Encephalomyelitis: Experimental Autoimmune Sulfatide-Mediated Immune Regulation in Dendritic Cells and Anergic Type I NKT Cells Play a Crucial Role in Experimental Autoimmune Encephalomyelitis

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Dendritic Cells and Anergic Type I NKT Cells Play a Crucial Role in Sulfatide-Mediated Immune Regulation in Experimental Autoimmune Encephalomyelitis

Igor Maricic,* Ramesh Halder,* Felix Bischof,† and Vipin Kumar*

CD1d-restricted NKT cells can be divided into two groups: type I NKT cells use a semi-invariant TCR, whereas type II express a relatively diverse set of TCRs. A major subset of type II NKT cells recognizes myelin-derived sulfatides and is selectively enriched in the CNS tissue during experimental autoimmune encephalomyelitis (EAE). We have shown that activation of sulfatide-reactive type II NKT cells by sulfatide prevents induction of EAE. In this article, we have addressed the mechanism of regulation, as well as whether a single immunodominant form of synthetic sulfatide can treat ongoing chronic and relapsing EAE in SJL/J mice. We have shown that the activation of sulfatide-reactive type II NKT cells leads to a significant reduction in the frequency and effector function of myelin proteolipid proteins 139–151/I-Aβ-tetramer+ cells in lymphoid and CNS tissues. In addition, type I NKT cells and dendritic cells (DCs) in the periphery, as well as CNS-resident microglia, are inactivated after sulfatide administration, and mice deficient in type I NKT cells are not protected from disease. Moreover, tolerized DCs from sulfatide-treated animals can adaptively transfer protection into naive mice. Treatment of SJL/J mice with a synthetic cis-tetracosenoyl sulfatide, but not α-galactosylceramide, reverses ongoing chronic and relapsing EAE. Our data highlight a novel immune-regulatory pathway involving NKT subset interactions leading to inactivation of type I NKT cells, DCs, and microglial cells in suppression of autoimmunity. Because CD1d molecules are nonpolymorphic, the sulfatide-mediated immune-regulatory pathway can be targeted for development of non-HLA-dependent therapeutic approaches to T cell–mediated autoimmune diseases. The Journal of Immunology, 2014, 193: 1035–1046.

Natural killer T cells that share the cell-surface receptors of NK cells (e.g., NK1.1) and in addition express an Ag receptor (TCR) generally recognize lipid Ags in the context of the CD1 molecules and bridge innate immune responses to adaptive immunity (1, 2). Their activation can influence the outcome of the immune response against tumors and infectious organisms, and in addition can modulate the course of several autoimmune diseases in experimental animal models and potentially in humans (3–7). Therefore, characterization of the biology and function of NKT cells is important for understanding their role in the entire spectrum of immune responses. CD1 molecules are nonpolymorphic, MHC class I-like, and associated with β2-microglobulin, and are expressed on APCs such as dendritic cells (DCs), macrophages, and subsets of B cells (1, 2). The CD1d pathway is highly conserved and is present in both mice and in humans.

Based on their TCR gene usage, CD1d-restricted NKT cells can be divided into two categories: one using a semi-invariant TCR (iNKT or type I) and the other expressing somewhat more diverse TCRs (type II NKT) (1, 4, 5, 8). The invariant receptor on type I NKT cells is encoded by the germline TCR α-chain (mouse Vα14Jα18, human Vα24-JαQ) and diverse TCR Vβ-chains (mouse predominantly Vβ8, human predominantly Vβ11). Type I NKT cells in mice and in humans recognize α-galactosylceramide (αGalCer), a marine sponge-derived glycolipid, and self-glycolipids such as isogalactobiohexosylceramide and β-glucoceramide. A major subset of type II NKT cells has been shown to recognize a self-glycolipid sulfatide (3′-sulfogalactosyl ceramide) in both mice and humans (9–13). Type I NKT cells can be identified using αGalCer/CD1d-tetramers, whereas a major subset of type II NKT cells can be identified using sulfatide/CD1d-tetramers. Because type I NKT cells use the invariant Vα14-Jα18 TCR, mice deficient in the Jα18 gene (Jα18−/−) lack these cells but possess normal levels of sulfatide-reactive type II NKT cells (10). Type I NKT cells upon activation with αGalCer rapidly secrete large quantities of cytokines, including IFN-γ and IL-4, which results in a cascade of events that includes activation of NK cells, DCs, and B cells. Thus, type I NKT-mediated cytokine secretion and modulation of NK cells and DCs profoundly alters immunity against both self-Ags and foreign Ags, including microbes and viruses.

Sulfatide or 3′-sulfogalactosyl ceramide is enriched in several membranes including myelin in the CNS, pancreatic islet cells, and kidney epithelium (3). Sulfatide is a sulfolipid in which the 3′-OH moiety on the galactose is sulfated and the carbohydrate moiety is attached to the ceramide in a β-linkage. The ceramide moiety has two long hydrocarbon chains, one of sphingosine and the other of a fatty acid. Several species of sulfatide are present that vary in the acyl chain length (C16-C24), unsaturation, and hydroxylation. It has been proposed that during chronic inflammation or tissue damage, self-glycolipids are presented by CD1d molecules. Indeed, in MS patients, increased serum levels of glycolipids (14, 15) and Abs directed against them have been reported (16, 17), and recently, T cells specific for glycolipids...
have been isolated from MS patients. Notably, their frequency in five active MS patients was three times higher compared with five normal individuals (12). Using cloned CD1d-restricted T cells in humans, it has been demonstrated that the ganglioside GM1 binds well to CD1b, whereas sulfatide binds promiscuously to the a, b, c, and d CD1 molecules (12, 18). The upregulation of CD1 protein in macrophages and astrocytes in areas of demyelination in chronic-active MS lesions, but not in silent lesions, is consistent with the notion that they can present relevant Ags to NKT cells (19), and thereby become involved in the disease process. Infiltrating macrophages or DCs can engulf sulfatide-enriched membrane fragments bound to anti-sulfatide Abs using FcRs or by complement receptor-mediated phagocytosis. When so activated, these tissue-resident microglia, infiltrating macrophages, or DCs can present not only proteins, but also glycolipids such as sulfatide locally in a CD1d-restricted manner to NKT cells. Therefore, sulfatide is a potential autoantigen, and sulfatide-reactive type II NKT cells might be modulated to influence the course of autoimmunity in the CNS. We have shown that although both subsets infiltrate into the CNS, only sulfatide-reactive type II, but not type I, NKT cells are enriched during experimental autoimmune encephalomyelitis (EAE) (10).

