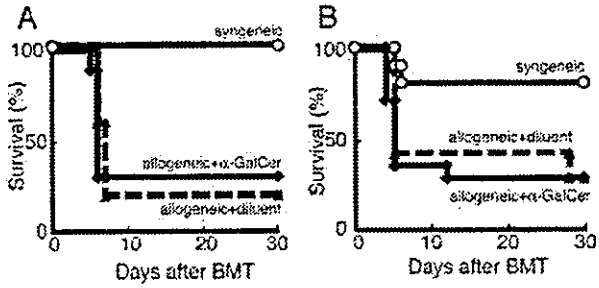

FIGURE 5. Host NKT cells and host IL-4 production are required for suppression of GVHD by α-GalCer. *A*, Lethally irradiated CD1d$^{-/-}$ B6 mice were transplanted as in Fig. 2. Survival curves of syngeneic control group (○, solid line; n = 6); allogeneic, diluent-treated recipients (▲, dotted line;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

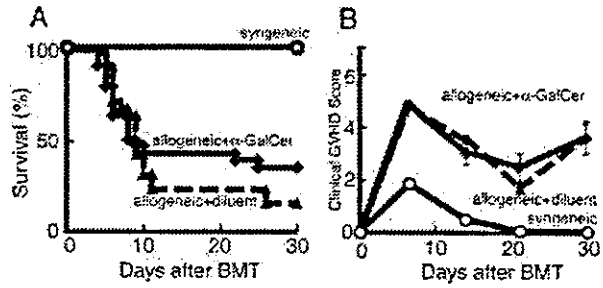

FIGURE 6. The protective effects of α-GalCer against GVHD are dependent upon the STAT6 pathway of donor T cells. Lethally irradiated B6 mice were transplanted with TCD-BM cells (4 × 10^6) from WT BALB/c mice and spleen cells (5 × 10^6) from STAT6$^{-/-}$ BALB/c mice. *A*, Survival curves of the syngeneic 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

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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.

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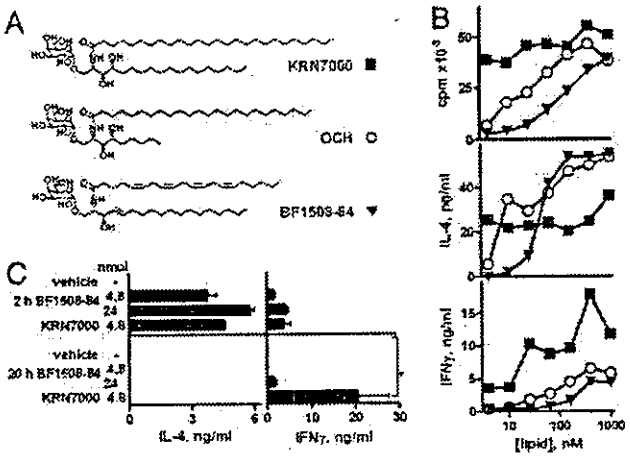

Fig. 1. Induction of a T_H2-polarized cytokine response by an unsaturated analogue of α-GalCer. (*A*) Glycolipid structures. (*B*) [^{3}H]thymidine incorporation and supernatant IL-4 and IFN-γ levels in 72-h splenocyte cultures with graded amounts of glycolipid. Means from triplicate cultures are shown; 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

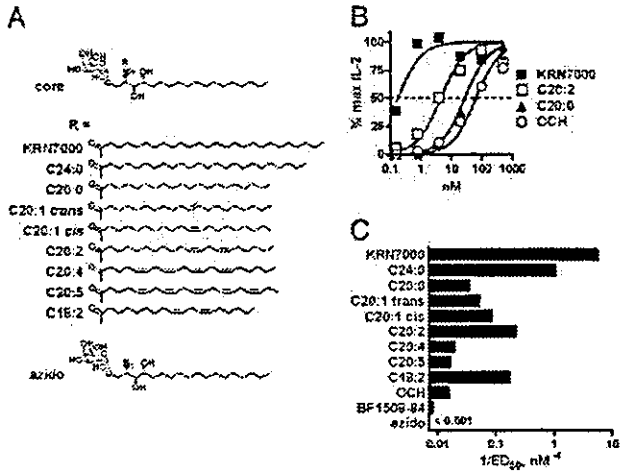

Fig. 2. Recognition of a panel of unsaturated analogues of KRN7000 by a canonical Vα14i NKT hybridoma. (*A*) Analogue structures. (*B*) Dose–response curves showing IL-2 production by hybridoma DN3A4–1.2 after stimulation with RMA-S.mCD1d cells pulsed with various doses of glycolipid. Maximal IL-2 concentrations 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.

