controversial result concerning the clinical features of anti-Mi-2 antibody. They analyzed 417 European patients with myositis for anti-Mi-2 using enzyme-linked immunosorbent assay (ELISA) for four overlapping fragments spanning the entire sequence of the Mi-2β molecule. Anti-Mi-2 was found in 48 patients, including those with dermatomyositis (50%), polymyositis (40%), and even IBM (8%). Myositis with anti-Mi-2 was characterized by relatively mild disease and treatment response was fair. Patients with antibody to the N-terminal fragment of Mi-2β appeared to have a high risk for malignancy, although no statistical difference was found. Although the reason for the discrepancy among these reports is unknown, the methods detecting autoantibodies may be critical for the results. Moreover, there may be some difference in reactivity against two Mi-2 molecules.

Pathogenic roles of autoantigens and autoantibodies in myositis

Both genetic and environmental factors are involved in the development of IIM and the production of MSA. Many reports [49–52] demonstrate the close associations between various MSAs and distinct HLA types. Such associations, however, vary among different ethnic groups, suggesting that other genetic and probably environmental backgrounds may also be important. There is significant evidence that various environmental factors such as infections may be involved in the development of myositis [41,53–55]. One possible hypothesis is that several myogenic viruses may interact with ARSs and virus–ARS complexes act as cryptic epitopes to break a tolerance to self-antigens. Certain RNA virus genomes have tRNA-like structures and can be aminoacylated by ARSs [19]. An alternative hypothesis is that molecular mimicry between autoantigen molecules and infectious agents may produce cross-reactive autoantibodies, as many examples of molecular mimicry between myositis autoantigens and viral proteins have been reported [56,57]. Once an antiviral antibody cross reacting with an autoantigen epitope is produced, the activated B cell, which expresses an immunoglobulin receptor to the common epitope, may process the autoantigen molecule and act as an antigen-presenting cell against autoreactive T cells. Then, epitopes on autoantigen molecules will be expanding. Another possible environmental factor is ultraviolet rays that exacerbate dermatomyositis skin rashes. An interesting report of a worldwide evaluation of geoclimatic factors by Okada *et al.* [58] suggested that surface ultraviolet radiation intensity most strongly correlated with the proportion of dermatomyositis and anti-Mi-2 antibody. This result supports a hypothesis that ultraviolet exposure may modulate the autoimmune response and development of autoimmune diseases.

The production of disease-specific autoantibodies may closely correlate with pathogenic mechanisms of inflammatory myopathy. For example, patients with antibodies to different ARSs show the same clinical symptoms known as antisynthetase syndrome. This fact strongly suggests that the immune response to molecules with analogous functions leads to a similar clinical syndrome. There is no evidence, however, that anti-ARS autoantibodies have direct pathogenic effects on developing myositis.

Recent reports have demonstrated that certain ARS molecules (histidyl, asparaginyl, and seryl-tRNA synthetases) and their proteolytic fragments have chemokine-like activities against inflammatory cells such as CD4+ and CD8+ T cells, activated monocytes and immature dendritic cells [59]. Mononuclear cells expressing chemokine receptors such as CCR3 and CCR5 infiltrate in muscle tissues of myositis patients but not in normal muscle, supporting the results that histidyl and asparaginyl-tRNA synthetases activate CCR5+ and CCR3+ cells, respectively [59]. These findings indicate that the liberation of autoantigenic ARSs from damaged muscle tissues may recruit inflammatory mononuclear cells and perpetuate the inflammation process of myositis, and also induce autoimmune responses to autoantigens.

Enhanced autoantigens in regenerating muscle fibers rather than normal muscle were suggested to be involved in autoimmune myositis as the source of autoantigen supply. Myositis-specific and myositis-associated autoantigens, such as Jo-1, Mi-2, U1-70K, Ku and DNA-PKcs, were expressed at very low levels in normal muscle, whereas they were increased at high levels in myositis muscle, especially in regenerating muscle cells [60]. It is also well known that damaged muscle fibers but not normal muscle express a high level of major histocompatibility class I antigens. These data strongly suggest that regenerating muscle cells after injury by inflammation, such as viral infection, may be the source of myositis-specific autoantigens and be targeted by autoreactive T cells in autoimmune myositis. Moreover, it was demonstrated that the expression of myositis autoantigens was also markedly increased in certain cancers associated with myositis, and the antigenic fingerprint of such cancers closely resembled that of regenerating muscle cells [60]. These results raise an interesting possibility that the autoimmune response in myositis may be initiated by an antitumor immune response. One of the weak points of this hypothesis is that MSAs and MAAs have rarely been detected in malignancy-associated myositis. The recent discoveries of anti-p155 and anti-p155/p140 antibodies with high prevalence in malignancy-associated myositis, however, may explain the linkage between cancer and myopathy.

Conclusion

During the past year great advances have been achieved, particularly in identifying novel MSAs in C-ADM and

malignancy-associated myositis. These findings are extremely important because it had previously been thought that MSAs and MAAs were not detected in those subgroups of IIM. The discovery of these new autoantibodies may enable IIM to be classified both clinically and serologically. Analysis of the detailed structure and function of target autoantigen molecules is particularly important for understanding the etiology and pathogenic mechanisms of myositis.

Acknowledgements

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

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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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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

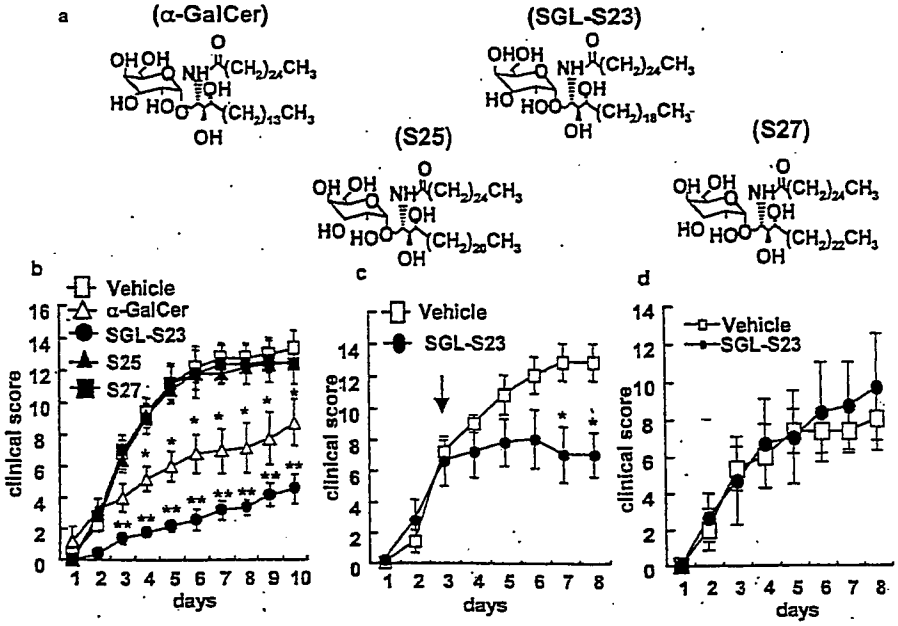

Figure 1. Effect of glycolipid antigens on K/BxN serum transfer arthritis in C57BL/6 (B6) and $J_\alpha 18$-knockout mice. a, Structure of the synthetic glycolipids used in this study. KRN7000 (α-GalCer) is the prototypical α-galactosylceramide and contains a C18 sphingosine base and a C26 acyl chain. 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