EAE is a CD4+ T cell–mediated autoimmune disease characterized by inflammation and demyelination in the CNS. EAE is a well-studied animal disease model for multiple sclerosis and is induced after immunization of susceptible animals with myelin proteins or their peptides in adjuvant. The immunizing peptides and the course of EAE from monophasic, relapsing and remitting to chronic varies according to the mouse strain used. In addition, adoptive transfer of myelin protein–reactive Th1/Th17-like CD4+ cells induces EAE, whereas Th2 myelin protein–reactive cells are generally protective (20–22). Th1 cells predominantly secrete IFN-γ, Th17 cells secrete IL-17, whereas Th2 cells secrete IL-4, IL-5, IL-10, and IL-13.

Previously we have shown that a single prophylactic i.p. injection of sulfatide in the absence of adjuvant can prevent the chronic EAE in C57BL/6J mice (10). Also, adjuvant-free administration of αGalCer or its analogs can prevent or enhance EAE depending on the mouse strain (23–26). A recent study has shown the presence of anti-sulfatide Abs in both SJL/J and BL/6 mice after the induction of EAE (27). SJL/J mice have a greater number of sulfatide-reactive NKT cells than BL/6 mice, making them a particularly favorable animal for investigation of EAE treatment (13). In this study, we have used peptide/MHC class II tetramers specific for myelin epitope myelin proteolipid proteins (PLP) 139–151, which is restricted by the I-Aα MHC molecule to directly investigate the fate of pathogenic T cells in SJL/J mice after sulfatide administration. We found that sulfatide administration in the absence of adjuvant ameliorates relapsing–remitting as well as chronic EAE by targeting the encephalitogenic Th1/Th17 CD4+ T cells. Furthermore, we found that a single synthetic long fatty acyl form of cis-tetrasacryenyl sulfatide is effective in the treatment of ongoing disease. Administration of sulfatide results in the inhibition of the effector function of the encephalitogenic PLP139–151–reactive CD4+ T cell population in the CNS and peripheral lymphoid organs. Interestingly, anergized type I NKT cells, as well as DCs, play an important role in the sulfatide-mediated regulation of EAE, and the microglial population is also inactivated in sulfatide-treated animals. Because CD1 molecules are nonpolymorphic, insight into the regulation mediated by type II NKT cells will be extremely valuable in the development of non-HLA–dependent therapeutic approaches to autoimmune demyelinating diseases.

### Materials and Methods

#### Animals

SJL/J and C57BL/6J female mice (6–8 wk) were purchased from The Jackson Laboratory. C57BL/6-Jo18+/− mice originally generated in the laboratory of Taniguchi (28) were kindly provided by Mitch Kronenberg (La Jolla Institute for Allergy and Immunology). All mice were bred and maintained in specific pathogen-free conditions in the Torrey Pines Institute for Molecular Studies animal facility. Treatment of animals was in compliance with federal and institutional guidelines, and was approved by the Torrey Pines Institute for Molecular Studies Animal Care and Use Committee.

#### Glycolipid Ags and CD1d-tetramers

Purified bovine myelin-derived sulfatide (>90% pure) and semisynthetic llysosulfatide and tetracosenoyl sulfatide was purchased from Matreya (Pleasant Gap, PA). The cis-tetracosenoyl sulfatide was chemically synthesized in collaboration with Prof. Chi-Huey Wong (The Scripps Research Institute, La Jolla, CA) as described earlier (13). Synthetic αGalCer was provided by Y. Kozuoka (Kirin Brewery, Tokyo, Japan). All lipids were dissolved in vehicle (0.5% polysorbate-20 [Tween 20] and 0.9% NaCl solution) and diluted in PBS. Murine CD1d was made in a baculovirus expression system as described earlier (10). PE-labeled mCD1d-tetramers loaded with αGalCer or PBS were generated as described earlier (10, 29).

#### MHC class II-tetramers

MHC class II tetramers were produced essentially as described previously (30–32). In brief, the cDNA for the I-Aα α-chain was elongated by overlapping PCR with sequences encoding for an acidic zipper sequence and a His6-tag and the I-Aα peptide/MHC class II tetramers for specific myelin epitope, PLP139–151, which is restricted by MHC molecule, I-Aα β-chain with the complementary basic zipper sequence and the BirA-dependent biotinylation substrate sequence (33). The cDNAs encoding for the peptides myelin basic protein (MBP)84–96 (VVFHHFKNIVTPRTP) and PLP139–151 (HCLGKWLGHPDKF) were attached to the 5′ end of the I-Aα β chain via a 6-aa linker. After cloning into baculovirus transfer vector pACD83 (BD Biosciences), recombinant baculoviruses were generated using the BaculoGold system (BD Biosciences) and grown suspension cultures of Sf9 cells in protein-free insect medium. Recombinant monomers were purified under native conditions from the supernatant and biotinylated, as described previously (33). Tetramers were formed by biotinylation with PE- or allophtycocyanin-labeled streptavidin (Invitrogen or BD Biosciences).

#### Isolation and tracking of lymphocytes from the CNS

Spinal cords were extracted from different groups (four to seven animals in each) of mice after anesthesia and whole-body perfusion with chilled PBS. Cell suspensions of infiltrating cells from the brain and spinal cords were separated by a discontinuous density gradient centrifugation, as described. After a low-speed (200 × g) centrifugation, the cell suspensions were suspended in 70% Percoll (Pharmacia) in HBSS. This was overlaid by equal volumes of 37 and 30% Percoll, and the gradient was centrifuged at 500 × g for 15 min. Mononuclear cells (MNCs) were harvested from the 37–70% interface, washed in HBSS. In C57BL/6J mice, we have routinely isolated 0.5–1 million cells from a single mouse at the peak of the disease. The number of infiltrating cells eventually decreased around 5- to 10-fold after recovery. The CNS infiltrating lymphocytes isolated as described earlier were subjected to staining with αGalCer/CD1d-lipid tetramers or standard PCR analysis using Vα14- and Jo18-specific primers: Vα14F, 5′-GTTCCTAGTCCTGCCGTGGTTGT-3′; Jo18R, 5′-CAAAAATGCAGCCTCCCTAAG-3′.