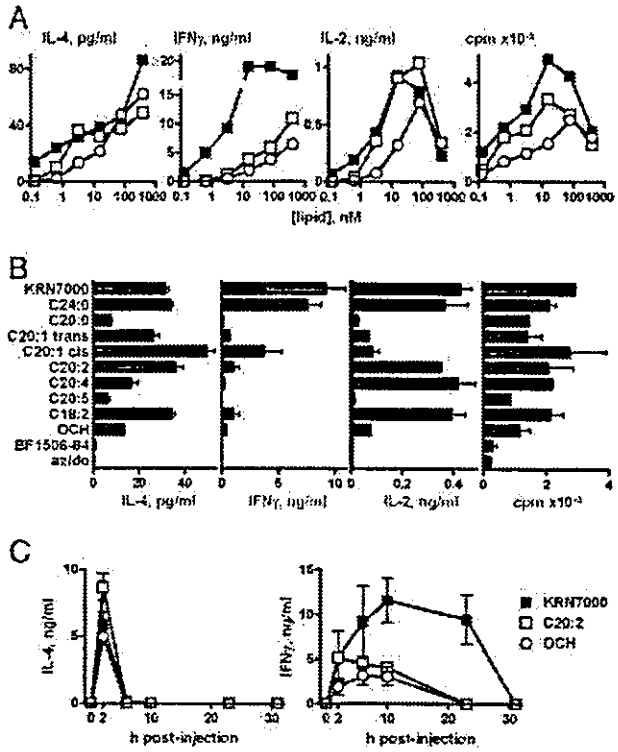

Fig. 3. T_H2-skewing of *in vitro* and *in vivo* cytokine responses to C20:2. (*A*) Dose–response curves reporting 48 h IL-4, IFN-γ, or IL-2 production, and cell proliferation of splenocytes in response to KRN7000, C20:2, and OCH. Means of duplicate cultures are shown; SEM were <10% of the means. (*B*) 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

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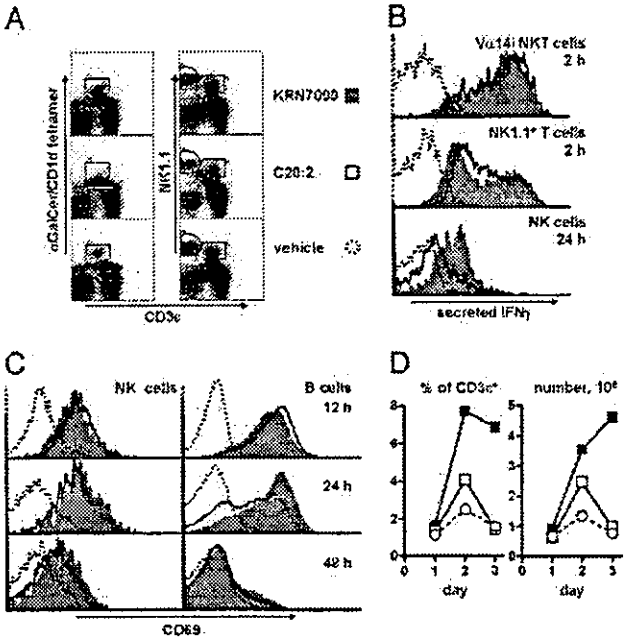

Fig. 4. Sequelae of KRN7000 and C20:2-induced Vα14i NKT cell activation. (*A*) Vα14i NKT cell (tetramer$^+$ CD3ε^{int}), NK cell (NK1.1$^+$ CD3ε^-), and NK1.1$^+$ T cell (NK1.1^{int} CD3ε^{int}) identification by FACS in splenocytes from mice given KRN7000, C20:2, or vehicle i.p. 2 h earlier. Lymphocytes 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.

