ferent system using TCR transgenic mice. The BDC2.5 TCR was cloned from an MHC class II-restricted T cell clone specific to an islet-derived antigen. Diabetogenic CD4 T cells from BDC2.5 NOD mice were unable to induce diabetes when transferred into mice with increased *NKT* cell numbers [33]. The presence of *NKT* cells inhibited the differentiation of BDC2.5 T cells into IFN-γ producing cells without a Th2 shift. BDC2.5 T cells were initially activated in pancreatic lymph nodes and then became anergic. The regulation of BDC2.5 T cells by *NKT* cells was not dependent on IL-4, IL-10, IL-13, or TGF-β [34]. A similar mechanism was implied in the transfer of diabetogenic transgenic $CD8^+$ AI4 T cells expressing the β cell-autoreactive TCR to sublethally irradiated NOD mice when *NKT* cells had been activated with α-GalCer [35]. Activation of *NKT* cells enhanced the apoptosis and induced anergy of AI4 T cells. Even though several different mechanisms might be involved, NKT cell seems to work as regulatory cells in diabetes models. Recently however, the opposing effect of *NKT* cells on $CD8^+$ MHC-restricted T cells was reported. In diabetes induced by the transfer of $CD8^+$ T cells specific for the influenza virus hemagglutinin into mice expressing the hemagglutinin antigen in pancreatic cells, a high frequency of *NKT* cells exacerbated diabetes by enhancing CD8 T cell activation, expansion, and differentiation into effector cells producing IFN-γ [36]. Acceleration of disease by the presence of *NKT* cells was also observed in other autoimmune models such as arthritis.

2.3 NKT Cells in Arthritis Models

Collagen-induced arthritis (CIA) is an animal model for human rheumatoid arthritis induced by immunizing susceptible mouse strains with type II collagen (CII) with adjuvant. When mice develop arthritis, the proportions of *NKT* cells in liver and peripheral blood have been reported to be increased, even though it is not clear what stimulates *NKT* cells to proliferate or if *NKT* cells are generated in situ or recruited from other organs [37]. Since it has been shown that activation of *NKT* cells can be enhanced by non-TCR stimulation such as IL-12 and possibly other cytokines [38], it might be possible that *NKT* cells proliferate in CIA by stimulation of inflammatory cytokines elevated in this model. The protective effect of blocking anti-CD1 monoclonal antibody on CIA has revealed that *NKT* cells contributed to the development of arthritis [37]. Furthermore, amelioration of arthritis is seen in mice lacking all *NKT* cells ($CD1d^{-/-}$) or invariant NK T cells ($J\alpha18^{-/-}$), further supported the important role of *NKT* cells in the development of arthritis [37, 39, 40]. The reduction of disease severity in $J\alpha18^{-/-}$ mice was associated with a decrease in IL-10 or IL-1β production in response to antigen stimulation, suggesting that NK T cells directly or indirectly control the levels of these cytokines [37, 40].

Recent advances in the use of anti-inflammatory drugs such as anti-TNF reagents reminded us of the importance of the later occurring inflammatory phase in the pathogenesis of arthritis in which cytokines amplify local tissue destruction. The K/BxN serum transfer model of arthritis allows arthritis to be induced in a manner that bypasses the initial T cell and B cell interactions necessary to promote autoantibodies and instead measures the downstream events involved in antibody-induced, cytokine-mediated joint destruction. To more specifically investigate the more distal inflammatory phase of arthritis, K/BxN serum transfer arthritis or anti-CII antibody-induced arthritis preferable to CIA [42, 43]. The proportion of *NKT* cells was increased in the lymph nodes and peripheral blood of mice receiving K/BxN serum similar to CIA [39]. Arthritis induced either by injection of K/BxN serum or CII antibody in *NKT*-deficient mice was ameliorated [39, 41]. Regarding the mechanisms that *NKT* cells contribute to the development of arthritis in K/BxN serum transfer model, production of IFN- γ and IL-4 from *NKT* cells has been implicated in the suppression of TGF-β1 [41]. TGF-β is known to inhibit arthritis, especially when administrated systemically [44–46]. TGF-β inhibits T cell proliferation and downregulates the expression of IL-1 receptor, which may result in the suppression of arthritis. Even though *NKT* cells appear to contribute to the pathogenesis of arthritis, activation of *NKT* cells by synthetic glycolipid ligands protected mice against CIA [37]. Repeated injections of OCH, the Th2 polarizing form of an αGalCer, inhibited the clinical course of CIA, whereas α-GalCer administration exhibited a mild suppression of disease. Interestingly, OCH treatment suppressed CIA in SJL mice that have defects in numbers and functions of *NKT* cells. Moreover, OCH treatment ameliorated disease even after arthritis had developed. Suppression of arthritis was associated with the elevation of the IgG1:IgG2a ratio, suggesting a Th2 bias of CII-reactive T cells in OCH-treated mice. Furthermore, neutralization of IL-10 or IL-4 by monoclonal antibody reversed the beneficial effect of OCH treatment. In contrast to CIA, the treatment of α-GalCer has been shown to exacerbate disease in K/BxN serum transfer arthritis [41]. In our study, however, administration of α-GalCer efficiently inhibited K/BxN serum transfer arthritis by a Th2-independent mechanism [75]. The protective effect of arthritis by synthetic glycolipid ligands in arthritis models seems inconsistent with the reduction of disease severity in *NKT*-deficient mice. It is possible that activation of *NKT* cells by synthetic glycolipid is different from the physiological activation of *NKT* cells with endogenous ligands under pathogenic conditions such as arthritis.

2.4
NKT Cells in Lupus Models

Early studies in mice strains which spontaneously develop lupus-like disease such as MRL lpr/lpr mice, C3H gld/gld mice, and (NZBxNZW)F1 mice demonstrated a decrease in *NKT* cell number before the onset of disease, suggesting a preventive effect of *NKT* cells, although NKT cells were not well defined in these studies [47, 48]. The studies using *NKT*-deficient mice revealed a functional role for *NKT* cells in lupus animal models. CD1d deficiency led to exacerbation of pristine-induced nephritis in Balb/c mice [49] and skin disease in MRL lpr/lpr mice without inducing significant differences in nephritis and production of autoantibodies to nuclear antigens [50]. Another group, however, demonstrated that CD1d deficiency neither accelerated skin disease nor ameliorated kidney disease in MRL/lpr mice [51]. Stimulation of *NKT* cells with α-GalCer ameliorated dermatitis in MRL lpr/lpr mice in association with expansion of *NKT* cells and increased Th2 responses, while treatment with α-GalCer had no effect on kidney disease and serum anti-DNA antibody level [52]. In pristine-induced nephritis models, the effect of α-GalCer differs, depending on the strains of mice used. In Balb/c mice, treatment with α-GalCer promoted Th2 responses and protected mice against nephritis. Conversely, treatment with α-GalCer promoted Th1 responses and exacerbated disease in SJL/J mice [53]. The difference in the effect of α-GalCer seems to correlate with the ability to produce Th2 cytokines by activated *NKT* cells, similar to the phenomenon observed in EAE models.