#### Flow cytometry

Leukocytes were suspended in FACS buffer (PBS containing 0.02% NaN3 and 2% FCS), blocked (and Mouse Fcγ-; BD Biosciences, San Diego, CA), and stained with loaded mCD1d-tetramer–PE or –FITC-, or PE-Cy5–labeled anti-mouse Abs (BD Biosciences, San Diego, CA), and stained with loaded mCD1d-tetramer–PE or –FITC-, or PE-Cy5–labeled anti-mouse Abs (BD Biosciences, San Diego, CA) as indicated. Intracellular cytokine staining of SF9 cells in protein-free insect medium. Recombinant monomers were purified under native conditions from the supernatant and biotinylated, as described previously (33). Tetramers were formed by biotinylation with PE- or allophtycocyanin-labeled streptavidin (Invitrogen or BD Biosciences).

#### Induction and clinical evaluation of disease

For active EAE induction, mice were immunized s.c. with 100–150 μg PLP139–151 or myelin oligodendrocyte glycoproteins (MOG) 35–55 emulsified in CFA; 0.15 μg pertussis toxin (List Biological, Campbell, CA) was injected i.p. in 200 μl saline on days 0 and 2. Mice were observed daily for the clinical appearance of EAE. Disease severity was scored on
a five-point scale (23): 1, flaccid tail; 2, hind-limb weakness; 3, hind-limb paralysis; 4, whole-body paralysis; 5, death.

Measurement of Ag-specific proliferative and cytokine responses

Draining lymph nodes of mice were removed 10 d after s.c. immunization with the myelin Ags, and single-cell suspensions were prepared. Lymph node cells (8 × 10^6 cells/well) were cultured in 96-well microtiter plates with peptides at concentrations ranging from 1.25 to 40 μg/ml. IFN-γ, IL-4, or IL-17 cytokine secretion in the assay supernatants were determined using standard sandwich ELISA technique, as described earlier (29). All capturing and detecting Ab pairs were purchased from BD Biosciences (San Diego, CA). Proliferation was assayed by the addition of 1 μCi [3H] thymidine (International Chemical and Nuclear, Irvine, CA) per well for the last 18 h of a 5-d culture. Incorporation of [3H]thymidine was measured by liquid scintillation counting.

Hepatic cell isolation and adoptive transfer

Hepatic lymphocytes were isolated using the Percoll gradient essentially as described earlier (29). Briefly following anesthesia with isoflurane (Baxter), mice were perfused with chilled PBS, and livers removed for the isolation of MNCs using 35% Percoll gradient. CD11c+ DCs were purified from liver MNCs by positive selection with CD11c microbeads on a Mini MACS column (Miltenyi Biotec) according to the manufacturer’s protocol (>90% pure). CD11c+ DCs (1 × 10^6), purified from livers of donors 1 d after sulfatide or vehicle/PBS administration, were injected i.v. into recipients that were then immunized 2 d later for the induction of EAE.

Isolation of microglia and macrophages from CNS

In brief, animals were sacrificed using CO2 inhalation and perfused with 40–50 ml chilled PBS. Single-cell suspensions were rapidly prepared from brain and spinal cord in 10% PBS in HBSS by mincing and filtering through a 70-μM cell strainer (BD Falcon). After washing, cell suspensions were enzymatically digested for 60 min at 37˚C with collagenase D (0.2 mg/ml; Roche) and DNase I (28 U/ml) in serum-free HBSS. After washing, cells were resuspended in serum-free HBSS, added to a 1.22-g/ml Percoll solution (Sigma), and overlaid with a 1.088-g/ml Percoll solution, then centrifuged at 2400 rpm for 20 min at room temperature. After removal of the fat and myelin layer at the top of the tube, cells were collected from the clear upper/interphase face. Cells were diluted in HBSS washed, centrifuged, and the pellet resuspended in FACS buffer for staining with indicated Abs and for cell sorting or flow cytometry analysis.

Statistical analysis

Data are expressed as mean ± SEM for each group. Statistical differences between groups were evaluated by unpaired, one-tailed Student’s t test using GraphPad Prism software (version 5.0a; GraphPad Software, La Jolla, CA).

Results

Adjuvant-free administration of bovine brain–derived sulfatide or a synthetic immunodominant form, cis-tetracosenoyl sulfatide, but not αGalCer reverses ongoing relapsing-remitting EAE in SJL/J mice

Although a single prophylactic injection of myelin-derived sulfatide and not other self-glycolipids at the time of MOG immunization or even later can significantly protect C57BL/6j mice from induction of EAE in a CD1d-dependent manner (10), it has not been shown whether ongoing disease can be reversed after activation of type II NKT cells by sulfatide. In this study, we determined whether sulfatide administration can ameliorate the ongoing chronic and relapsing form of PLP139–151–induced disease in SJL/J mice. Groups of female SJL/J mice were administered i.p. 20 μg sulfatide in vehicle/PBS or vehicle/PBS only, after antigenic challenge and after the onset of disease at around days 11 and 30. As shown in Fig. 1A, clinical signs of disease were significantly reduced and remained low in mice treated with sulfatide in comparison with those in the control or vehicle/PBS-treated group.

We found that sulfatide administered s.c. in adjuvant is unable to stimulate type II NKT cells and accordingly fails to cross-regulate type I NKT cells (R. Halder, unpublished observations). Similarly, when a higher dose of sulfatide is administered emulsified with the PLP peptide in adjuvant, the induced disease may be potentiated (27). In this study, we have determined whether the protective effect of an optimum dose (20 μg/animal) of sulfatide, as shown in Fig. 1A, is lost when it is administered emulsified with the adjuvant. As shown in Fig. 1B, sulfatide given with adjuvant does not reduce clinical symptoms of disease, but rather appears to exacerbate EAE.