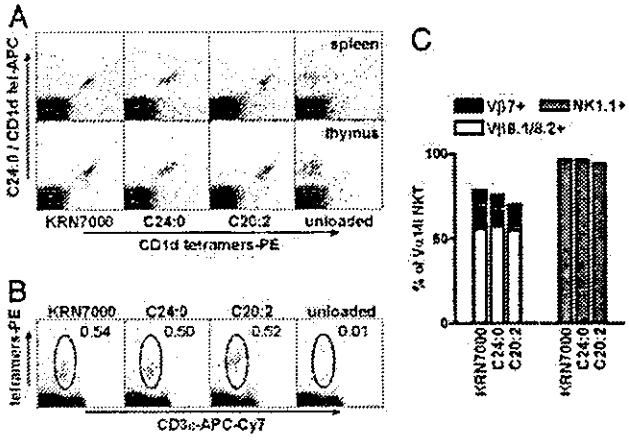

Fig. 5. Recognition of KRN7000, C24:0, and C20:2 by the same population of Vα14i NKT cells. (*A*) Costaining of C57BL/6 splenocytes or thymocytes with allophycocyanin-conjugated CD1d tetramers assembled with C24:0, and phycoerythrin-labeled CD1d tetramers assembled with various analogues. (*B*) Thymocytes 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.

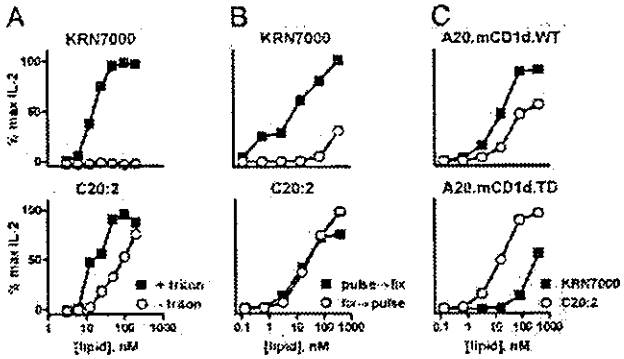

Fig. 6. Differential requirements for CD1d loading with KRN7000 and C20:2. IL-2 response of hybridoma DN3A4–1.2 to glycolipid presentation in three *in vitro* CD1d presentation systems: platebound CD1d loaded with varying amounts of KRN7000 or C20:2 in the presence or absence of the detergent Triton 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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Suppression of Collagen-Induced Arthritis by Natural Killer T Cell Activation With OCH, a Sphingosine-Truncated Analog of α-Galactosylceramide

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Objective. **OCH, a synthetic analog of α-galactosylceramide with a truncated sphingosine chain, stimulates natural killer T (NKT) cells to produce predominantly Th2 cytokines. Thus, OCH may be a potential agent for the treatment of Th1-mediated autoimmune diseases. This study was designed to evaluate the protective effects of OCH on collagen-induced arthritis (CIA) in mice.**

Methods. **Mice were immunized with type II collagen (CII) and injected intraperitoneally twice per week with OCH, before or after the onset of CIA. They were monitored to assess the effect of OCH treatment on the severity of disease. Anti-CII antibodies and cytokine production were measured by enzyme-linked immunosorbent assay. Expression of cytokine genes was determined by quantitative reverse transcriptase–polymerase chain reaction.**

Results. **OCH inhibited CIA in wild-type C57BL/6 (B6) mice but not in NKT-deficient mice. OCH suppressed CIA in SJL mice, which are prone to autoimmune diseases and have a deficiency in the number and function of NKT cells which is similar to that in patients with autoimmune diseases, even after disease has already developed. Disease protection conferred by OCH correlated with its ability to selectively induce Th2 cytokine production mediated by NKT cells and to promote collagen-specific Th2 responses. Neutralization of interleukin-4 (IL-4) or IL-10 with monoclonal antibodies abolished disease protection by OCH, indicating a critical role for these cytokines.**

Conclusion. **Taken together, our findings suggest that OCH holds possibilities as a therapeutic agent for autoimmune diseases such as rheumatoid arthritis.**

Rheumatoid arthritis (RA) is a common autoimmune disease characterized by persistent inflammation of joints resulting in progressive destruction of cartilage and bone. Although its precise etiology is not clearly understood, cumulative evidence suggests that Th1 cells secreting interferon-γ (IFNγ) and tumor necrosis factor α (TNFα) exacerbate disease, whereas Th2 cells producing interleukin-4 (IL-4) and IL-10 suppress arthritis (1). Studies with animal models have demonstrated that systemic or locally administered IL-4 and IL-10 can effectively protect against arthritis in mice (2–11).