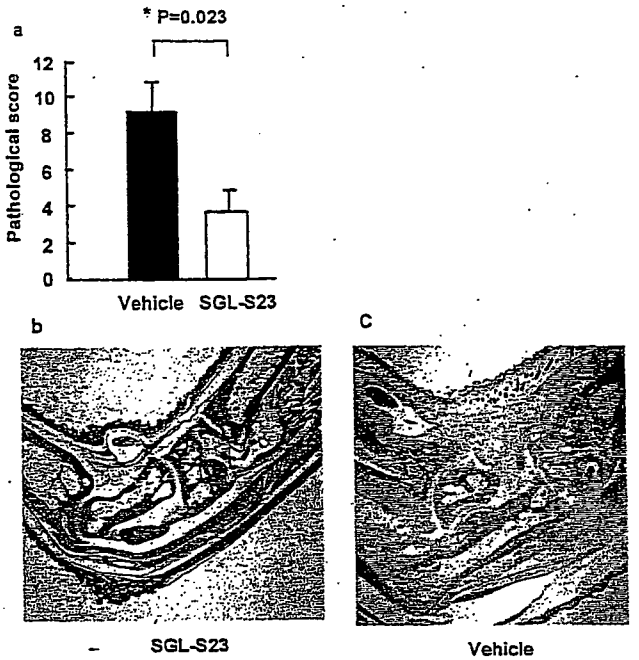

Figure 2. Histopathologic assessment of arthritic joints. a, Quantification of histopathologic findings in the joints of C57BL/6 mice, 10 days after K/BxN serum transfer. Mice were treated with 100 μg/kg of SGL-S23 or vehicle 3 times, starting on the day of serum transfer. Values are the mean and SEM 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.

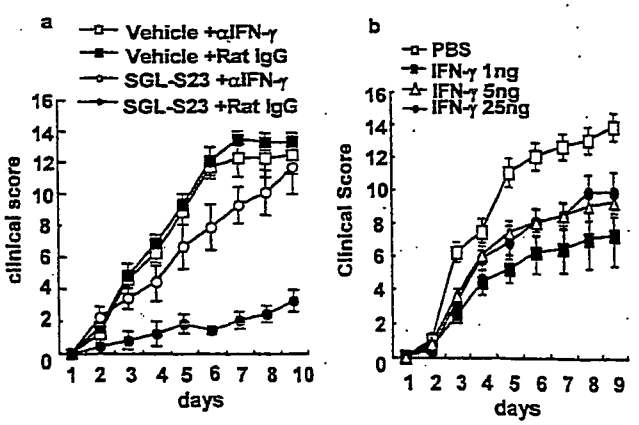

Figure 3. Role of interferon-γ (IFNγ) in SGL-S23–mediated suppression of arthritis. a, Clinical score of K/BxN serum transfer arthritis in C57BL/6 (B6) mice treated with 100 μg/kg of SGL-S23 or vehicle together with anti-IFNγ neutralizing monoclonal antibody (αIFNγ) or control rat IgG 3 times, starting 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.

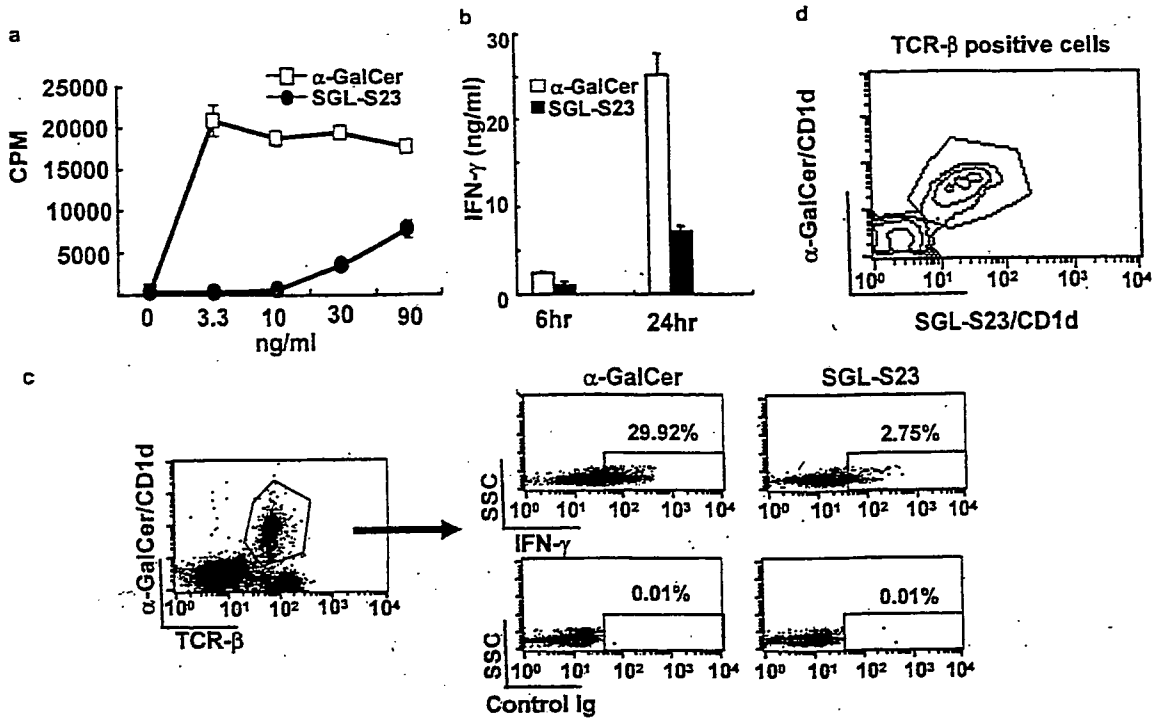

Figure 4. Interferon-γ (IFNγ) production by invariant natural killer T (iNKT) cells stimulated with SGL-S23 in vitro and in vivo. a, Effect of glycolipids on liver-derived NKT cells. NK1.1+,CD3+ cells were sorted from liver mononuclear cells and were cultured with various concentrations of α-galactosylceramide (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