Bovine brain–derived sulfatide is a mixture of several different forms of sulfatide that vary in hydroxylacyl, fatty acid chain length, and unsaturation. We have recently identified the most immunodominant form of sulfatide using proliferation, cytokine secretion, CD1d binding, and CD1d tetramer staining (13). In this study, we have determined whether this single immunodominant form of sulfatide alone can be used for the treatment of chronic-relapsing EAE in SJL/J mice. Groups of SJL/J mice with ongoing disease were injected i.p. with a synthetic cis-tetracosenoyl sulfatide in vehicle/PBS or vehicle/PBS only. As shown in Fig. 1C and Table I, the immunodominant cis-tetracosenoyl sulfatide administered after disease onset is more effective than the brain-derived mixture of sulfatides in ameliorating clinical signs of ongoing relapsing-remitting disease in SJL/J mice. Other forms of sulfatide with shorter or no fatty acid chain (lysosulfatide or C16:0), as well as tetracosenoyl sulfatide, did not have any significant effect on the course of disease (Table I). Interestingly, inflammatory lesions as visualized by H&E staining of cross sections of lumbar region of spinal cords also decreased significantly in sulfatide-treated animals (Fig. 1D, 1E). Also, sulfatide-treated mice have a reduced number of inflammatory MNC infiltrates in comparison with sections from the control PBS/vehicle group (Fig. 1E). These data suggest that the adjuvant-free administration with an immunodominant synthetic form of sulfatide is superior to bulk sulfatide for the treatment of ongoing chronic and relapsing forms of EAE in SJL/J mice.

Although prophylactic administration of αGalCer at the time of myelin protein immunization has been shown to prevent EAE, it has not been shown whether activation of type I NKT cells with αGalCer during ongoing chronic disease has a similar effect. In this study, we examined whether αGalCer injection can treat the ongoing chronic and relapsing form of EAE. Interestingly, treatment with αGalCer at the time of disease onset has no significant effect on the course of EAE in SJL/J mice (Fig. 1C, Table II). Similarly, inflammatory lesions and MNC infiltrates in spinal cords of αGalCer-treated mice did not differ significantly from the control PBS/vehicle group (Fig. 1D, 1E). It has been shown earlier (23) that αGalCer given prophylactically before disease onset can prevent EAE in BL/6 mice. However, it is notable that although C57BL/6 mice are protected by prophylactic injection of αGalCer (2 μg/animal) before onset, a similar dose of αGalCer at the time of disease onset repeatedly caused rapid death of most of the mice in <24 h (see Table II). Even a lower dose of αGalCer injection during ongoing EAE did not have a significant influence on the course of EAE. These data suggest that targeting activation of type II NKT cells with cis-tetracosenoyl sulfatide is a reliable method for the treatment of ongoing chronic and relapsing EAE.

Reduced numbers of pathogenic PLP-reactive CD4+ T cells in draining lymph nodes, as well as in CNS-infiltrating cells, in mice treated with adjuvant-free sulfatide

During the course of PLP139–151–induced relapse-remitting EAE in SJL/J mice, there is an increased level of PLP-reactive CD4+ T cells in the periphery, as well as in the target organ, the CNS. First, we determined whether the decrease in clinical symptoms of
disease in sulfatide-treated mice results in a decrease in the number of encephalitogenic CD4+ T cells in both the draining lymph nodes and the CNS. We have used PLP139–151/IAs–tetramers for the identification of encephalitogenic T cells. In parallel, MBP84–96/IAs–tetramers were used as a negative control. As shown in Fig. 2A, there is a 10-fold reduction in the percentage of disease-causing T cells in sulfatide-treated mice.

Table I. Treatment of ongoing chronic and relapsing EAE in SJL/J mice with the immunodominant cis-tetracosenoyl sulfatide

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Incidence</th>
<th>Mean Maximum Score (No. of Mice)</th>
<th>Mean Day of Onset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10/13</td>
<td>2.38 (2 [0], 2 [1], 2 [2], 4 [3], 2 [4], 1 [5])</td>
<td>12.9</td>
</tr>
<tr>
<td>cis-Tetracosenoyl</td>
<td>8/17</td>
<td>1.06 (9 [0], 4 [1], 2 [3], 2 [4])</td>
<td>14.4</td>
</tr>
<tr>
<td>Tetracosenoyl</td>
<td>4/4</td>
<td>2.75 (1 [2], 3 [3])</td>
<td>12.3</td>
</tr>
<tr>
<td>Lyso</td>
<td>8/10</td>
<td>2.3 (1 [1], 1 [2], 4 [3], 2 [4])</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Groups of female SJL/J mice were injected with 20 μg cis-tetracosenoyl sulfatide (C24:1), tetracosenoyl sulfatide (C24:0), or lysosulfatide (C16:0) after the onset of EAE (days 11, 29) and disease monitored.
PLP139–151/I-As–tetramer+ CD4+ T cells in draining lymph nodes in sulfatide-treated mice in comparison with that in vehicle/PBS-treated animals. There was no significant difference in the number of MBP84–96/I-As–tetramer+ cells.

Because sulfatide is enriched in the CNS and can be presented in the context of CD1d to infiltrating type II NKT cells, we wanted to examine whether encephalitogenic conventional CD4+ T cells are also decreased in the CNS. Groups of SJL/J mice were immunized s.c. with PLP139–159/CFA/PT for the induction of disease and injected i.p. with sulfatide in vehicle/PBS or vehicle/PBS only. At the peak of disease during days 15–19, CNS-infiltrating MNCs were isolated and subjected to flow cytometry after staining with tetramers and the indicated Abs. In sulfatide-treated mice, encephalitogenic PLP139–151/I-As–tetramer+ CD4+ T cells were about a third (0.11–0.30%) in comparison with those in the vehicle/PBS-treated mice (Fig. 2A). These data demonstrate that

Table II. Survival rate of mice treated during ongoing EAE with different glycolipids

<table>
<thead>
<tr>
<th>Treatment of Ongoing Disease (No. of Animal Deaths/Total No. of Animals)</th>
<th>Mice</th>
<th>αGalCer</th>
<th>Sulfatide</th>
<th>cis-Tetracosenoyl</th>
<th>Mono-GM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>8/9</td>
<td>0/16</td>
<td>0/4</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>SJL/J</td>
<td>0/9*</td>
<td>0/22</td>
<td>0/42</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

*No death, but no significant amelioration of disease.