Enhancement of disease rather than protection was observed in (NZB x NZW)F1 mice. In wild-type mice, *NKT* cells increased in number after the onset of disease [54, 55], and the transfer of $NK1.1^+$ T cells from diseased mice to young F1 mice (before the onset of renal failure) induced proteinuria and swelling of the glomeruli [56]. Moreover, treatment with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a anti-double-stranded DNA (dsDNA) antibodies, and ameliorated lupus [56]. Consistent with these results, activation of *NKT* cells with α-GalCer accelerated nephritis in correlation with enhancement of Th1 responses [56]. Despite the differences in the outcome of disease treatment following *NKT* cell activation, one consistent finding in the above studies is that *NKT* cell-driven Th1 responses lead to disease exacerbation, whereas *NKT* cell-driven Th2 responses lead to disease amelioration. One future direction may be to concentrate on which strain-dependent factors promote *NKT* cell-induced Th1 or Th2 responses.

2.5
NKT Cells in Colitis

Dextran sodium sulfate (DSS) -induced colitis is an experimental model for Crohn's disease mediated by Th1 cells. Activation of *NKT* cells with α-GalCer or OCH has been shown to protect mice against DSS-induced colitis [57, 58]. While treatment with both glycolipids induced higher amounts of IFN-γ and IL-4 than controls, the IFN-γ:IL-4 ratio was decreased compared to the control group [58]. The level of IL-10 in the supernatants of colon organ cultures after the injection of OCH was increased [58]. Treatment with OCH induced higher IL-10 production than did α-GalCer, which is consistent with the stronger inhibitory effect of OCH in this model of colitis [58]. Conversely, *NKT* cells have been proposed to act as effector cells in oxazalone-induced colitis [59]. Oxazalone-induced colitis is an experimental colitis model of human ulcerative colitis and is dependent on IL-13. This model of colitis was effectively blocked by neutralizing the IL-13 or depleting *NKT* cells. Moreover, the colitis did not develop in mice deficient in *NKT* cells, indicating the crucial pathological role of *NKT* cells in this model, similar to asthma models which *NKT* cells play an important role in the pathogenesis through IL-13 production.

3
NKT Cells in Human Autoimmune Diseases

Previous studies have documented a reduced number of *NKT* cells in the peripheral blood of patients suffering from systemic sclerosis [60], type 1 diabetes [61,62], multiple sclerosis (MS) [63–65], and other autoimmune disease conditions [66–68]. However, in type 1 diabetes, inconsistent results (decreased [61, 62], normal [69] or increased [70] number of *NKT* cells) were obtained by three independent groups, which led to considerable argument on the role of *NKT* cells in autoimmunity. To identify *NKT* cells, recent studies used α-GalCer-loaded CD1d tetramers, the most reliable tool for staining *NKT* cells, and confirmed a reduced number of *NKT* cells in the peripheral blood from untreated MS patients in remission [64] compared to healthy subjects. Unexpectedly, the number of *NKT* cells tended to increase in the relapse phase of MS and that the deficiency of *NKT* cells may become normalized in the patients treated with low doses of oral corticosteroids (unpublished results). These results imply that disease activity as well as prescribed medications would greatly influence on the number of *NKT* cells, which may provide some clue to designing future studies.

Production of Th2 cytokines was previously described as a cardinal feature of *NKT* cells. Now it is widely accepted that anti-inflammatory Th2 cytokines are secreted from $CD4^+$ *NKT* cells, but not from $CD4^-$ *NKT* cells [71, 72]. Namely, $CD4^+$ *NKT* cells are able to produce both Th1 and Th2 cytokines, whereas $CD4^-$ *NKT* cells selectively produce proinflammatory Th1 cytokines TNF-α and IFN-γ. Given this important dichotomy of *NKT* cells, we have re-analyzed the number and functions of *NKT* cells from untreated MS patients after sorting the cells into $CD4^+$ and $CD4^-$ populations [64]. $CD4^-$ *NKT* cells were greatly reduced in the peripheral blood of the untreated patients in remission, whereas a reduction of $CD4^+$ *NKT* cells was only marginal. Moreover, $CD4^+$ *NKT* cells from the patients were Th2-biased, as evidenced by enhancement of IL-4, whereas $CD4^-$ *NKT* cells were not. As both of the observed changes, Th2 bias of $CD4^+$ and a great reduction of $CD4^-$ *NKT* cells, could be regarded as disease stabilizing changes, we interpreted that this finding to suggest that the role of *NKT* cells in remission of MS is protective against disease development. In contrast, Th1 bias, as evidenced by loss of IL-4 secretion, was confirmed in *NKT* cell clones derived from peripheral blood [61] as well as draining lymph nodes [73] of type 1 diabetes. Although it remains unclear why *NKT* cells from MS and type 1 diabetes are biased toward opposing directions, it is possible that it may reflect the differential disease activity at the time of examination or due to differences in disease pathogenesis.

4 Concluding Remarks and Future Research

The research of the last decade has firmly established that *NKT* cells have the potential to either drive or suppress autoimmune conditions, although we know very little about the precise rules of how the regulation of *NKT* cells in vivo. Despite previous controversies, it now seems that numerical or functional changes of NKT cells may be associated with certain stages of autoimmune diseases, as has been revealed in MS [64] Therapeutic effects of glycolipid ligands for *NKT* cells have indicated that these lymphocytes bridging innate and adaptive immunity may be an excellent target for immune intervention. In particular, the design of "altered" CD1 glycolipid ligands has been shown in many models to alter the outcomes of disease. Meanwhile, it is much less clear how *NKT* cells regulate unwanted autoimmunity taking place in the context of the physiological immune network. It is likely that *NKT* cells produce Th2 cytokines to achieve this, and that the trigger of cytokine production may be an encounter of *NKT* cells with an endogenous ligand bound to CD1d. However, one known endogenous ligand, iGb3, seems to provoke

Th1 cytokines from *NKT* cells [74]. In this regard, there is room for a seeking Th2-inducing natural ligand. Not mutually exclusive to this is the theory that cytokines produced in the inflammatory site together with endogenous antigen and TCR-mediated signals may play a key role in conducting *NKT* cells [38] to secrete Th2 cytokines (Sakuishi et al., unpublished data). To gain deeper insights into the role of *NKT* cells in autoimmunity, the details of natural regulation needs to be clarified in the future.

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Activation of Invariant Natural Killer T Cells by Synthetic Glycolipid Ligands Suppresses Autoantibody-Induced Arthritis

Shinjiro Kaieda,[1] Chiharu Tomi,[2] Shinji Oki,[2] Takashi Yamamura,[2] and Sachiko Miyake[2]

Objective. **Stimulation of invariant natural killer T (iNKT) cells with SGL-S23, a novel synthetic glycolipid analog of α-galactosylceramide with an elongated sphingosine chain, has been shown to strongly suppress K/BxN serum transfer arthritis. This study was designed to evaluate the protective effects of SGL-S23 in an effector phase of arthritis.**

Methods. **To induce arthritis, C57BL/6 mice were injected with 150 μl of serum from K/BxN mice (KRN TCR–transgenic mice crossed with nonobese diabetic mice). Subsequently, synthetic glycolipid ligands were administered intraperitoneally twice, either 3 times starting on day 0 (the day of K/BxN serum injection) or twice starting on day 3. Neutralizing antibody against interferon-γ (IFNγ) interleukin-4 (IL-4), IL-10, or transforming growth factor β was administered 4 hours before injection of SGL-S23. Recombinant IFNγ was administered subcutaneously every day. The severity of arthritis was monitored using a macroscopic scoring system. Cytokine production and plasma histamine levels were measured by enzyme-linked immunosorbent assay.**