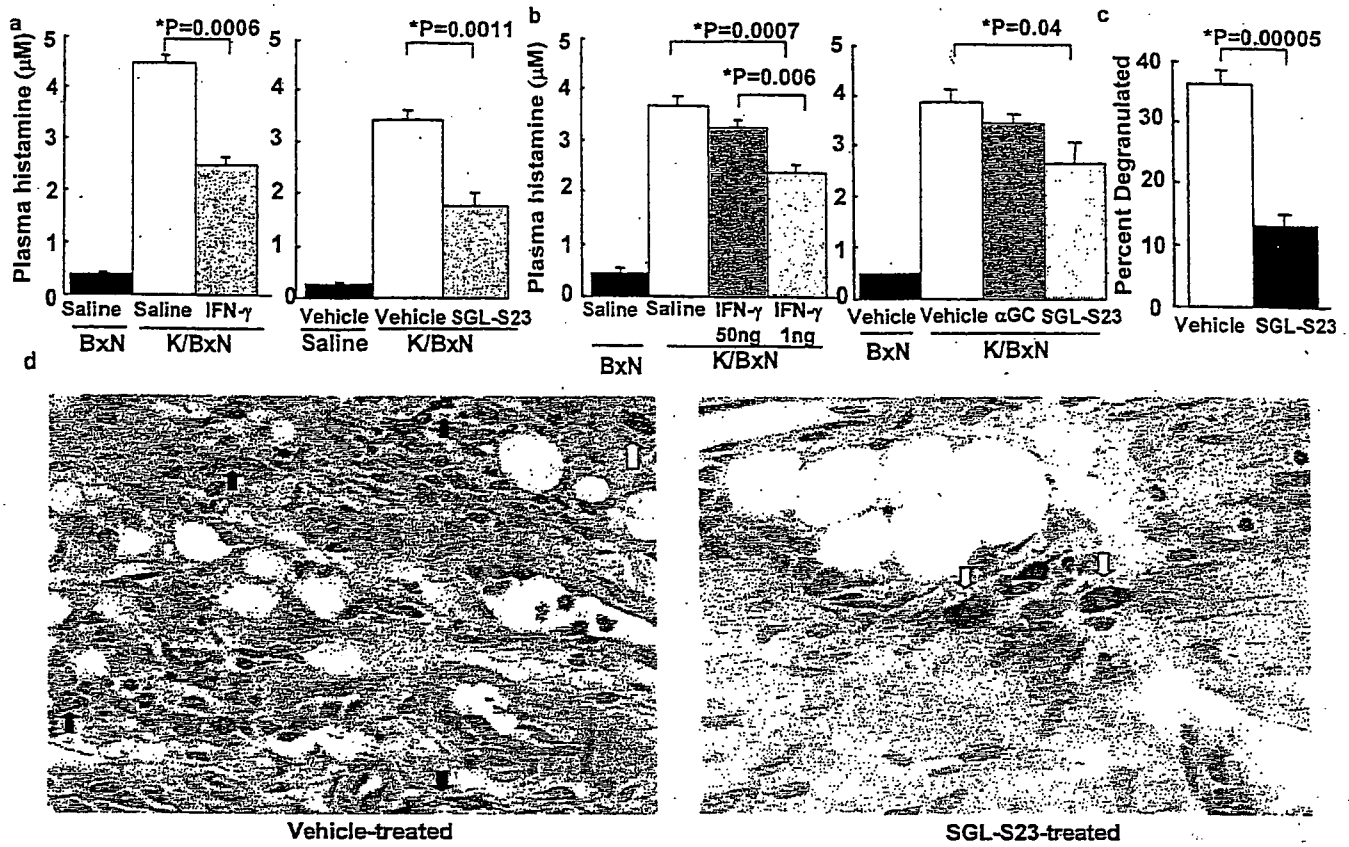

Figure 5. Effect of administration of SGL-S23 on mast cell activation induced by K/BxN serum injection. a (left panel), Plasma histamine concentrations 5 minutes after injection of K/BxN serum in C57BL/6 (B6) mice pretreated for 3 days by subcutaneous injection of phosphate buffered saline (PBS) or interferon-γ (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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THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Human Th17 Cells Are Identified as Bearing CCR2$^+$CCR5$^-$ Phenotype[1]

Wakiro Sato, Toshimasa Aranami, and Takashi Yamamura[2]

Recent reports have shown that IL-17-producing CD4$^+$ T cells (Th17 cells) belong to a distinct helper T cell lineage and are critically involved in the pathogenesis of autoimmune diseases and allergies. However, the chemokine receptor profile of Th17 cells remains to be clarified. In this study, we report that human Th17 cells are identified as CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells. Analysis of PBMC from healthy donors showed that CCR2$^+$ cells produce much larger amounts of IL-17 than CCR2$^-$ cells, indicating the preferential expression of CCR2 on Th17 cells. Notably, CCR2$^+$CCR5$^-$ memory CD4$^+$ T cells produced a large amount of IL-17 and little IFN-γ, whereas CCR2$^+$CCR5$^+$ cells reciprocally produced an enormous amount of IFN-γ but little IL-17. Moreover, a higher expression of T-bet was seen in the CCR5$^+$ memory T cells. These results indicate that absence of CCR5 distinguishes human Th17 cells from Th1 cells. The Journal of Immunology, 2007, 178: 7525–7529.

CD4$^+$ Th cells are essential regulators of adaptive immune responses. Th cells have been classified as either Th1 or Th2 according to the cytokine production profile and functional properties. However, recent studies have demonstrated that IL-17-producing T cells, rather than Th1 cells, play a pivotal role in the pathogenesis of autoimmune disease models, including experimental autoimmune encephalomyelitis (EAE)[3] (1–4). IL-17 is a cytokine mainly produced by activated memory T cells and could recruit and expand neutrophils through induction of various chemokines and GM-CSF (5–7).

Numerous studies have provided evidence that IL-17-producing T cells belong to a distinct lineage of Th cells whose development is severely hampered in IL-23 knockout but not in IL-12 knockout mice (8, 9). Although IL-23 was initially thought to induce differentiation of the IL-17-producing cells, it now seems that IL-23 is not involved in differentiation but propagation of Th17 cells (10). In fact, recent studies have shown that a combination of TGF-β1 plus IL-6 promotes the differentiation of Th17 cells in vitro (11–13). Differentiation of Th17 cells is prohibited by IFN-γ or IL-4 (11–13), further supporting the concept that Th17 cells comprise a distinct population cross-regulated by Th1 or Th2 cells. Notably, the independent nature of Th17 cells has been further highlighted by the recent discovery that the transcription factor RORγt is critically involved in the development of Th17 cells (14).