Natural killer T (NKT) cells are a unique subset of T cells that coexpress T cell receptor α/β (TCRα/β) and receptors from the NK lineage. NKT cells express an invariant TCRα chain (encoded by a $V_\alpha14$–$J_\alpha281$ rearrangement in mice and a homologous $V_\alpha24$–$J_\alpha Q$ rearrangement in humans). Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens bound to the nonpolymorphic class I MHC-like protein, CD1d. NKT cells have been implicated in a variety of immune responses such as infection and tumor immunity. One striking feature of NKT cells is their capacity to secrete a large amount of cytokines,

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including IL-4 and IFNγ, in response to TCR stimulation (12–14). Recently, a number of reports have indicated that NKT cells play a critical role in the regulation of autoimmune responses. Abnormalities in the numbers and functions of NKT cells have been observed in patients with autoimmune diseases (15–18) as well as in a variety of mouse strains that are genetically predisposed to the development of autoimmune diseases (19–23).

While the natural ligand for NKT cells remains to be determined, α-galactosylceramide (α-GC) (Figure 1A), a derivative of marine sponge, has been shown to bind to CD1d and strongly stimulate NKT cells to produce IFNγ and IL-4, both in humans and in mice (24–26). Previously, we have shown that OCH (Figure 1A), an analog of α-GC with a truncated sphingosine chain, efficiently inhibits induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 (B6) mice, due to its ability to stimulate NKT cells to selectively produce Th2 cytokines; in contrast, α-GC had little effect on EAE (27,28).

In the present study, we found that OCH inhibits collagen-induced arthritis (CIA), a murine experimental model for RA, in wild-type B6 but not NKT-deficient $J_{\alpha}281$-knockout mice. We also demonstrated that OCH inhibits CIA in SJL mice even after arthritis has already developed. Experiments with anti–IL-4 or anti–IL-10 administration revealed that IL-4 and IL-10 are critical for OCH-mediated suppression of CIA. These results suggest that stimulation of NKT cells with OCH could be an attractive means of intervention in autoimmune diseases such as RA.

MATERIALS AND METHODS

Mice. B6 mice were purchased from Clea Laboratory Animal Corp. (Tokyo, Japan). SJL mice were obtained from Charles River Japan (Yokohama, Japan). $J_{\alpha}281$-knockout mice were kindly provided by Dr. Masaru Taniguchi (Chiba University Graduate School of Medicine, Chiba, Japan). The animals were kept under specific pathogen-free conditions and studied at 7–10 weeks of age.

Induction of CIA. Mice were immunized intradermally at the base of the tail with 100 μg of either chicken type II collagen (CII) (for B6 mice) or bovine CII (for SJL mice) (Collagen Research Center, Tokyo, Japan) emulsified with an equal volume of Freund's complete adjuvant (CFA), containing 250 μg of H37RA *Mycobacterium tuberculosis* (Difco, Detroit, MI). The animals were boosted by intradermal injection with the same antigen preparation on day 21. Mice were examined for signs of joint inflammation 3 times per week, and joint involvement was scored as follows: 0 = no change, 1 = focal redness of the limb or swelling and redness of 1 digit, 2 = mild swelling and erythema of the limb or swelling of >2 digits, 3 = marked swelling and erythema of the limb, 4 = maximal