Results. **SGL-S23 strongly suppressed K/BxN serum transfer arthritis by inhibiting inflammatory cell infiltration and subsequent destruction of cartilage and bone. The inhibitory effect mediated by SGL-S23 was abolished by neutralization of IFNγ. Systemic administration of IFNγ prevented the development of inflammatory arthritis. Histamine release was suppressed by administration of SGL-S23 or IFNγ. Degranulated mast cells in the synovium were significantly reduced in SGL-S23–treated mice, suggesting that suppression of mast cell activation contributed to the inhibition of arthritis.**

Conclusion. **These findings suggest that activation of iNKT cells with glycolipid ligands holds promise with regard to the treatment of autoimmune diseases such as rheumatoid arthritis. SGL-S23 has clinical benefit over α-galactosylceramide since it induces a weaker cytokine production response in iNKT cells, therefore reducing potential side effects caused by excessive cytokine release.**

Rheumatoid arthritis is a common autoimmune disease characterized by chronic inflammation and progressive destruction of joints. Although antigen-specific T cells in the joints have been thought to be important in inciting an inflammatory cascade, triggering activation of macrophages and synoviocytes, recent advances in antiinflammatory drugs such as anti–tumor necrosis factor (anti-TNF) agents serve as a reminder of the importance of the later inflammatory phase in the pathogenesis and control of arthritis (1,2).

K/BxN mice (KRN TCR–transgenic mice crossed with nonobese diabetic [NOD] mice) spontaneously develop a polyarthritis with many of the hallmarks of human rheumatoid arthritis, including cellular infiltration, synovial hyperplasia, and bone and cartilage destruction (3). The disease depends on recognition of glucose-6-phosphate isomerase (GPI) presented by I-A^{g7} in the periphery (4). Furthermore, arthritis can be induced in most strains of mice by transfer of K/BxN

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[1]Shinjiro Kaieda, MD: National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan, and Kurume University School of Medicine, Kurume, Japan; [2]Chiharu Tomi, Shinji Oki, PhD, Takashi Yamamura, MD, PhD, Sachiko Miyake, MD, PhD: National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

Address correspondence and reprint requests to Sachiko Miyake, MD, PhD, Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. E-mail: miyake@ncnp.go.jp.

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mouse serum or affinity-purified anti-GPI antibodies (4,5). Recent studies with the K/BxN serum transfer model have led to new understanding of the importance of autoantibodies and mediators of innate immunity in the pathogenesis of arthritis. This adoptive transfer process requires the presence of proinflammatory cytokines such as interleukin-1 (IL-1) and TNFα and activation of alternative-pathway complement including C5a (6,7). It has also been shown that mast cells, neutrophils, macrophages, and Fcγ receptor III (FcγRIII) are critical for disease development (7–10).

CD1d-restricted invariant natural killer T (iNKT) cells are a unique subset of T cells that express an invariant T cell receptor (TCR) α-chain and recognize glycolipid antigens presented by CD1d (11,12). Invariant NKT cells are an attractive target for the development of immunotherapies, not only because they contribute to various types of immunoregulation, but also because several synthetic glycolipid ligands specifically activate these cells (13–17). The lack of polymorphism in the antigen-presenting molecule indicates that the ligand has potential for widespread use among individuals, unlike major histocompatibility complex–restricted antigens.

The glycolipid α-galactosylceramide (α-GalCer) is a synthetic glycolipid originally isolated from marine sponge *Agelas mauritanius* and used as a component of anticancer agents, and subsequently a synthetic analog of this compound, KRN7000 (referred to herein as α-GalCer), has been used in experimental studies and in several cancer clinical trials (15–20). Glycolipid α-GalCer and its analogs such as OCH, a sphingosine truncated form, have been shown to suppress autoimmune disease in animal models by inducing a Th2 response to autoantigen (13–17,21–25). In autoantibody-mediated arthritis such as the K/BxN serum transfer model, in which innate immune cells rather than lymphocytes are critical in the pathogenesis (6), iNKT cells have been shown to exaggerate the disease, probably by a mechanism other than modulation of the Th1/Th2 balance (26,27). These findings led us to test a panel of analogs of α-GalCer for their ability to suppress antibody-mediated arthritis.

In the present study, we found that activation of iNKT cells with a novel synthetic glycolipid ligand strongly suppressed K/BxN serum transfer arthritis. Furthermore, we demonstrated the critical role of interferon-γ (IFNγ) in iNKT cell–mediated inhibition of antibody-mediated inflammation.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice were purchased from Clea (Tokyo, Japan). J_α18-knockout mice (28) were kindly provided by Dr. Masaru Taniguchi (Riken Research Center for Allergy and Immunology, Yokohama, Japan). These mice were generated on the 129 strain and backcrossed 10 times to the B6 background. KRN TCR–transgenic mice (3) were kindly provided by Drs. Christophe Benoist and Diane Mathis (Joslin Diabetes Center, Boston, MA). Animals were kept under specific pathogen–free conditions. Animal care and use were in accordance with institutional guidelines.

Glycolipids. Glycolipids SGL-S23, S25, S27, and α-GalCer (KRN7000) were synthesized by reacting 4,5-anhydro-1,3-*O*-benzylidene-D-arabitol with alkyl metal reagents that correspond to the length of sphingosine side chain, and performing subsequent transformations as previously described (29).

Induction of arthritis by K/BxN serum transfer. As previously described, KRN TCR–transgenic mice maintained on the B6 background were crossed with NOD mice to generate K/BxN mice, which spontaneously develop arthritis (3). K/BxN serum pools were prepared from 8-week-old arthritic mice, and arthritis was induced in B6 mice by intraperitoneal injection of 150 μl serum. Serum from nontransgenic littermate mice crossed with NOD mice (BxN mice) was used as control serum.

Clinical assessment of arthritis. Mice were examined for signs of joint inflammation, scored as follows: 0 = no change, 1 = significant 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 swelling and redness of the limb and subsequent ankylosis. The macroscopic score was expressed as the sum of the scores in all paws, with a maximum possible score of 16.

Histopathologic analysis. B6 mice were killed 10 days after K/BxN serum transfer. All 4 paws were removed and then fixed in buffered formalin, decalcified, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or toluidine blue. Histologic features of joint inflammation were scored as follows: 0 = normal joint, 1 = mild arthritis (minimal synovitis without cartilage/bone erosions), 2 = moderate arthritis (synovitis and erosions but joint architecture maintained), 3 = severe arthritis (synovitis, erosions, and loss of joint integrity) (30). The histologic score was expressed as the sum of the scores in all paws, with a maximum possible score of 12. Mast cells in synovium were visually assessed for intact versus degranulating phenotype, using morphologic criteria. Mast cells were identified as cells that contained toluidine blue–positive granules. Only cells in which a nucleus was present were counted. Degranulating cells were defined by the presence of granules outside the cell border with coincident vacant granule space within the cell border, as described previously (8).