During the critical process whereby naive CD4$^+$ T cells differentiate, they acquire reciprocal sets of chemokine receptors (15), which would endow them a unique character of homing or migration to corresponding ligand chemokines. Namely, Th1 cells preferentially express CCR5 and CXCR3 and migrate to inflammatory milieu expressing the corresponding ligand chemokines, whereas Th2 cells express CCR4, CCR8, and CRTh2 indicative of a distinctive homing property (16–19). It is conceivable that Th17 cells may also possess unique chemotactic and migratory property. However, chemokine receptor expression by Th17 cells has not been characterized yet, at least to our knowledge.

In this study, we attempted to identify chemokine receptor expression by human Th17 cells by examining cytokine production profiles of T cell subpopulation-bearing chemokine receptor(s) of interest (16, 20). We started by comparing CCR2$^+$ and CCR2$^-$ memory CD4$^+$ T cells, because CCR2 and its ligand CCL2 were shown to be essential for development of EAE (21, 22). We found that only the CCR2$^+$ subpopulation would produce IL-17. Further analysis has demonstrated that CCR5$^-$ cells among the CCR2$^+$CD4$^+$ memory T cells produce IL-17, whereas a CCR5$^+$ subpopulation produces IFN-γ. Thus, human Th17 cells are identified as uniquely bearing the CCR2$^+$CCR5$^-$ phenotype.

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[3] Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DN, double negative; DP, double positive; SP, single positive; MFI, mean fluorescence intensity.

Materials and Methods

Reagents

Anti-CCR2-biotin mAb, anti-CCR5-FITC mAb, and goat anti-IL-23R polyclonal Abs were purchased from R&D Systems. Streptavidin-PE, anti-CD4-PC5 mAb, and anti-CD45RA-energy-coupled dye (ECD) mAb were obtained from Beckman Coulter, anti-CCR5-allophycocyanin mAb from BD Pharmingen, and donkey anti-goat IgG-FITC from Jackson ImmunoResearch Laboratories. Anti-T-bet mAb and isotype control Ab (mouse IgG1a) purchased from Santa Cruz Biotechnology were used for intracellular staining. RPMI 1640 medium (Invitrogen Life Technologies) was supplemented with 0.05 mM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% FBS.

Cell preparation

Peripheral blood was obtained from healthy human volunteers (24–42 years of age) from whom informed consent was obtained. The Ethics Committee of the National Center of Neurology and Psychiatry approved the study. PBMC were freshly isolated by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). To purify whole T cells or memory $CD4^+$ T cells from PBMC, we used a Pan T cell isolation kit II or Memory $CD4^+$ T cell isolation kit (Miltenyi Biotec), respectively. Briefly, PBMC were labeled with a mixture of biotin-conjugated mAbs directed against either non-T or non-memory $CD4^+$ T cells and then reacted with magnetic microbead-conjugated anti-biotin mAbs. The magnetically labeled non-T or non-memory $CD4^+$ T cells were depleted with autoMACS (Miltenyi Biotec), which yielded >95% purity of whole T cells or memory $CD4^+$ T cells as assessed by flow cytometry for the proportion of $CD3^+$ cells or $CD4^+CD45RA^-$ cells.

To further separate the purified cells according to CCR2 or CCR5 expression, they were labeled with anti-CCR2-biotin, streptavidin-PE, and anti-PE microbeads (Miltenyi Biotec) or anti-CCR5-FITC and anti-FITC microbeads (Miltenyi Biotec). The magnetically labeled cells were separated into positive ($CCR2^+$ and $CCR5^+$) and negative ($CCR2^-$ and $CCR5^-$) fractions with autoMACS (>99% purity of $CCR2^+$ or $CCR5^+$ cells and >90% purity of $CCR2^+$ or $CCR5^+$ cells). To obtain $CCR2^+CCR5^-$ and $CCR2^-CCR5^-$ memory $CD4^+$ T cells, $CCR2^+$ memory $CD4^+$ T cells were labeled with anti-CCR5-allophycocyanin and separated into $CCR2^+CCR5^-$ (>80% purity) and $CCR2^-CCR5^+$ cells (>95% purity) by flow cytometric cell sorter Epics Altra (Beckman Coulter).

Cell culture and cytokine measurement by ELISA

Purified T cell populations were resuspended at 5×10^5/ml and stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) in 96-well U-bottom plates for 24 h. The concentrations of IFN-γ and IL-17 in the supernatants were measured by using a Human IFN-γ ELISA Set (BD Pharmingen) and a Human IL-17 DuoSet (R&D Systems).

Flow cytometric analysis of chemokine receptors

To evaluate the expression of chemokine receptors, purified memory $CD4^+$ T cells were stained with anti-CD4-PC5, anti-CD45RA-ECD, anti-CCR5-FITC and PE-conjugated mAbs against anti-CCR2-biotin were analyzed with Epics flow cytometry (Beckman Coulter). To examine the expression of IL-23R, memory $CD4^+$ T cells were stained with goat anti-IL-23R and anti-goat-IgG-FITC and were analyzed with a FACSCalibur (BD Pharmingen).

Intracellular staining of T-bet

Purified memory $CD4^+$ T cells were first stained with biotin-conjugated anti-CCR2, streptavidin-PE, and allophycocyanin-CCR5, then fixed in PBS containing 2% paraformaldehyde and permeabilized with 0.1% saponin solution. Subsequently, the cells were stained with FITC-anti-T-bet. Mouse IgG1a was used as an isotype control.

Statistics

An unpaired Student's *t* test or one-way ANOVA was used for statistical analysis. We considered $p < 0.01$ as significant.