FIGURE 2. Decrease in numbers of pathogenic PLP-reactive CD4+ T cells in the draining lymph nodes and in the CNS after treatment with sulfatide. (A) A representative flow-cytometric profile of lymph node cells and CNS-infiltrating cells isolated ex vivo from SJL/J mice and stained with PLP139–151/I-As–tetramers or an irrelevant MBP84–96/I-As–tetramers and anti-CD4 mAb. The flow-cytometric profile shown is derived from the gate that includes all of the MNCs and excludes the dead cells and RBCs. Groups of animals were treated with sulfatide in PBS/vehicle or sulfatide emulsified in CFA or with PBS/vehicle only, as in Fig. 1. Single-cells suspensions of draining lymph node cells or the CNS-infiltrating lymphocytes were stained with tetramers or other indicated Abs and analyzed by flow cytometry. Lymph node cells and CNS infiltrates were isolated 6 and 19 d, respectively, after immunization with PLP139–151/CFA. (B) A summary of data from sulfatide-treated and control mice is shown. **p < 0.01. These data are representative of three independent experiments. (C) Flow-cytometric profile of in vitro cultured lymph node cells stained with PLP139–151/I-As–tetramers and anti-CD4 mAb. Groups of PLP139–151/CFA–immunized SJL/J mice were treated with PBS/vehicle (PBS), sulfatide/PBS (sulf/PBS), or sulfatide in adjuvant (sulf/adj). Lymph node cells were isolated 10 d after immunization and cultured with PLP139-151 peptide in vitro for 4–6 d and stained with indicated Abs. Representative flow-cytometry profiles are shown from each group.
sulfatide treatment reduces the number of encephalitogenic CD4+ T cells in the CNS (Fig. 2A). Accordingly, as summarized in Fig. 2B, the absolute number of the pathogenic PLP139-151–reactive CD4+ T cells is significantly reduced both in the draining lymph nodes and in the CNS tissues from sulfatide-treated animals.

In parallel, a comparison of the number of PLP139–151/IA2–tetramer+ cells both in the lymph nodes and in the CNS between adjuvant-free versus adjuvant-emulsified sulfatide-treated mice revealed that the encephalitogenic T cells are diminished in animals treated with adjuvant-free sulfatide, but not in mice injected with sulfatide emulsified in adjuvant (see Fig. 2B). To further confirm tetramer staining and a decrease in PLP139–151–reactive encephalitogenic T cells in sulfatide-treated mice, we cultured draining lymph node cells in vitro with the immunizing peptide and stained them with PLP139–151/IA–tetramers. Fig. 2C clearly shows a substantial decrease in the percentage of tetramer+ cells only in adjuvant-free sulfatide, but not in adjuvant emulsified sulfatide-treated mice, in comparison with mice in the control (PBS) group.

Treatment of SJL mice with adjuvant-free sulfatide also leads to a significant decrease in PLP139–151–reactive Th1/Th17 cells

We next determined the proliferation and secretion of cytokines IFN-γ (Th1) or IL-17 (Th17) by PLP-reactive CD4+ T cells after treatment of mice with sulfatide. Because the frequency of tetramer+ cells was very low, levels of both cytokines were determined directly ex vivo in PLP139–151–reactive T cells isolated from draining lymph nodes of mice treated with adjuvant-free or adjuvant-emulsified sulfatide, or PBS. As shown in Fig. 3, although the proliferative response to PLP139–151 was similar in different groups, there was a significant decrease in both IFN-γ and IL-17 secretion by PLP-reactive T cells isolated from adjuvant-free, sulfatide-treated animals in comparison with the untreated group. Notably, although there was some reduction in IFN-γ secretion in adjuvant-emulsified, sulfatide-treated mice, IL-17 secretion by PLP-reactive T cells was significantly enhanced. These data indicate a significant inhibition of both the Th1 and the Th17 responses only after adjuvant-free administration of sulfatide in SJL/J mice. These data also explain why sulfatide administered with adjuvant is able to potentiate disease because of an increase in IL-17–securing encephalitogenic CD4+ T cells.

Induction of anergy in type I NKT cells after sulfatide administration in SJL/J mice

Recently, we have identified a novel immunoregulatory pathway in which activation of sulfatide-reactive type II NKT cells leads to anergy induction in type I NKT cells mediated by DCs and a significant protection from liver injury after Con A injection or after ischemic reperfusion-induced in C57BL/6J mice (29, 34). In this study, we determined whether type I NKT cells are also anergized after sulfatide administration in SJL/J mice. Groups of SJL/J mice were administered with adjuvant-free sulfatide, and anergy induction in type I NKT cells was examined by proliferation of αGalCer/CD1d–tetramer+ cells by CFSE dilution (Fig. 4A), as well as by [3H] thymidine incorporation (Fig. 4B) in response to an vitro challenge with αGalCer. As shown in Fig. 4A and 4B, whereas type I NKT cells (αGalCer/CD1d–tetramer+) proliferate in response to the cognate ligand αGalCer in the PBS-treated group, their proliferative response is significantly inhibited in sulfatide-treated animals. This inhibition of proliferation of type I NKT cells can be reversed by the addition of IL-2 (data not shown). These data suggest that type I NKT cells are anergized after activation of sulfatide-reactive type II NKT cells in SJL/J mice.

Sulfatide-mediated inhibition of EAE is lost in mice that lack type I NKT cells

Anergic type I NKT cells have been recently shown to limit inflammation-induced damage in the liver disease mediated by NKT cells (29, 34). In this study, we examined the role of anergic type I NKT cells in the regulation of autoimmune disease mediated by myelin protein–reactive, MHC class II–restricted CD4+ T cells. Because SJL/J mice deficient in type I NKT cells are not available, we have used Jα18−/− in the C57BL/6 background to examine whether anergic type I NKT cells are required for sulfatide-mediated protection from EAE. Groups of WT (C57BL/6J) and type I NKT-deficient (Jα18−/−) mice were immunized for induction of EAE and were treated either with sulfatide/PBS or PBS only, and clinical signs of disease were monitored daily. As shown in Fig. 4C, sulfatide treatment did not significantly protect Jα18−/− mice from EAE, although the disease was delayed in comparison with the control group. As expected, EAE was significantly suppressed in the WT mice. These data suggest that anergic type I NKT cells behave like regulatory cells and play an important role in regulation of autoimmunity.