In vivo antibody treatment. Mice were administered SGL-S23 at a dose of 100 μg/kg, in 3 intraperitoneal injections on day 0 (the day of immunization), day 3, and day 7 or in 2 intraperitoneal injections on day 3 and day 7. Control mice were injected with vehicle alone (10% DMSO in phosphate buffered saline [PBS]). To neutralize IFNγ, IL-4, IL-10, or transforming growth factor β (TGFβ), 500 μg of anti-IFNγ

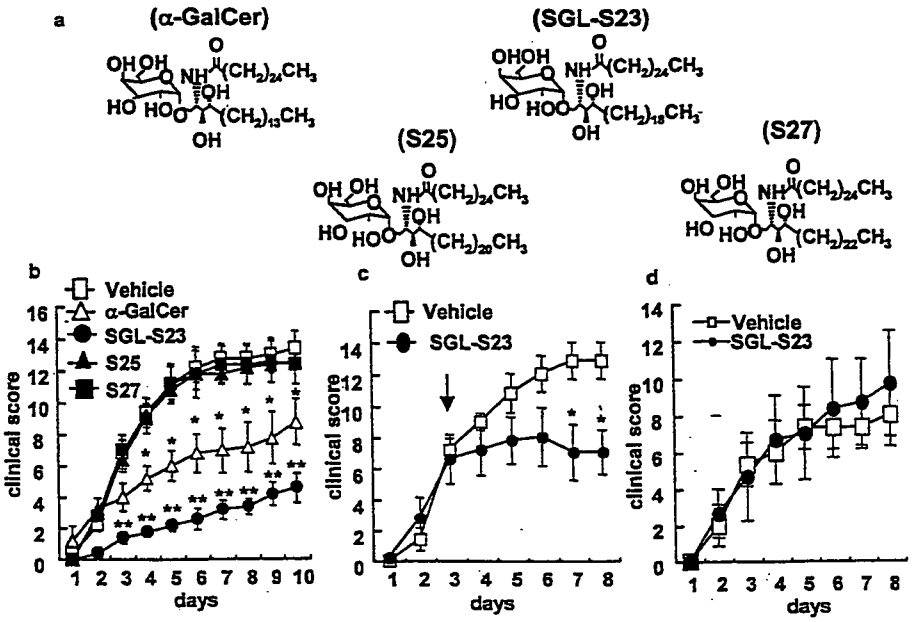

Figure 1. Effect of glycolipid antigens on K/BxN serum transfer arthritis in C57BL/6 (B6) and $J_\alpha 18$-knockout mice. a, Structure of the synthetic glycolipids used in this study. KRN7000 (α-GalCer) is the prototypical α-galactosylceramide and contains a C18 sphingosine base and a C26 acyl chain. SGL-S23, S25, and S27 differ from α-GalCer in the length of the sphingosine base. b, Clinical score of K/BxN serum transfer arthritis in B6 mice treated with 100 μg/kg of α-GalCer, SGL-S23, S25, S27, or vehicle 3 times, starting on the day of K/BxN serum transfer (day 0). * = $P < 0.05$; ** = $P < 0.01$, versus vehicle-treated mice. c, Clinical score of K/BxN serum transfer arthritis in B6 mice treated with 100 μg/kg of SGL-S23 or vehicle twice, starting on day 3 (arrow). * = $P < 0.05$ versus vehicle-treated mice. d, Clinical score of K/BxN serum transfer arthritis in $J_\alpha 18$-knockout mice treated with 100 μg/kg of SGL-S23 or vehicle 3 times, starting on day 0. Values in b–d are the mean ± SEM of 5 mice per group, from a single experiment representative of 2 similar experiments.

monoclonal antibody (mAb) (R4-6A2), anti–IL-4 mAb (11B11), anti–IL-10 mAb (JES052A5), or anti-TGFβ mAb (1D11.16.8) was injected intraperitoneally 4 hours before administration of glycolipid. Non–isotype-matched whole rat IgG or mouse IgG (Sigma, St. Louis, MO) was used as control antibody.

In vitro stimulation. Liver mononuclear cells from B6 mice were isolated by Percoll density-gradient centrifugation and stained with phycoerythrin (PE)–conjugated NK1.1 and fluorescein isothiocyanate (FITC)–conjugated CD3 mAb. Dendritic cells from spleen cells were purified using anti-CD11c microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of sorted NKT cells and dendritic cells was >95%. NKT cells were cultured for 48 hours with irradiated dendritic cells and glycolipid ligands in RPMI 1640 medium supplemented with 50 μ*M* 2-mercaptoethanol, 2 m*M* L-glutamine, 100 units/ml penicillin/streptomycin, and 10% fetal calf serum. The content of cytokines in the culture supernatants was measured by cytometric bead array (BD PharMingen, San Jose, CA), and proliferative responses were assessed based on incorporation of ^{3}H-thymidine (1 μCi/well) in the final 16 hours (analyzed with an αβ-1205 counter [Pharmacia, Uppsala, Sweden]).

Assessment of in vivo responses of iNKT cells to glycolipid antigen. B6 mice were injected intraperitoneally with 100 μg/kg of glycolipid ligands and serum was collected 6 hours and 24 hours after injection. Serum levels of IFNγ were measured by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry and intracellular cytokine staining. Liver mononuclear cells harvested after stimulation with glycolipids in vivo were cultured in complete media containing GolgiStop (BD PharMingen). Cells were then incubated with Fc blocker (anti-mouse FcγIII/IIR mAb clone 2.4G2) and stained with α-GalCer–loaded Dimer X recombinant soluble dimeric mouse CD1d (BD PharMingen), washed with PBS, and then stained with FITC-conjugated mAb A85-1 (anti-mouse IgG1) and allophycocyanin (APC)–conjugated anti-TCR β-chain (BD PharMingen). Subsequently, cells were washed twice with PBS and fixed in Cytofix/Cytoperm (BD PharMingen) solution for 20 minutes at 4°C. After fixation, cells were washed with Perm/Wash (BD PharMingen) solution and resuspended in the same solution containing either PE-conjugated anti-IFNγ mAb XMG1.2 or PE-conjugated isotype control Ig, for 30 minutes at 4°C. Then samples were washed and stained cells analyzed using a FACSCalibur (Becton Dickinson, Mountain View, CA) with CellQuest software

(Becton Dickinson). Liver mononuclear cells stained with α-GalCer–loaded dimeric mouse CD1d followed by FITC-conjugated mAb A85-1 were then stained with SGL-S23–loaded dimeric mouse CD1d followed by PE-conjugated mAb A85-1 and APC-conjugated anti–TCR β-chain. PE-conjugated mAb A85-1 was confirmed not to react with the previously used α-GalCer–loaded dimeric mouse CD1d/FITC-conjugated A85-1 mAb (results not shown).

Measurement of plasma histamine concentrations. Five minutes after intravenous administration of 200 μl K/BxN serum, BxN serum, or PBS, blood from the mice was collected into heparin-containing microtubes. Samples with obvious hemolysis during blood collection were excluded in order to avoid an artificial increase in the histamine concentration due to platelet lysis. The plasma level of histamine was examined by ELISA (Research Diagnostics, Flanders, NJ).

Statistical analysis. The nonparametric Mann-Whitney U test was used to calculate significance levels for all measurements. *P* values less than 0.05 were considered significant.