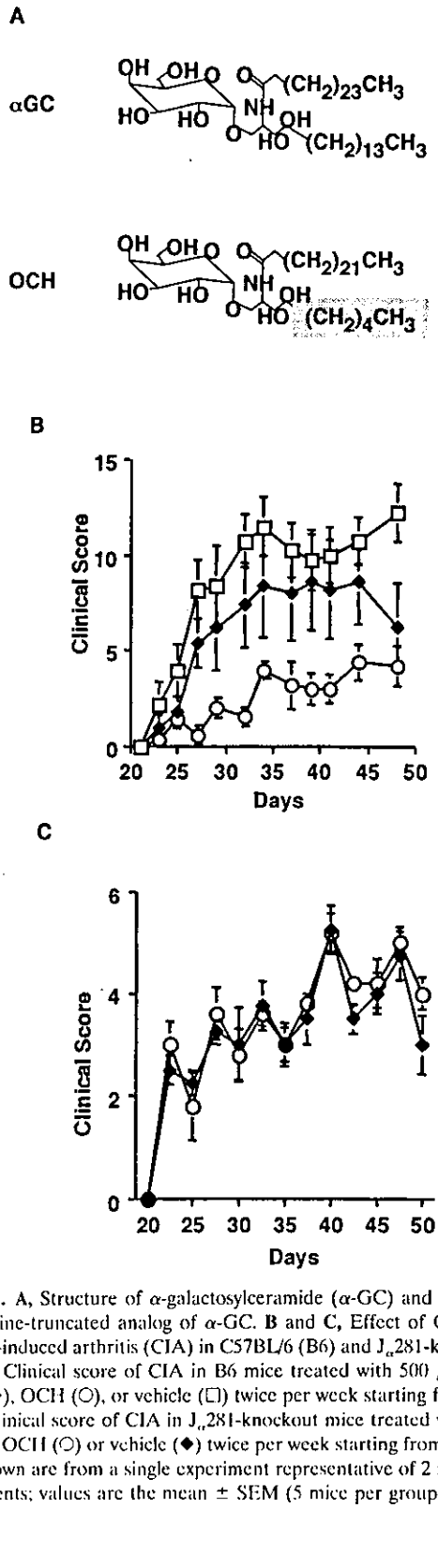

Figure 1. A, Structure of α-galactosylceramide (α-GC) and OCH, a sphingosine-truncated analog of α-GC. B and C, Effect of OCH on collagen-induced arthritis (CIA) in C57BL/6 (B6) and $J_{\alpha}281$-knockout mice. B, Clinical score of CIA in B6 mice treated with 500 μg/kg of α-GC (◆), OCH (○), or vehicle (□) twice per week starting from day 21. C, Clinical score of CIA in $J_{\alpha}281$-knockout mice treated with 500 μg/kg of OCH (○) or vehicle (◆) twice per week starting from day 21. Data shown are from a single experiment representative of 2 identical experiments; values are the mean ± SEM (5 mice per group).

swelling and redness of the limb and later, ankylosis. The average of the macroscopic score was expressed as a cumulative value for all paws, with a maximum possible score of 16 per mouse. The in vivo experiments were performed with 10 mice per group and repeated twice to ensure reproducibility.

In vivo glycolipids treatment and antibody treatment. Synthetic glycolipids were used to treat CIA. Starting from the indicated day, mice were injected intraperitoneally twice per week with either OCH or α-GC at a dose of 500 μg/kg. The control mice were injected with vehicle alone (10% DMSO in phosphate buffered saline [PBS]). To neutralize IL-4 or IL-10, anti–IL-4 monoclonal antibody (mAb) (11B11) or anti–IL-10 mAb (JES-2A5) (500 μg per mouse) was injected intraperitoneally 2 hours before glycolipid administration.

Histopathologic study. Forepaws were removed from mice killed 50 days after the first immunization of CII, then fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histopathologic analysis.

NKT cell preparation. NK1.1-positive T cells were purified from the liver of 6–8-week-old B6 mice. Liver mononuclear cells were prepared by Percoll density-gradient centrifugation. Prepared cells were then incubated with phycoerythrin-conjugated NK1.1 mAb and fluorescein isothiocyanate–conjugated CD3 mAb (BD PharMingen, San Jose, CA). The stained cells were sorted into $NK1.1^+$,$CD3^+$ cells. The purity of the sorted cells was >95%.