Adoptive transfer of CD11c+ DCs from sulfatide-treated animals prevents disease in naive recipients

We have earlier shown that activation of sulfatide-reactive type II NKT cells leads not only to inactivation of type I NKT cells, but also to tolerization of myeloid DCs (mDCs) (29, 34). We inves-
tigated whether tolerized DCs also participate in inhibiting expansion of the encephalitogenic myelin protein-reactive CD4+ T cells. Liver CD11c+ DCs were isolated from groups of mice that were administered sulfatide (20 mg/mouse) or PBS/vehicle. One day later, positively selected CD11c+ cells (1 × 10^6) were isolated and adoptively transferred into naive recipients that were subsequently immunized for the induction of EAE. Notably, mice in the group that received CD11c+ DCs from sulfatide-treated animals were significantly protected from induction of EAE in comparison with animals that received DCs from PBS/vehicle-injected mice (Fig. 5). These data suggest that tolerized DCs after sulfatide administration play an important role in this immune-regulatory pathway.

Inhibition of costimulatory molecules and CD1d expression on the CNS-resident microglia/macrophages after sulfatide administration

Because sulfatide-reactive type II NKT cells are enriched in the CNS during EAE and in situ activation of microglia is required for CNS demyelination, we examined whether activation of sulfatide-reactive type II NKT cells also influences activation of microglia. Several studies have shown upregulation of costimulatory molecules on microglia following their activation during EAE. In this study, we examined whether expression of CD1d, MHC, and other costimulatory molecules on microglia is modulated during disease and whether treatment with sulfatide influences their expression. Using a Percoll gradient, we isolated microglia-enriched MNCs from diseased SJL/J mice at days 0, 7 (preonset), 20 (peak disease), and 35 (remission), and F4/80+ cells were analyzed for their expression.

FIGURE 4. Induction of anergy in type I NKT cells after adjuvant-free sulfatide administration in SJL/J mice. (A) CFSE dilution profiles of αGalCer/CD1d-tetramer+ cells in splenocytes from sulfatide- or PBS/vehicle-treated mice are shown. Splenocytes isolated 6 h after sulfatide/PBS/vehicle injection were labeled with CFSE and stimulated in vitro with αGalCer, as described earlier (29). (B) In parallel, incorporation of [3H]thymidine in triplicate cultures of splenocytes from sulfatide or PBS/vehicle-injected mice were measured in response to the in vitro challenge with αGalCer. Data are representative of two individual experiments. (C) Loss of sulfatide-mediated protection from EAE in mice deficient in type I NKT cells. Groups of C57BL/6 (B6) or type I NKT-deficient Io18−/− (Io18−/−) mice (four to five mice in each group) were injected with sulfatide in PBS/vehicle or PBS/vehicle alone at days 1 and 20 in relation to MOG35–55/CFA/PT for the induction of EAE. Disease symptoms were monitored daily until days 32–35. Data are representative of two independent experiments. *p < 0.05.

FIGURE 5. Adoptive transfer of hepatic DCs from sulfatide-treated but not from control mice prevents EAE in recipient animals. Groups of BL/6 mice were injected with sulfatide (20 μg i.p.) or PBS/vehicle. One day later, purified liver CD11c+ DCs (1 × 10^6 cells/mouse) from both groups of donor mice were injected i.v. into recipients. Two days later, recipients were injected with MOG35–55/CFA/PT for the induction of EAE. Disease severity was monitored every day and until day 35. Data are representative of two independent experiments. *p < 0.05.
pression of CD1d using dual-color flow cytometry. Although both microglia and macrophage populations express a low level of CD1d at day 0, by days 7–20 expression increases significantly and then decreases during remission (Fig. 6A). Next, we used four-color flow cytometry to carefully analyze CD1d expression on the infiltrating macrophages and resident microglial populations individually in naïve mice, untreated mice with EAE, and sulfatide-treated mice on day 20. Data presented in Fig. 6B–D show a significant increase in CD1d–, MHC class II–, CD80–, CD86–expressing CD45+ intermediate/F4/80+ microglial cells, as well as CD45+high/F4/80+ macrophage/DC populations during EAE. Notably, in mice treated with sulfatide, a significantly reduced level of costimulatory molecules, MHC, and CD1d expression was found on both macrophages and microglial populations (Fig. 6). These levels were similar to those in mice before disease onset.

Loss of the CNS-infiltrating type I NKT cells during EAE in mice treated with sulfatide

Next, we examined the number of infiltrating type I NKT cells into the CNS during EAE (Fig. 7). Because sulfatide administration leads to anergy induction in type I NKT cells in the periphery, we wanted to determine whether their numbers are reduced in the CNS at the peak of the demyelination. As shown in Fig. 7A, αGalCer/CD1d-tetramer+ cells are significantly reduced in CNS-infiltrating cells in mice treated with sulfatide in comparison with those in the control group (0.08 versus 2.1%). Using PCR, we further investigated whether the reduced staining of type I NKT cells with αGalCer/CD1d-tetramers was due to their reduced number in the CNS or due to downregulation of their TCR. As shown in Fig. 7B, a comparison of the PCR product amplified using TCR-specific primers for type I NKT cells in the CNS infiltrate from the sulfatide-treated mice showed a substantial reduction (~3.5-fold) in relation to PBS/vehicle-injected animals. Collectively, these data indicate a significantly reduced number of type I NKT cells infiltrating into the CNS during EAE in sulfatide-treated animals.