RESULTS

Activation of iNKT cells by synthetic glycolipid ligands suppresses K/BxN serum transfer arthritis. Through screening of a panel of analogs of α-GalCer for the ability to suppress K/BxN serum transfer arthritis, we found that (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-tricosanetriol, an α-GalCer analog with a 5-carbon longer sphingosine base compared with α-GalCer (referred to as SGL-S23 [suppressor glycolipid S23]) (Figure 1a) had a strong ability to suppress arthritis. Administration of SGL-S23 almost completely inhibited the development of arthritis (Figure 1b); α-GalCer also inhibited arthritis, but to a lesser extent. Arthritis was not suppressed by (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-pentacosanetriol or (2S,3S,4R)-1-*O*-(α-D-galactopyranosyl)-*N*-hexacosanoyl-2-amino-1,3,4-heptacosanetriol, α-GalCer analogs with a 7- or 9-carbon longer sphingosine base (referred to as S25 and S27, respectively) (Figures 1a and b).

To examine the potential therapeutic effect of SGL-S23 on established arthritis, we injected SGL-S23 on day 3 after serum injection, when arthritis had already developed (Figure 1c). In contrast to findings in vehicle-treated mice, in which the severity of arthritis gradually increased, disease severity did not increase, and even decreased, in SGL-S23–treated mice. These results suggest that SGL-S23 has a therapeutic effect in established arthritis.

To confirm that SGL-S23–mediated suppression of K/BxN serum transfer arthritis depends on iNKT cells, we examined the ability of SGL-S23 to modulate

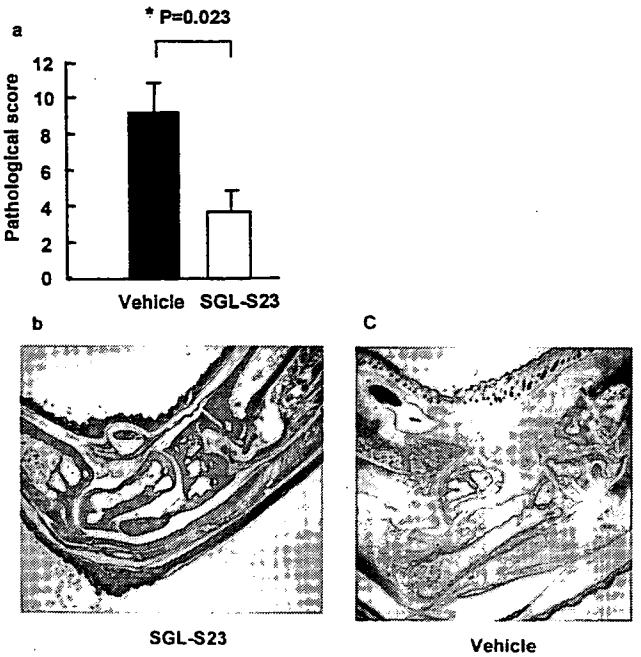

Figure 2. Histopathologic assessment of arthritic joints. **a**, Quantification of histopathologic findings in the joints of C57BL/6 mice, 10 days after K/BxN serum transfer. Mice were treated with 100 μg/kg of SGL-S23 or vehicle 3 times, starting on the day of serum transfer. Values are the mean and SEM of 3 mice per group, from a single experiment representative of 2 similar experiments. **b** and **c**, Histopathologic findings in the joints of representative SGL-S23–treated (b) and vehicle-treated (c) mice (hematoxylin and eosin stained; original magnification × 40).

disease in $J_\alpha 18$-knockout mice, in which iNKT cells are absent (28). Administration of SGL-S23 did not modulate the clinical course of arthritis induced in these mice by injection of K/BxN serum, compared with that in mice treated with vehicle alone (Figure 1d), confirming that SGL-S23–mediated suppression of K/BxN serum transfer arthritis requires iNKT cells.

In addition to visual scoring, we analyzed the histologic features in the joints of all 4 paws in vehicle-treated or SGL-S23–treated mice, on day 10 after disease induction. Quantification of the histologic severity of arthritis is shown in Figure 2a, and typical histologic features are demonstrated in Figures 2b and c. Arthritis was not apparent in the joints of mice treated with SGL-S23 (Figure 2b), in contrast to the severe arthritis with massive cell infiltration, cartilage erosion, and bone destruction seen in the joints of animals treated with vehicle (Figure 2c).

Necessity of IFNγ for SGL-S23–mediated suppression of arthritis. To investigate the mechanism of SGL-S23–mediated suppression of K/BxN serum trans-

Table 1. Clinical arthritis scores in mice with K/BxN serum transfer arthritis*

Treatment	Maximum score	Day of onset
Control rat IgG/vehicle	12.6 ± 1.78	2.6 ± 0.24
Control rat IgG/SGL-S23	5.2 ± 1.24†	4.0 ± 0.55
Anti-IFNγ mAb/vehicle	12.4 ± 1.21	2.4 ± 0.24
Anti-IFNγ mAb/SGL-S23	11.6 ± 1.69	2.2 ± 0.2
Anti-IL-4 mAb/vehicle	12.4 ± 1.67	2.8 ± 0.2
Anti-IL-4 mAb/SGL-S23	6.4 ± 1.12‡	3.0 ± 0.32
Anti-IL-10 mAb/vehicle	14.8 ± 1.15	2.8 ± 0.25
Anti-IL-10 mAb/SGL-S23	6.0 ± 1.38§	3.2 ± 0.66
Control mouse IgG/vehicle	12.0 ± 1.78	1.6 ± 0.25
Control mouse IgG/SGL-S23	6.8 ± 0.97¶	3.6 ± 0.51
Anti-TGFβ mAb/vehicle	13.4 ± 1.17	1.8 ± 0.2
Anti-TGFβ mAb/SGL-S23	6.2 ± 0.92#	2.8 ± 0.73

* C57BL/6 mice were treated with 100 μg/kg of SGL-S23 or vehicle, together with neutralizing monoclonal antibodies (mAb) 3 times, starting on day 0. Values are the mean ± SEM of 5 mice per group, from a single experiment representative of 2 similar experiments. Anti-IFNγ = anti–interferon-γ.
† $P = 0.028$ versus control rat IgG plus vehicle.
‡ $P = 0.027$ versus anti–interleukin-4 (anti–IL-4) mAb plus vehicle.
§ $P = 0.0013$ versus anti–IL-10 mAb plus vehicle.
¶ $P = 0.022$ versus control mouse IgG plus vehicle.
# $P = 0.009$ versus anti–transforming growth factor β (anti-TGFβ) mAb plus vehicle.

fer arthritis, we examined the effect of neutralization of cytokines that have been reported to be implicated in iNKT cell–mediated suppression of experimental autoimmune disease. Previous studies showed that Th2 cytokines such as IL-4 and IL-10 were involved in the suppression of Th1-mediated autoimmune disease in models such as collagen-induced arthritis (CIA), type 1 diabetes in NOD mice, and experimental autoimmune encephalomyelitis (13–17). However, in mice with K/BxN serum transfer arthritis, neutralization of IL-4 or IL-10 did not reverse the protective effect of SGL-S23 against arthritis (Table 1). TGFβ has been reported to be involved in the regulation of K/BxN serum transfer arthritis by iNKT cells (27). However, as was found with IL-4 and IL-10, neutralization of TGFβ did not alter the disease course or the inhibitory effect of SGL-S23 on arthritis (Table 1). In contrast, neutralization of IFNγ unexpectedly, almost completely abolished the inhibitory effect of SGL-S23 (Figure 3a and Table 1).