Results and Discussion

Both Th17 cells and Th1 cells are enriched in $CCR2^+CD4^+$ memory T cells

Previous reports on the CCR2 requirement for development of EAE (21, 22) prompted us to compare the cytokine-producing ability of $CCR2^+$ and $CCR2^-$ cells isolated from whole T cells. The results showed that $CCR2^+$ cells produced a larger amount of IFN-γ and IL-17 as compared with $CCR2^-$ cells, whereas unseparated whole T cells showed intermediate values (Fig. 1*A*, *upper panels*). This indicates that $CCR2^-$ cells contain the vast majority of Th1 and Th17 cells. We next separated the whole T cells into $CCR5^+$ and $CCR5^-$ populations to compare the cytokine profile. Although $CCR5^+$ cells produced a larger amount of IFN-γ as compared with $CCR5^-$ or the whole T

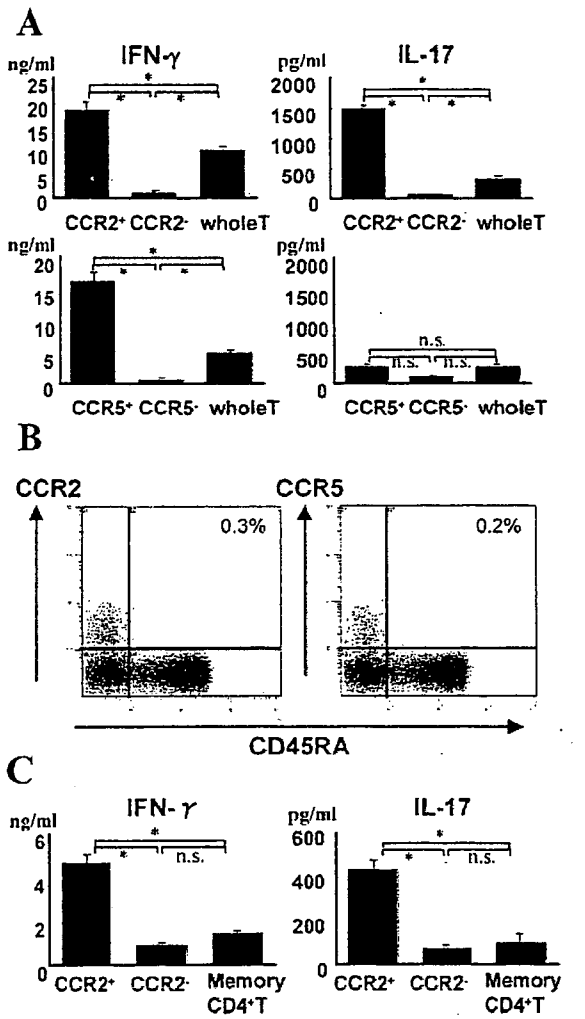

FIGURE 1. Th17 cells are enriched in $CCR2^+CD4^-CD45RA^-$ cells. *A*, IL-17-producing cells are selectively enriched in the $CCR2^+$, but not in a $CCR5^+$ population. From PBMC of healthy donors, whole T cells were purified magnetically by negative selection using a Pan T cell isolation kit II with autoMACS. The purity of the cells was generally >98%, as determined by FACS analysis. Purified $CCR2^+$ and $CCR2^-$ T cells (or $CCR5^+$ and $CCR5^-$ T cells) were stimulated with PMA and ionomycin for 24 h before the supernatants were collected. The IFN-γ and IL-17 protein in each supernatant was measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *B*, Chemokine receptor (CCR2 or CCR5) expressing T cells are largely confined to $CD45RA^-$ memory T cells. PBMC from healthy subjects were stained with anti-CCR2 (PE), anti-CCR5 (FITC), anti-CD4 (PC5), and anti-CD45RA (ECD) and analyzed after being gated for CD4. Shown is a representative of five individual data sets. *C*, Th17 cells are enriched in $CCR2^+CD4^+CD45RA^-$ cells. Memory $CD4^+$ T cells were purified by a memory $CD4^+$ T cell isolation kit with autoMACS. $CCR2^+$ and $CCR2^-$ T cells were further isolated by anti-CCR2-biotin, streptavidin-PE, anti-PE microbeads, and autoMACS. Purified cells were stimulated with PMA and ionomycin for 24 h before collecting supernatants. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$.

cells (Fig. 1*A*, *lower panels*), production of IL-17 did not increase after enrichment for $CCR5^+$ cells. These results suggest that Th17 cells may be selectively enriched in $CCR2^-$, but not in $CCR5^-$ populations. However, because the $CCR2^+$ T cell preparation also contains $CD8^+$ T cells and $\gamma\delta$T cells, capable of producing IL-17 (23–25), it remained possible that the major source of IL-17 could be $CD8^-$ T or $\gamma\delta$T cells. Therefore, we next needed to assess the production of IFN-γ and IL-17 from purified $CD4^+$ T cells. Preparatory experiments showed that $CCR2^+$ or $CCR5^+CD4^+$ populations are mainly confined to the $CD45RA^-$ memory T cell population (Fig. 1*B*). Consequently, we decided to use memory $CD4^+$ T cells that could be obtained after deleting $CD8^+$, $\gamma\delta$, and naive $CD4^+$ T cells for further analysis. Analysis of the purified memory $CD4^+$ T cells has also demonstrated that the $CCR2^+$ population produced a significantly larger amount of both IFN-γ and IL-17 compared to the $CCR2^-$ population, with the values of unseparated cells being intermediate (Fig. 1*C*). These results strongly indicate that Th17 cells as well as Th1 cells are enriched in $CCR2^+CD4^+$ memory T cells. However, since Th1 and Th17 cells are thought to belong to distinct T cell lineages, we speculated that they might be further divided into two subpopulations based on expression of chemokine receptors.

$CCR2^+$ $CCR5^-$ memory $CD4^+$ T cells predominantly produce IL-17 but not IFN-γ

Simultaneous staining of CCR2 and CCR5 showed that the $CCR2^+$ memory T cell population could be divided into $CCR5^-$ (CCR2 single positive (SP)) and $CCR5^+$ (CCR2 and CCR5 double positive (DP)) subpopulations (Fig. 2). Since CCR5 is reported to be expressed predominantly on Th1 cells (16–18), we hypothesized that SP and DP cells might correspond to Th17 and Th1 cells, respectively. To correlate cytokine production profile and chemokine receptor expression in T cell populations, we first thought of staining total unseparated T cells to detect intracellular cytokines as well as surface CCR; however, the cell activation process required for intracellular cytokine staining was found to down-regulate CCR2 and CCR5 significantly (data not shown), as reported previously (26). To accurately correlate the expression of CCR2 or CCR5 with the cytokine profile ex vivo, we decided to first isolate SP and DP cells from memory $CCR2^+$ T cells by using a flow cytometric cell sorter and stimulate them with PMA and ionomycin. We then measured IFN-γ and IL-17 in the supernatant (Fig. 3*A*). Remarkably, the sorted T cell subpopulations exhibited different cytokine production patterns: SP cells produced a large amount of IL-17 and a small amount of IFN-γ, whereas DP cells produced a small amount of IL-17 and a large amount of IFN-γ (Fig. 3*B*). These results suggest that Th17 cells are largely confined to SP cells, whereas DP cells contain a majority of Th1 cells.