Discussion

The data presented in this article demonstrate that the administration of bulk purified sulfatide or a synthetic immunodominant species, cis-tetracosenoyl sulfatide, in the absence of adjuvant, but not in an adjuvant-emulsified form, leads to reversal of ongoing chronic-relapsing EAE in SJL/J mice. Furthermore, our data begin to identify the cellular mechanism(s) by which activation of sulfatide-reactive type II NKT cells control autoimmunity. As shown using PLP139–151/I-As–tetramers, pathogenic class II MHC–restricted Th1 and Th17 responses are significantly inhibited after treatment with sulfatide. Consistent with our earlier studies of NKT-mediated liver diseases in BL/6 mice (29, 34), anergic type I NKT cells and tolerized DCs are involved in this sulfatide-mediated regulation of EAE in SJL/J mice. Furthermore, sulfatide administration also leads to a significant inhibition in the expression of several activation markers on the CNS-resident microglial population, including CD1d molecules that are involved in autoimmune demyelination. Collectively, our data suggest that the regulation of EAE after activation of sulfatide-reactive type II NKT cells modulates key cellular players in peripheral and lymphoid compartments, including inhibition of the effector MHC class II–restricted myelin-protein reactive T cells, type I NKT cells, DCs, and microglial activation in the CNS.

Sulfatide-reactive, CD1d-restricted T cells represent a major subset of type II NKT cells in both H-2b and H-2d haplotypes as examined by staining with sulfatide/CD1d-tetramers in C57BL/6J and SJL/J mice, respectively (13). Also, cis-tetracosenoyl sulfatide (C24:1) is an immunodominant form in both mouse haplotypes (13). Interestingly, cis-tetracosenoyl sulfatide/CD1d-tetramer+ cells are around 2-fold higher (1.3 versus 2.5%) in SJL/J mice in comparison with those in C57BL/6J mice (13). In contrast, αGalCer/CD1d-tetramer+ cells in spleen of SJL/J mice are about half (0.74 versus 1.6%) of those in BL/6 mice (V. Kumar, unpublished observations) consistent with the earlier report in the thymus (35).

This may explain the effectiveness of C24:1 sulfatide in stimulating type II NKT cells and control of EAE in SJL/J mice despite the presence of a lower number of type I NKT cells. A lower number of type I NKT cells in SJL/J mice may also explain the reduced cytotoxicity and incidence of death in SJL/J mice in comparison with BL/6 animals after αGalCer administration during ongoing EAE (Fig. 1, Table II).

It is clear from our data that activation of type II NKT cells using brain-derived sulfatide or a synthetic immunodominant species reverses ongoing chronic and relapsing course of disease in SJL/J mice. However, this occurs only if the sulfatide is administered in PBS/vehicle and not when it is emulsified in CFA/IFA. These data are consistent with an earlier report showing that sulfatide emulsified in adjuvant did not protect mice from EAE but appeared to exacerbate the clinical symptoms caused by an anti-sulfatide Ab response (27). It is not yet known whether the anti-sulfatide Ab response or the potentiation of EAE uses a CD1d-dependent pathway. It is important to note that the activation of sulfatide-reactive type II NKT cells, as well as the subsequent anergy induced in the type I NKT population, occurs only when sulfatide is administered i.p. and not i.v. or s.c. in the absence of adjuvant. This is distinct from the case of αGalCer, which can be administered i.v., i.p., or intranasally for the activation of type I NKT cells. Studies are ongoing to understand the necessity of an i.p. injection of sulfatide for the activation of these type II NKT cells.

We have noted that although treatment of chronic EAE in BL/6 mice with sulfatide completely ameliorates disease in some mice, it has only a partial effect in others. This effect may result from a difference in number or activation state of type I NKT cells in different animals. In several experiments, we have found that sulfatide administration provides the best protection in those C57BL/6 animals that have a lower (~20% in the liver) and not a higher (>35%) number of type I NKT cells, as delineated with αGalCer/CD1d-tetramers in naïve conditions. We are investigating whether the higher number of type I NKT cells in some animals represents a differential activation/expansion that might be present in seemingly identically housed animals of the same strain.

Consistent with our data in this study in the EAE model, long fatty acyl analogs of sulfatide, including tetracosenoyl sulfatide, activate a subset of type II NKT cells in NOD mice and significantly inhibit the development of type 1 diabetes in a CD1d-dependent fashion (36). Despite the prevalence of C16:0 sulfatide in the pancreatic tissue, administration of the C16:0 isofrom does not suppress diabetes. In contrast, C24:0 and C24:1 sulfatides are predominant in myelin. These data suggest that the long fatty acyl form of sulfatide or similar analogs may be better suited for intervention in autoimmune diseases. Recently, we have determined the crystal structure of the lysosulfatide/CD1d/TCR trimolecular complex and found that different sulfatide isoforms may bind in a similar fashion to CD1d, as well as to the TCR (9). In addition, we have shown that there is an overlap in the TCR repertoire of type II NKT cells reactive to different isoforms of sulfatide (37). Therefore, it is likely that the differential ability of different sulfatide isoforms to activate responsive type II NKT cells may relate to the stability of the various Ag-receptor complexes in vivo,
strength of Ag presentation, or their ability to efficiently activate CD1d-restricted T cells in the naive repertoire.

An important aspect of our results combined with others is that the manner and timing of the activation of both type I and type II NKT cells affects the results observed. Although activation of sulfatide-reactive type II NKT cells as described ameliorates ongoing EAE, a GalCer-mediated activation of type I NKT cells fails to reverse clinical symptoms of ongoing chronic or chronic and relapsing disease (Fig. 1, Table II). In fact, injection of a GalCer during ongoing EAE appears to exacerbate clinical symptoms of disease in both H-2\(^d\) and H-2\(^b\) mouse haplotypes. Particularly in BL/6 mice, administration of αGalCer during ongoing disease leads to rapid death within 24 h, perhaps because of a cytokine storm (Table II). This is in contrast with earlier studies demonstrating prevention of disease when a GalCer is administered prophylactically before or simultaneously with immunization with myelin protein Ags (23, 25, 38). Several studies have shown that activation of type I NKT cells with a GalCer under different conditions results in production of various cytokines that can either ameliorate or exacerbate disease. For example, a GalCer can induce IL-4, IL-13, and IL-6, which are known to be proinflammatory cytokines, whereas activation of type II NKT cells with a sulfatide can induce IFN-γ and IL-4, which are known to be anti-inflammatory cytokines. This suggests that the balance of cytokines produced by NKT cells in response to different Ags can have a significant impact on disease outcome.