Inhibition of K/BxN serum transfer arthritis by systemic administration of IFNγ. Since it had not previously been reported that IFNγ suppresses autoantibody-mediated inflammation, we next examined whether administration of IFNγ would ameliorate K/BxN serum transfer arthritis. As shown in Figure 3b, injection of IFNγ reduced the clinical severity of arthritis. Interestingly, there was a tendency for IFNγ administered at a relatively low dose (1 ng) to inhibit arthritis more effectively compared with higher-dose IFNγ (5 ng or 25 ng).

Biologic function of SGL-S23 in vitro and in vivo. We next compared the ability of SGL-S23 and α-GalCer to stimulate NKT cells isolated from liver mononuclear cells. As observed previously (21), α-GalCer at the lowest dose induced a maximum proliferative response (Figure 4a). In contrast, SGL-S23 was able to induce proliferation only when used at higher doses, and its efficacy was lower than that of α-GalCer (Figure 4a). In addition, SGL-S23 at a dose of 90 ng/ml was able to induce IFNγ production (mean ± SEM 21.3 ± 1.40 ng/ml), even though its efficacy was much lower compared with α-GalCer stimulation (830 ± 72.7 pg/ml). The response of liver-derived mononuclear cells to SGL-S23 or to α-GalCer was completely abolished in iNKT cell–deficient TCR $J_\alpha 18$–knockout mice (data not shown), indicating that the response to SGL-S23 is mediated by iNKT cells. S25 or S27 did not induce IFNγ production (data not shown).

Next we examined the response of iNKT cells to SGL-S23 in vivo. We injected SGL-S23, S25, S27, or α-GalCer into B6 mice and measured serum levels of IFNγ by ELISA. Consistent with data obtained in vitro, SGL-S23 induced IFNγ to a lesser extent compared with induction by α-GalCer (Figure 4b). IFNγ was not induced in vivo by either S25 or S27.

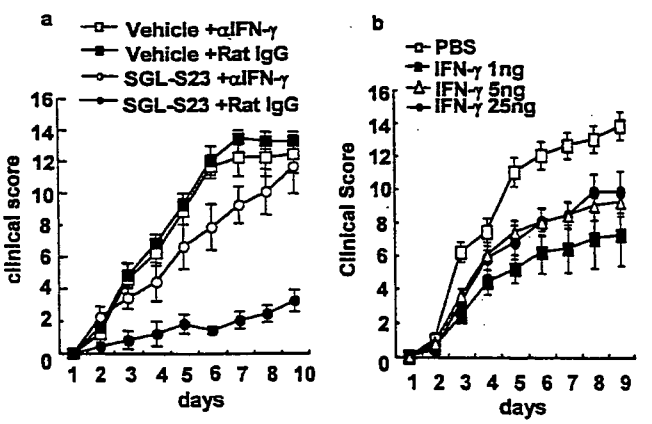

Figure 3. Role of interferon-γ (IFNγ) in SGL-S23–mediated suppression of arthritis. a, Clinical score of K/BxN serum transfer arthritis in C57BL/6 (B6) mice treated with 100 μg/kg of SGL-S23 or vehicle together with anti-IFNγ neutralizing monoclonal antibody (αIFNγ) or control rat IgG 3 times, starting on the day of K/BxN serum transfer (day 0). b, Clinical score of K/BxN serum transfer arthritis in B6 mice treated with phosphate buffered saline (PBS) or with IFNγ administered subcutaneously every day starting on day 0. Values are the mean ± SEM of 5 mice per group, from a single experiment representative of 2 similar experiments.

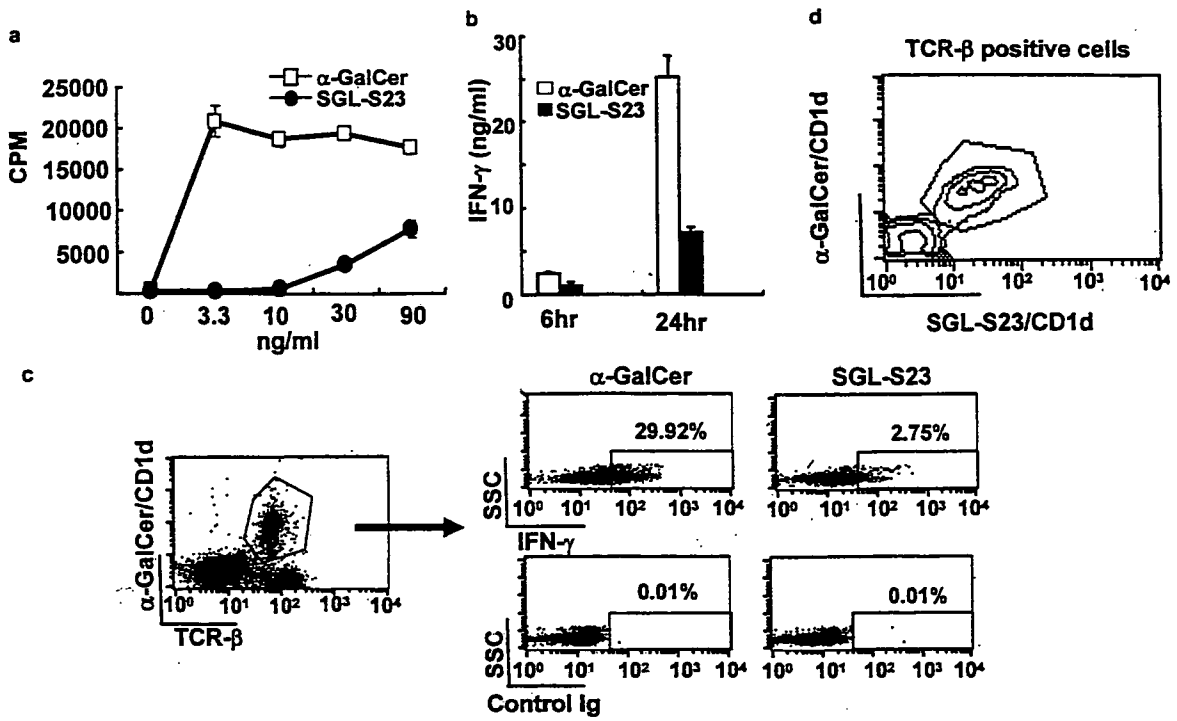

Figure 4. Interferon-γ (IFNγ) production by invariant natural killer T (iNKT) cells stimulated with SGL-S23 in vitro and in vivo. **a**, Effect of glycolipids on liver-derived NKT cells. NK1.1+,CD3+ cells were sorted from liver mononuclear cells and were cultured with various concentrations of α-galactosylceramide (α-GalCer) or SGL-S23 in the presence of irradiated dendritic cells. Proliferative responses were assessed based on ^{3}H-thymidine incorporation. **b**, Change in serum IFNγ levels in C57BL/6 mice after injection of glycolipids. Serum levels of IFNγ 6 hours and 24 hours after intravenous injection of glycolipids were measured by enzyme-linked immunosorbent assay. Values in **a** and **b** are the mean ± SEM of 3 mice per group, from a single experiment representative of 3 similar experiments. **c**, IFNγ production by iNKT cells 2 hours after intraperitoneal injection of α-GalCer or SGL-S23. Intracellular IFNγ-containing cells among CD1d–Dimer X–positive, T cell receptor β (TCRβ)–positive iNKT cells were quantified by flow cytometry. **d**, Results from double staining of TCRβ-positive cells with α-GalCer–loaded CD1d and SGL-S23–loaded CD1d. Data in **c** and **d** are from a single experiment representative of 3 similar experiments. SSC = side scatter.