T-bet and IL-23R expression in memory $CD4^+$ T cells

Finally, we assessed whether SP and DP cells are distinctive in expression of transcription factor T-bet and IL-23R. T-bet is an essential transcription factor for Th1 differentiation (27), whereas it was reported to be redundant for Th17 cells (3, 8, 9, 11, 14). IL-23 has been shown to play a pivotal role in the survival and expansion of Th17 cells (2, 10). Magnetically purified memory $CD4^+$ T cells were first stained with biotin-conjugated CCR2, streptavidin-PE, and allophycocyanin-CCR5, and then were intracellularly stained with FITC-anti-T-bet or were stained with goat anti-IL-23R Ab and anti-goat IgG-FITC. We compared T-bet expression in SP vs DP cells by evaluating the mean fluorescence intensity (MFI) (Fig. 4, *A* and *B*). T-bet was significantly expressed by SP as well as $CCR2^-CCR5^-$ double-negative (DN) cells, but its expression was much higher in DP cells and $CCR2^-CCR5^+$ cells, suggesting that Th1 cells may be confined to $CCR5^+$ populations. On the other hand, the frequency of $IL\text{-}23R^-$ cells was highest in the SP fraction, compared with the others (Fig. 4, *C* and *D*).

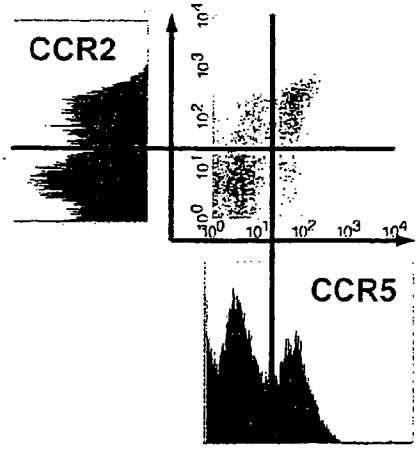

FIGURE 2. $CCR2^+$ memory $CD4^+$ T cells consist of $CCR5^+$ and $CCR5^-$ subsets. Purified memory $CD4^+$ T cells were stained with anti-CCR2-biotin and streptavidin-PE as well as anti-CCR5-FITC. The separation of positive and negative populations was determined by histogram plots.

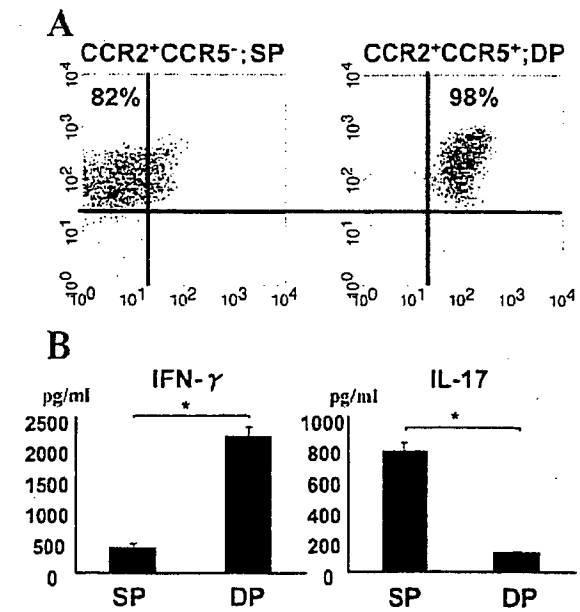

FIGURE 3. $CCR2^+CCR5^-$ (SP) and $CCR2^+CCR5^+$ (DP) cells correspond to Th17 and Th1 cells, respectively. SP and DP subsets were sorted from memory $CD4^+$ T cells by flow cytometry and stimulated with PMA/ionomycin. *A*, A representative of five individual data sets showing the purity of the sorted cells. *B*, SP and DP cells were stimulated with PMA/ionomycin for 24 h. Then the amounts of IFN-γ and IL-17 in the supernatant were measured using ELISA. Results are expressed as mean ± SD of a representative of five independent experiments. *, $p < 0.01$

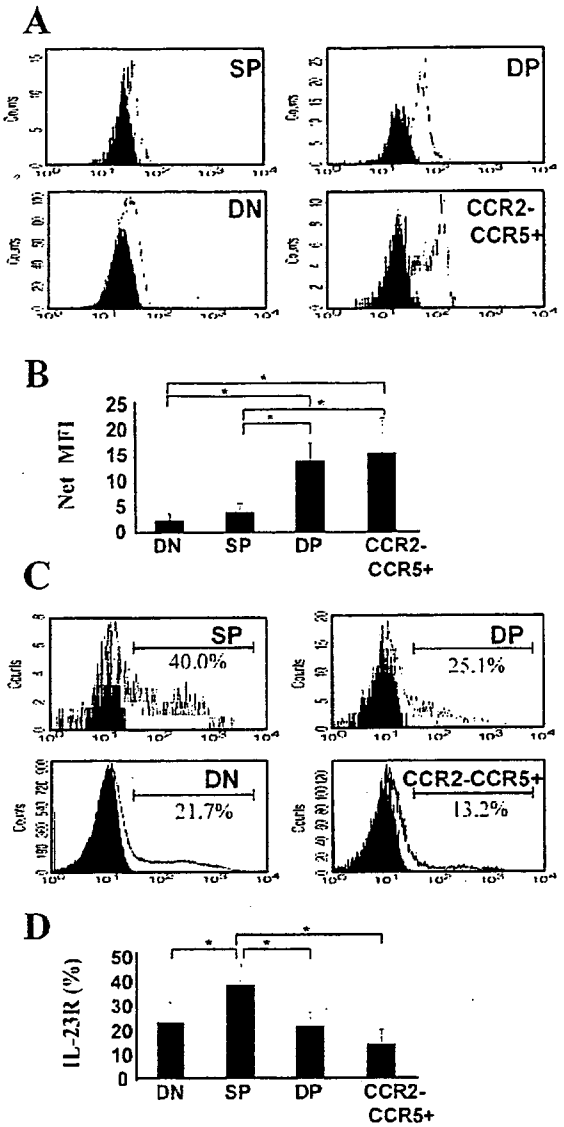

FIGURE 4. T-bet expression of memory $CD4^+$ T cells. *A*, Histogram plots of T-bet expression within memory $CD4^+$ T cells. SP, DP, DN ($CCR2^-CCR5^-$), and $CCR2^-CCR5^+$ T cells were stained with anti-T-bet or isotype control Ab. Shown is a representative of five individual data sets. *B*, The MFI of each histogram plot. Data for the MFI of T-bet expression subtracted by that of control Ab are calculated and shown as bar graphs with error bars showing the SD of four individual data sets. *C*, Histogram plots of IL-23R expression within memory $CD4^+$ T cells. The cells were stained with goat anti-IL-23R polyclonal Ab and anti-goat-FITC Ab. A representative of seven individual data sets is shown. *, $p < 0.01$. *D*, The frequency of IL-23R-positive cells of each histogram plot. Data are shown as bar graphs with error bars showing the SD of seven individual data sets. *, $p < 0.01$.