![Figure 6](http://www.jimmunol.org/) Modulation of CD1d expression on macrophages and microglial populations during EAE and after treatment with sulfatide. (A) Upregulation of CD1d expression in CNS during ongoing chronic-relapsing EAE in SJL/J mice. Total CNS cells were isolated from brain and spinal cord in the indicated time points from SJL/J mice (two to three mice in each time point) immunized with PLP139–151/CFA for the induction of EAE. Two-color staining was performed using mAbs, for example, CD1d-PE and F4/80-FITC, and analyzed by flow cytometry. The percent positive cells are indicated in each quadrant. One of two representative experiments is shown. (B) Inhibition of CD1d expression on CNS-resident microglia and macrophage populations after sulfatide administration. (Top panels) MNCs specifically enriched in microglia (CD45\(^{int}\)/F4/80\(^{+}\), gate R3) and macrophages (CD45\(^{hi}\)/F4/80\(^{+}\), gate R2) were isolated on day 19 from the CNS of diseased SJL/J mice (n = 3) using Percoll gradients. Cells were pooled from naïve unimmunized, as well as PBS or sulfatide-treated PLP139–151/CFA-immunized mice, and stained with the indicated CD45-PE, F4/80-FITC Ab, and CD1d-allophycocyanin, and analyzed by flow cytometry. Numbers indicate mean fluorescence intensity (MFI). All of the flow cytometric profiles shown are derived from the gate that includes all of the MNCs and excludes the dead cells and RBCs. Sulfatide (20 μg) was injected on days 14 and 17. EAE scores were lower in sulfatide-treated mice (2, 1) in comparison with the control PBS/vehicle-injected mice (4, 3). One of two independent experiments is shown. (C) A summary from the data related to the CD1d expression (MFI) in macrophages and microglia from groups of mice in Fig. 1B treated with sulfatide/PBS (sulfatide) or with PBS as control are shown. ***p < 0.001. (D) Reduced expression of CD1d, MHC class II, CD80, and CD86 molecules on microglial populations after treatment with sulfatide. MNCs were isolated from the spinal cords of mice treated with sulfatide or PBS (control) day 19 after induction of EAE. The cells were gated on F4/80 and CD45 intermediate expression and percentage of F4/80\(^{+}\)/CD45\(^{int}\) cells expressing CD1d, MHC class II, CD80, and CD86 are shown in bar graph. Results are representative of two experiments with four to five mice per group. \(p < 0.05\).
controls. It is also interesting to note that during disease, it is likely that type II NKT cells are activated enriched. Because CD1d expression is also enhanced in the CNS of type II NKT cells, but not the αGalCer-reactive type I NKT cells (43, 44). Anergy induction in type I NKT cells after repeated injections with αGalCer also leads to induction of non-inflammatory DCs that inhibit autoimmune diseases (45, 46). Indeed, the transformation of type I NKT cells from promoters to suppressors of the inflammatory response requiring DCs or monocytes has recently been shown in autoimmune diabetes and EAE (45, 47, 48). Consistent with the role of tolerized DCs, our data clearly show that adoptive transfer of DCs alone from sulfatide-treated animals is able to protect recipients from EAE. We are investigating potential pathways including ERK1/2 signaling (49) or IL-10 secretion (50) or ICOS/PD-1–dependent signaling (51, 52) by which DCs are tolerized after NKT cell subset interactions leading to control of inflammatory response. Active tissue injury in CNS demyelination in MS and EAE is associated with the inflammatory infiltrate containing CD4+ and CD8+ T cells, as well as activated microglia/macrophages (53). During EAE, inflammation starts with the infiltration of Th1/TH17 cells and is followed by microglial activation and recruitment of macrophages and CD8+ T cells into the CNS. Activated microglia are a potent source of inflammatory cytokines and chemokines, and actively participate in propagation of autoimmune in the CNS (54, 55). These cells play a major role in the recruitment of lymphocytes, as well as in presenting myelin-derived Ags locally. Because NKT cells also infiltrate the CNS (10) (Fig. 7), it is not surprising that their interaction locally influences the activation of microglial cells. Our data indicate that sulfatide treatment tolerizes not only DCs in the periphery, but also the microglial population in the CNS (Fig. 6). In vitro treatment with higher concentrations of sulfatide can also result in the activation of microglial cells and astrocytes in a CD1d-independent manner (56). It is important to emphasize that in most of the vivo studies performed by us and others, administration of 20 µg sulfatide results in activation of type II NKT cells and modulation of inflammatory condition in a CD1d-dependent manner (6, 10, 34, 40, 41, 57). Because of the paucity of sulfatide-reactive T cells, attempts are being made to generate cis-tetracosenoyl sulfatide-reactive TCR transgenic lines for further careful analysis of type II NKT cells. Our preliminary data also suggest that CD1d expression on
microglial cells from mice with EAE is functional, because they are able to stimulate NKT cells in Ag-presentation assays in vitro (data not shown). It will be interesting to investigate whether pathways by which these two Ag-presenting populations are inactivated are similar or involve different signaling molecules. We have begun to undertake a critical study using a microRNA screening strategy to investigate the mechanism of tolerance induction in mDCs and in the microglial population.

Collectively, data presented in this article further shed light on a novel immunoregulatory mechanism by which activation of sulfatide-reactive type II NKT cells suppress inflammatory class II MHC–restricted pathogenic CD4+ T cell responses in a T cell–mediated autoimmune disease. In this regulatory pathway, mDCs play an important role leading to inactivation of type I NKT cells, as well as inhibition of the activation and/or effector cytokine secretion by conventional MHC class II–restricted encephalitogenic T cells. Furthermore, inactivation of microglia in the CNS contributes to an efficient limitation of the inflammatory response and protection from EAE. Recently, a delicate balance between activation of sulfatide-reactive type II NKT cells, type I NKT cells, and Foxp3+ CD4+ regulatory T cells has also been shown to be critical for the suppression of tumor immunity (58). Therefore, understanding the molecular events leading to activation or inactivation of type I versus type II NKT cell subsets by self-lipids is important for autoimmunity and antitumor immunity.

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