Production of IFNγ by SGL-S23–activated iNKT cells was further confirmed by intracellular staining of IFNγ in iNKT cells derived from liver mononuclear cells after SGL-S23 injection. It has been shown that iNKT cells produce IFNγ mainly at an early time point, such as ~1–2 hours after injection of glycolipid ligands, and lower induction of IFNγ by iNKT cells results in lower levels of IFNγ in serum, mainly mediated by other cells such as NK cells, at later time points, such as ~6–24 hours after ligand injection (23). Therefore, it seemed reasonable that lower initial IFNγ production by iNKT cells would result in lower induction of IFNγ at later time points. We confirmed that SGL-S23–reactive iNKT cells could recognize α-GalCer as well as α-GalCer–reactive iNKT cells, as shown in Figure 4d. This was consistent with the finding that SGL-S23, a weak inducer of IFNγ, was most effective in suppressing arthritis.

Inhibition of mast cell activation by administration of SGL-S23 or IFNγ. A profound decrease of cellular infiltration into the joints of SGL-S23–treated-mice suggested that the mechanisms of SGL-S23–mediated suppression of arthritis involve inhibition of inflammation at an early phase. We examined the expression of C5a receptor, lymphocyte function–associated antigen 1 (LFA-1), and FcγRIII, which have been previously reported to be necessary for the development of K/BxN serum transfer arthritis, on granulocytes and macrophages, critical components of this model of arthritis (6,9,10,31). The expression of C5a receptor, LFA-1, and FcγRIII on these cells was not reduced during the course of arthritis in SGL-S23–treated mice compared with vehicle-treated mice (data not shown).

Mast cells have been shown to be essential for the

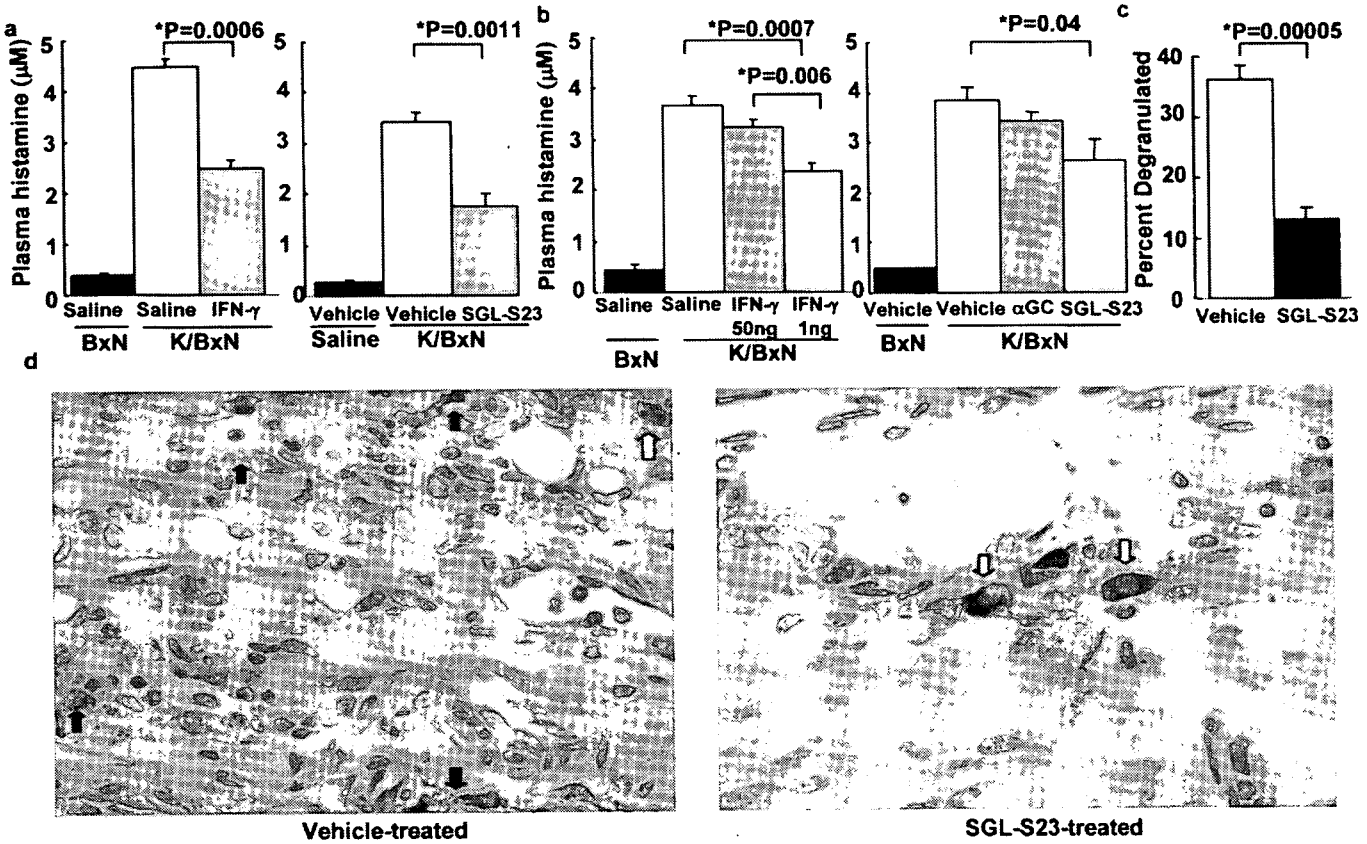

Figure 5. Effect of administration of SGL-S23 on mast cell activation induced by K/BxN serum injection. **a (left panel),** Plasma histamine concentrations 5 minutes after injection of K/BxN serum in C57BL/6 (B6) mice pretreated for 3 days by subcutaneous injection of phosphate buffered saline (PBS) or interferon-γ (IFNγ). BxN serum was injected as a control. **a (right panel),** Plasma histamine concentrations 5 minutes after injection of K/BxN serum in B6 mice treated with SGL-S23 or vehicle. PBS was injected as a control. **b (left panel),** Plasma histamine concentrations 5 minutes after injection of K/BxN serum in B6 mice pretreated for 3 days by subcutaneous injection of IFNγ at the indicated concentrations. **b (right panel),** Plasma histamine concentrations 5 minutes after injection of K/BxN serum in B6 mice pretreated with SGL-S23 or α-galactosylceramide (αGC). **c,** Quantification of histopathologic assessment of mast cell activation 10 days after K/BxN serum transfer in SGL-S23–treated or vehicle-treated mice. Consecutive tissue sections (shown in Figure 2) were stained with toluidine blue. Values in **a–c** are the mean and SEM of 5 mice per group (**a** and **b**) or 3 mice per group (**c**), from a single experiment representative of 2 similar experiments. **d,** Histopathologic features of degranulated or intact mast cells in the joints of representative vehicle-treated and SGL-S23–treated mice. **Solid arrows** indicate degranulated mast cells; **open arrows** indicate intact mast cells (original magnification × 600).

development of arthritis in the K/BxN serum transfer model (8). More recently, vascular leak following mast cell activation induced by K/BxN serum transfer has been shown to be critical for the initiation of arthritis (32). To assess the effect of IFNγ on mast cell activation by serum transfer, we first examined serum level of histamines. B6 mice were treated with 1 ng of IFNγ subcutaneously for 3 consecutive days, and then injected with K/BxN serum intravenously. Five minutes after serum injection, blood was collected and assayed for histamine concentration. As shown in Figure 5a, the plasma histamine concentration increased after treatment with K/BxN serum compared with treatment with control serum. The elevation of the plasma histamine level was inhibited by administration of IFNγ but not by vehicle administration.