Given the distinguished ability to produce IL-17 as well as higher IL-23R and lower T-bet expression, we propose that Th17 cells are confined to SP cells, whereas Th1 cells are either DP or $CCR2^-CCR5^+$. It has recently been reported that T-bet directly regulates the transcription of IL-23R in mice (28). It is possible that weak expression of IL-23R by non-Th17 cells (DP, DN, and $CCR2^-CCR5^+$) may result from baseline activation of T-bet.

Additional remarks

Using freshly isolated healthy human lymphocytes, we showed here that $CCR2^+CCR5^-$ memory T cells would produce a large amount of IL-17 but not IFN-γ, whereas $CCR2^-$ memory T cells produced IFN-γ, but not IL-17. Although we presented the data obtained after stimulation with PMA/ionomycin, polyclonal stimulation by anti-CD3/CD28 also gave similar results (data not shown). Moreover, when we stimulated $CCR2^+CD4^+$ memory T cells by IFN-γ, IL-4, IL-2, or IL-23 in addition to PMA/ionomycin, IL-17 production was not changed (data not shown).

The frequency of Th17 cells among this subset is an important issue to be investigated. By using the ELISPOT assay, we found that ~200 spots of IL-17-producing cells could be detected among 1×10^5 memory $CCR2^+CD4^+$ T cells (~0.2%), whereas the numbers of IFN-γ-producing cells were about 5-fold higher (~1.0%). Although this needs to be systematically verified, the lower frequency of IL-17-producing cells is consistent with the lower value of IL-17 than IFN-γ in supernatants detected by ELISA.

The unique chemokine receptor expression pattern of Th17 cells provides a basis for their recruitment to specialized inflammatory conditions in vivo, which should be relevant for understanding the pathogenesis of autoimmune diseases.

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Disclosures

The authors have no financial conflict of interest.

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Invariant NKT Cells Biased for IL-5 Production Act as Crucial Regulators of Inflammation[1]

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Although invariant NKT (iNKT) cells play a regulatory role in the pathogenesis of autoimmune diseases and allergy, an initial trigger for their regulatory responses remains elusive. In this study, we report that a proportion of human CD4$^+$ iNKT cell clones produce enormous amounts of IL-5 and IL-13 when cocultured with CD1d$^+$ APC in the presence of IL-2. Such IL-5 bias was never observed when we stimulated the same clones with α-galactosylceramide or anti-CD3 Ab. Suboptimal TCR stimulation by plate-bound anti-CD3 Ab was found to mimic the effect of CD1d$^+$ APC, indicating the role of TCR signaling for selective induction of IL-5. Interestingly, DNA microarray analysis identified *IL-5* and *IL-13* as the most highly up-regulated genes, whereas other cytokines produced by iNKT cells, such as IL-4 and IL-10, were not significantly induced. Moreover, iNKT cells from BALB/c mice showed similar IL-5 responses after stimulation with IL-2 ex vivo or in vivo. The iNKT cell subset producing IL-5 and IL-13 could play a major role in the development of allergic disease or asthma and also in the immune regulation of Th1 inflammation. *The Journal of Immunology*, **2007, 179: 3452–3462.**

Invariant NKT (iNKT)[3] cells are a nonconventional population of T cells, expressing a canonical invariant TCR α-chain (Vα14-Jα18 for mice and Vα24-Jα18 for human) and TCR β-chains using limited Vβ segments (Vβ8.2, 2, and 7 in mice and Vβ11 in humans) (1–4). They are selected and restricted by CD1d, a nonclassical MHC class I-like molecule, and proliferate vigorously in response to α-galactosylceramide (αGC), a prototypical iNKT cell ligand, originally isolated from marine sponge (5). Although most iNKT cells express NK cell markers such as CD161, they also contain a small population of cells that are negative for NK cell markers (6). Importantly, CD1d-restricted T cells also contain T cells that neither express the canonical TCR α-chain nor respond to αGC (7, 8). To avoid confusion, it has recently been recommended that iNKT cells should be defined by their reactivity to αGC loaded onto CD1d multimers, instead of expression of NK cell markers (6). iNKT cells comprise CD4$^+$ and CD4$^-$ cells, which show differential expression of regulatory cytokines. In humans, studies have shown that the former produce both Th1 and Th2 cytokines, whereas the latter predominantly produce proinflammatory cytokines such as IFN-γ and TNF-α (9, 10). Accordingly, the CD4$^+$ cells are thought to be the major source of Th2 cytokines for controlling Th1 cell-mediated inflammation or promoting Th2-dependent pathologies.

Although earlier studies have tended to focus on the ability of iNKT cells to down-modulate inflammatory responses, more recent works have shown that they could promote joint inflammation in models of arthritis (11–13) or mediate airway inflammation in bronchial asthma (14, 15). The divergent effects of iNKT cells in inflammatory pathologies are thought to reflect a broad spectrum of their functions in vivo. In fact, iNKT cells explosively produce a number of pro- and anti-inflammatory cytokines after nonphysiological stimulation with αGC (2, 5, 16) or anti-CD3 mAb (17), although stimulation with alternative ligands such as αGC analogues may lead to selective Th1 (18) or Th2 cytokine production (19, 20). Regarding the molecular mechanism for iNKT cell-mediated immune regulation, previous studies have suggested the role of iNKT cell-derived IL-4 or IL-10 in controlling Th1-mediated inflammation (16, 19, 20), whereas the role of IL-13 secreted by iNKT cells has recently been highlighted in the pathogenesis of asthma (14, 15) and ulcerative colitis (21). The published results, however, do not exclude the possible role of other cytokines secreted by iNKT cells. In fact, it is not clear whether iNKT cells could produce specific cytokines that are truly needed to exert regulatory functions or whether they produce cytokines in a redundant way. Another important question is what would trigger the regulatory iNKT cells to promote a cytokine response in vivo during the natural course of disease. Although TCR and/or costimulatory molecule signaling are likely to be the triggers involved, direct evidence for this postulate so far has not been provided.