Quantitative reverse transcriptase–polymerase chain reaction (RT-PCR). Total RNA was extracted with an RNeasy kit (Qiagen KK, Tokyo, Japan) from purified $NK1.1^+$ T cells. Random hexamer-primed complementary DNA was prepared with the First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). For quantitative analysis of cytokines, we used the LightCycler quantitative PCR system (Roche Molecular Biochemicals, Mannheim, Germany) and performed quantitative PCR with a commercial kit (LightCycler-DNA Master SYBR Green I; Roche Molecular Biochemicals). The PCR amplification was repeated 40 times (for 15 seconds at 95°C, 5 seconds at 60°C, and 10 seconds at 72°C). All PCR reactions were normalized by GAPDH expression.

Enzyme-linked immunosorbent assay (ELISA). To detect CII-specific IgG1 and IgG2a, chicken CII or bovine CII (5 μg/ml) was coated onto ELISA plates (Sumitomo Bakelite, Tokyo, Japan) at 4°C overnight. After blocking with 3% bovine serum albumin in PBS, serially diluted serum samples were added onto CII-coated wells. The plates were incubated with biotin-labeled anti-IgG1 and anti-IgG2a (Southern Biotechnology, Birmingham, AL) or anti-IgG antibody (CN/Cappel, Aurora, OH) for 1 hour and then incubated with streptavidin–peroxidase. After addition of substrate, the reaction was evaluated. The levels of IL-4, IL-10, and IFNγ in serum were measured by standard sandwich ELISA, using purified and biotinylated mAb pairs and standards (BD PharMingen).

RESULTS

Suppression of CIA development by OCH. In order to determine whether stimulation of NKT cells modulates arthritis, we first examined the effect of α-GC, a prototypic ligand for NKT cells, on the development of CIA. Early studies showed that CIA is restricted to mouse strains bearing the $H\text{-}2^q$, $H\text{-}2^r$, or $H\text{-}2^s$ haplotype (29) and is generally induced in DBA/1 mice. More recently, modified immunization conditions that are sufficient to induce CIA in B6 mice have been developed; this allowed us to study CIA in knockout mice with a B6 background, which eliminated the need to backcross the knockout mice onto a DBA/1 background (30,31). The histologic and immunologic characteristics of the disease induced in B6 mice have been shown to be similar to those in DBA/1 mice, even though B6 mice have a slightly delayed onset and less uniformly severe disease (31). We immunized B6 mice with chicken CII in CFA to elicit CIA as described previously (30) and then injected mice intraperitoneally with either α-GC or vehicle alone twice per week starting from the day of the second immunization. As shown in Figure 1B, α-GC treatment did not improve the arthritis score significantly. We next examined the effect of OCH on CIA. The mean maximum clinical CIA score was profoundly reduced in OCH-treated B6 mice (Figure 1B and Table 1). The incidence and the time of onset of disease were not significantly different between the OCH-treated group and the control group.

Table 1. Clinical scores of collagen-induced arthritis in C57BL/6 and $J_\alpha281$-knockout mice*

Mice, treatment	Incidence, %	Maximum score, mean ± SEM	Days to onset, mean ± SEM
C57BL/6 (wild-type)			
Vehicle	100	13.0 ± 0.83	23.8 ± 0.48
α-GC	100	9.8 ± 2.37	24.2 ± 0.48
OCH	100	4.6 ± 0.92†	24.2 ± 0.48
$J_\alpha281$-knockout			
Vehicle	100	5.5 ± 0.5	24 ± 0.58
OCH	100	5.75 ± 0.25	23.5 ± 0.5

* C57BL/6 mice or $J_\alpha281$-knockout mice were sensitized with chicken type II collagen for induction of arthritis.
Vehicle or 500 μg/kg of α-galactosylceramide (α-GC) or OCH was injected intraperitoneally twice per week from day 21. Data are from 5 mice per group.
† $P < 0.05$ versus control vehicle, by Mann-Whitney U test.

To investigate the role of $V_\alpha14$ NKT cells in the suppression of CIA by OCH, we examined the ability of OCH to modulate disease in $J_\alpha281$-knockout mice, in which $V_\alpha14$ NKT cells are absent (32). Administration of OCH did not modulate the clinical course of CIA in $J_\alpha281$-knockout mice compared with mice treated with vehicle alone (Figure 1C and Table 1). These results indicate that OCH-mediated suppression of CIA requires NKT cells.

In addition to visual scoring, on day 50 after