We next examined the effect of SGL-S23 on the increase in plasma histamine levels induced by K/BxN serum injection. B6 mice were pretreated with 100 μg/kg of SGL-S23, and K/BxN serum was injected 24 hours later. Consistent with the result obtained with IFNγ treatment, administration of SGL-S23 also suppressed the plasma level of histamine after transfer of K/BxN serum (Figure 5a). To further investigate the hypothesis that treatment with low-dose IFNγ contributes to the suppression of arthritis, we next determined the differ-

ence in histamine levels after administration of low-dose versus high-dose IFNγ. As shown in Figure 5b, lower-dose IFNγ was more effective in suppressing histamine release. Furthermore, we examined whether SGL-S23 is more effective in suppressing histamine release compared with α-GalCer. As shown in Figure 5b, SGL-S23 suppressed histamine levels more effectively than did α-GalCer after K/BxN serum injection. These results indicate that low-dose IFNγ or SGL-S23 inhibits the release of histamine after K/BxN serum injection, suggesting that suppression of mast cell activation contributes to the inhibition of arthritis development.

To further examine whether mast cells were suppressed in SGL-S23–treated arthritic mice, we performed histologic analysis to evaluate mast cell activation. Because degranulation is the clearest histologic hallmark of mast cell activation, mast cells in the joint sections were visually assessed for intact versus degranulating phenotype in tissues stained with toluidine blue. The proportion of degranulated mast cells was significantly lower in SGL-S23–treated mice compared with vehicle-treated control mice. Taken together, these results indicate that mast cell activation is suppressed in mice treated with SGL-S23.

DISCUSSION

We demonstrated in the present study that a newly synthesized glycolipid ligand for iNKT cells, SGL-S23 (a sphingosine chain elongated analog of α-GalCer), strongly suppressed K/BxN serum transfer arthritis by inhibiting inflammatory cell infiltration and the resultant destruction of cartilage and bone. SGL-S23–mediated suppression of arthritis was dependent on IFNγ. Consistent with this, administration of IFNγ inhibited the development of arthritis. Administration of either SGL-S23 or IFNγ suppressed the K/BxN serum transfer–induced histamine release from mast cells that is critical for the initiation of arthritis.

It has been reported that iNKT cells are involved in the pathogenesis of K/BxN serum transfer arthritis (26,27). Activation of iNKT cells by α-GalCer has been previously shown to exacerbate K/BxN serum transfer arthritis (27), which is inconsistent with our finding in the present study that α-GalCer inhibited arthritis to some extent. It is clear that newly synthesized SGL-S23 inhibited arthritis to a greater extent than did α-GalCer, however, and the reason for the discrepancy between the previous result and ours is not known.

IFNγ has been shown to have biphasic functions in several murine arthritis models, such as CIA, adjuvant-induced arthritis, and group B streptococcal arthritis (33–36). Administration of IFNγ exacerbated arthritis and blocking of IFNγ inhibited arthritis at an early stage in these models; however, when IFNγ was administered at a later time point, arthritis was not exacerbated, and was in fact suppressed. IFNγ appears to enhance the immune response in the early phase, and to down-regulate arthritis in the later phase in these models. Moreover, acceleration of CIA has been reported in both IFNγ-knockout and IFNγ receptor–knockout mice (37,38). K/BxN serum transfer arthritis is considered to represent the inflammatory process of arthritis; therefore, the suppression of disease by IFNγ observed in the present study is not inconsistent with these previous findings.

Although IFNγ has been shown to have both an enhancing and a suppressive effect on autoimmune inflammation, the mechanisms that underlie the inhibition of inflammation are not clearly understood (39,40). The suppressive effect of IFNγ on osteoclastogenesis has been implicated as one of the mechanisms of inhibition of arthritis (41). Activation-induced cell death of T cells has also been suggested to be involved in IFNγ-mediated suppression of inflammation (42,43). More recently, IFNγ has been shown to suppress the development of IL-17–producing T cells, which are critical for autoimmune inflammation (44–47).

However, it is unlikely that SGL-S23–mediated suppression of K/BxN serum transfer arthritis involves these mechanisms, because lymphocytes are not required for the development of arthritis in this model. Furthermore, the inhibition of arthritis by SGL-S23 is accompanied by suppression of inflammatory cell infiltration into the joints prior to bone destruction by osteoclasts. Therefore, our results suggest that IFNγ-regulated inflammatory cells are involved in innate immune responses. IFNγ is well known as a stimulator of monocyte/macrophages and granulocytes, which are important for the development of K/BxN serum transfer arthritis. In support of this, administration of SGL-S23 did not induce any suppressive effect on these cells. SGL-S23 did, however, reduce the release of histamine induced by K/BxN serum transfer, suggesting suppression of the mast cell activation that is crucial for the development of arthritis.

Interestingly, SGL-S23 is less potent than α-GalCer in stimulating iNKT cells and inducing IFNγ production, which is consistent with the finding that IFNγ suppressed arthritis more effectively at a lower dose than at a higher dose. Similarly, low-dose IFNγ has been demonstrated to prevent migration of T cells and B

cells (48–50). IFNγ might suppress inflammation only if administered at a very precise dosage level, and SGL-S23 may thus have greater utility as an inhibitor of inflammatory arthritis. High, nonphysiologic doses of IFNγ may induce negative feedback loops, thereby limiting any potential protective effects.

We have previously shown that OCH, another analog of α-GalCer with a truncated sphingosine chain, preferentially induces Th2 cytokines (21–23). SGL-S23 administered in vivo induces more IFNγ and less IL-4 compared with OCH and possesses a stronger ability to suppress inflammatory arthritis compared with α-GalCer or OCH (Kaieda S, et al: unpublished observations), indicating a unique property of this ligand. SGL-S23 may have clinical benefit over α-GalCer since it induces a weaker cytokine response in iNKT cells, thereby reducing potential side effects caused by excessive cytokine release. In addition, because repeated administration of SGL-S23 stimulates iNKT cells to a lesser extent than does α-GalCer administration, recovery from nonresponsiveness may be more rapid with SGL-S23 than with α-GalCer.

Manipulation of regulatory cells is a new strategy for immunotherapy, and iNKT cells would serve as one of the most suitable cell types for in vivo stimulation, due to the availability of specific ligands. The lack of polymorphism in the antigen-presenting molecule further indicates that the ligand has potential for widespread use among individuals, unlike major histocompatibility complex–restricted peptide antigens.

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AUTHOR CONTRIBUTIONS

Dr. Miyake had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Miyake.
Acquisition of data. Kaieda, Tomi.
Analysis and interpretation of data. Kaieda, Oki, Yamamura, Miyake.
Manuscript preparation. Kaieda, Miyake.
Statistical analysis. Kaieda.

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