Based on the observation of neonatal iNKT cells expressing memory-activated phenotype (CD45RO$^+$CD62L$^-$CD25$^+$) (22, 23) and resting adult iNKT cells containing preformed transcripts of IFN-γ and IL-4 (24), it has been suggested that iNKT cells are preactivated by endogenous ligands. If endogenous ligands for iNKT cells are to exist in vivo, we speculate that they transmit a relatively weak signal through TCR (25). Supportive of this idea,

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[3] Abbreviations used in this paper: iNKT, invariant NKT; αGC, α-galactosylceramide; iGb3, isoglobotrihexosylceramide; HS, healthy subject; MS, multiple sclerosis; DN, double negative; DC, dendritic cell; iDC, immature DC; CBA, cytometric bead array.

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Table I. *IL-5 versus IFN-γ secretion profile of CD4⁻ iNKT cell clones generated from HS and MS*[a]

	Clone	Age	Primary Stimulation	IL-5	IL-5-IFN-γ Ratio	Medication
HS						
1	Kai.1	32	αGC	7336.5	26.29	
2	Sk	32	αGC	1950.0	9.81	
3	Ot.1	34	αGC	80.0	0.001	
4	Ok.1	39	αGC	3.3	0.09	
5	Kai.2	32	OCH	2796.1	160.70	
6	Ar	35	OCH	191.7	0.78	
7	Ot.2	34	OCH	97.7	0.07	
8	Nn	28	OCH	87.3	0.04	
9	Ln	35	OCH	79.6	0.08	
10	Yk	31	OCH	70.1	0.98	
11	Ok.2	39	OCH	23.1	0.17	
MS						
12	Kn.1	22	αGC	2998.3	59.53	PSL[b]
13	Oz	31	αGC	2252.3	14.62	IFN-β
15	Kk	31	αGC	1095.6	20.67	PSL
14	Og	32	αGC	176.9	0.21	IFN-β
16	Sd	37	αGC	95.0	0.19	None
17	Ich	37	αGC	59.7	0.26	None
18	Nkj.1	61	αGC	48.4	0.18	None
19	Tj	31	αGC	44.0	0.05	None
20	Mtz.1	47	αGC	41.2	0.65	None
21	Yta	35	αGC	15.1	0.17	None
22	Kn.2	22	OCH	4636.0	2.26	PSL
23	Nkj.2	61	OCH	2353.4	3.32	None
24	Mtz.2	47	OCH	133.7	0.25	None
25	Ag	34	OCH	6.3	0.01	None
26	Ko	35	OCH	0.0	0.0000052	None

[a] The clone cells were cocultured with iDCs in the presence of exogenous IL-2. The amount of IL-5 and IFN-γ from day 2 supernatant was measured by CBA.
[b] PSL, Prednisolone.

Brigl et al. (26) have recently shown that human iNKT cell clones as well as freshly separated rodent iNKT cells could exert an enormous IFN-γ response, when they react to an endogenous ligand in the presence of costimulatory IL-12 (26). As such, a very weak autoreactive iNKT cell response to CD1d-positive cells could be remarkably augmented by various additional signals such as cytokines and costimulatory molecules. Several candidates for endogenous ligands have been previously reported (27–29). More recent studies have demonstrated that lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) is a possible endogenous ligand naturally presented to iNKT cells in the context of CD1d (30, 31). Notably, Mattner et al. (31) has shown that iNKT cell activation following bacterial infection could be elicited either by stimulation with bacterial glycolipids or by endogenous iGb3 bound to CD1d, depending on the strain of bacteria. This indicates that recognition of endogenous ligand may be critical in triggering at least certain iNKT cell responses in vivo. However, it is unclear whether recognition of endogenous ligand by iNKT cells may lead to production of Th2 cytokines required for iNKT cell-mediated Th2 immune deviation. Taking these into consideration, we have attempted to re-examine the functional properties of human $CD4^+$ iNKT cell clones by exploring the effects of cytokines on the autoreactive iNKT cell responses to $CD1d^+$ DCs.

We report here that although none of the iNKT cell clones responded to $CD1d^+$ DCs after coculture, addition of exogenous IL-2 could trigger the production of enormous amounts of IL-5 and IL-13 from some of the clones. Comprehensive analysis using DNA microarray has shown that *IL-5* and *IL-13* Th2 cytokine genes are almost exclusively and robustly induced from the clones in response to $CD1d^+$ DCs and IL-2. IL-2 alone did not induce IL-5 production, but IL-2 together with suboptimal TCR stimulation by anti-CD3 Ab could provoke a striking IL-5 response. Because a similar Th2 bias was reproducibly demonstrated by using iNKT cells freshly isolated from BALB/c mice, we propose that the combination of IL-2 and a weak TCR stimulus by endogenous ligand/CD1d could be a mechanism by which $CD4^+$ iNKT cells could start producing Th2 cytokines IL-5 and IL-13 in autoimmune diseases and allergy.

Materials and Methods

Subjects

Venous samples of nine healthy subjects (HS) and 13 multiple sclerosis patients (MS) were used for study (Table I). All the patients had conventional MS, fulfilled standard criteria for the diagnosis of relapsing-remitting MS, and were in remission at examination based on clinical and magnetic resonance imaging assessment. Four patients were on medication for >3 mo: two on low-dose corticosteroids and the other two on IFN-β. HS (33.9 ± 2.2 years old) and MS (37.53 ± 11.8 years old) were age matched. Written informed consent was obtained from all subjects and the Ethics Committee of the National Center of Neurology and Psychiatry approved this study.

Abs and reagents

PE-labeled anti-Vα24, FITC-anti-Vβ11, phycoerythrin-Texas Red X-anti-CD4, PC5-anti-CD8, PE-anti-CD206, and anti-mouse IgM were purchased from Immunotech and PE-anti-iNKT cells (specific for invariant Vα24-Jα18 TCR; 6B11) (32), PE-anti-human IL-4, FITC-anti-human IFN-γ, mouse CD1d dimer (dimer X), FITC-anti-mouse TCRβ, PE-anti-mouse NK1.1, and PE-rat anti-mouse IgG1 were purchased from BD Biosciences/BD Pharmingen. All human recombinant cytokines were obtained from PeproTech and microbeads coated with anti-CD14, anti-CD45RO, or anti-PE and the CD4 T cell isolation kit were obtained from Miltenyi Biotec. Flow cytometry was performed on an Epics XL and analyzed with EXPO 32 software (Coulter). Cell sorting was conducted on an Epics Altra (Coulter) or autoMACS cell sorter (Miltenyi Biotec). αGC and OCH (19) were solubilized in DMSO (100 μg/ml). Anti-CD1d mAb (aCD1d 59; IgM) was prepared in the laboratory of S. A. Porcelli.