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Lipid microarrays identify key mediators of autoimmune brain inflammation

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Recent studies suggest that increased T-cell and autoantibody reactivity to lipids may be present in the autoimmune demyelinating disease multiple sclerosis. To perform large-scale multiplex analysis of antibody responses to lipids in multiple sclerosis, we developed microarrays composed of lipids present in the myelin sheath, including ganglioside, sulfatide, cerebroside, sphingomyelin and total brain lipid fractions. Lipid-array analysis showed lipid-specific antibodies against sulfatide, sphingomyelin and oxidized lipids in cerebrospinal fluid (CSF) derived from individuals with multiple sclerosis. Sulfatide-specific antibodies were also detected in SJL/J mice with acute experimental autoimmune encephalomyelitis (EAE). Immunization of mice with sulfatide plus myelin peptide resulted in a more severe disease course of EAE, and administration of sulfatide-specific antibody exacerbated EAE. Thus, autoimmune responses to sulfatide and other lipids are present in individuals with multiple sclerosis and in EAE, and may contribute to the pathogenesis of autoimmune demyelination.

Multiple sclerosis is presumed to be an autoimmune disease targeting the myelin sheath in the central nervous system (CNS). Although researchers have shown both T-cell and autoantibody reactivity to myelin proteins including myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG)[1,2], the breadth and specificity of autoimmune responses in multiple sclerosis remain incompletely characterized. Lipids comprise over 70% of the myelin sheath[3], and a growing number of reports have shown T-cell and antibody reactivity to lipids in multiple sclerosis[4–9]. Autoimmune responses to lipids have been studied much less extensively than responses to proteins largely as a result of lack of enabling technologies. Existing methods to study immune responses against lipids are hindered by the large number of potential lipid antigens, the hydrophobicity of lipids and the technical difficulty of detecting B- and T-cell responses directed against lipids.

Lipids are important targets of immune responses in a variety of microbial and autoimmune diseases. Autoimmune responses directed against phospholipids and gangliosides contribute to the pathogenesis in systemic lupus erythematosus and Guillain-Barré syndrome, respectively[10]. Despite reports of myelin-specific lipid responses in multiple sclerosis, the role of lipid-specific autoimmunity in multiple sclerosis remains controversial[11]. Most lipids are presented to T cells bound to CD1 molecules[12] and CD1 expression is increased in CNS lesions in both multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)[13–15]. These observations led us to hypothesize that myelin lipids may be target autoantigens in individuals with multiple sclerosis and to develop lipid-array technology to investigate this hypothesis.

We developed simple, large-scale lipid microarrays for detection of autoantibodies present in biological fluids such as serum and cerebrospinal fluid (CSF). Using lipid microarrays we show that antibodies to sulfatide and other lipids are present in CSF samples from individuals with multiple sclerosis and in sera from mice with EAE. Furthermore, we show that immunization of mice with sulfatide or transfer of sulfatide-specific antibody results in more severe clinical manifestations of EAE.

RESULTS

Lipid arrays

We created ordered arrays with 100 features containing duplicate spots of 50 distinct brain, myelin and microbial lipids and glycolipids that represent potential targets of the autoimmune response in multiple sclerosis. The lipids printed included ganglioside, sulfatide, cerebroside, sphingomyelin, total brain lipid fractions and microbial lipids (**Supplementary Table 1** online). Arrays were produced using a Camag Automatic TLC Sampler 4 to draw the lipids from sealed vials and spray them under nitrogen gas in spatially addressable locations on PVDF membranes affixed to microscope slides. We incubated lipid arrays with diluted serum or CSF samples from individuals with multiple sclerosis or mice with EAE, and used chemiluminescence to detect autoantibody binding to specific lipids and glycolipids on the arrays. Images of representative arrays are presented (**Fig. 1a**,**b**). As compared to antibody reactivities in CSF derived from other neurological disease (OND) control, antibodies in CSF derived from an individual with multiple sclerosis reacted with

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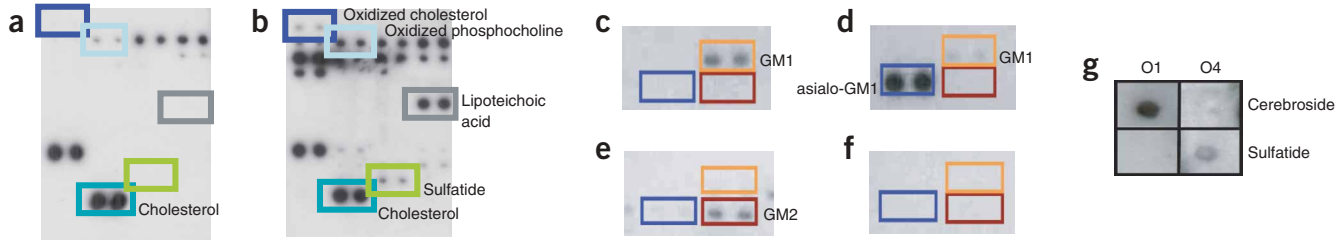


Figure 1 Lipid microarrays. (**a**,**b**) Individual lipid arrays were probed with 1:10 dilutions of CSF from OND control subject (**a**) and a subject with relapsing-remitting multiple sclerosis (**b**). (**c**–**g**) Array validation. Individual arrays were incubated with polyclonal antibodies specific for GM1 (**c**), asialo-GM1 (**d**), GM2 (**e**) or secondary antibody alone (**f**). Individual arrays were also incubated with the monoclonal antibodies O1 specific for cerebroside and O4 specific for sulfatide (**g**).

lipoteichoic acid and at lower levels with sulfatide, oxidized cholesterol and oxidized phosphocholine (**Fig. 1a**).

Array validation and sensitivity analysis

We validated our lipid arrays using polyclonal and monoclonal antibodies with defined specificities. The polyclonal antibodies specific for GM1 bound specifically to GM1, but not to the closely related gangliosides GM2 or asialo-GM1 (**Fig. 1c**). The polyclonal antibodies raised against asialo-GM1 have previously shown low-level reactivity to GM1, and incubation with lipid arrays showed high-level reactivity against asialo-GM1 and low-level reactivity against GM1 (**Fig. 1d**). Polyclonal antibodies raised against GM2 specifically bound GM2 (**Fig. 1e**). The secondary antibody did not show reactivity against lipids (**Fig. 1f**). The sulfatide-specific antibody O4 and cerebroside-specific antibody O1 specifically bound their corresponding antigen features, differentiating antibody reactivity against lipids that differ by only a sulfate group (**Fig. 1g**).

To assess array sensitivity, we performed a direct comparison between lipid arrays and conventional enzyme-linked immunosorbent assay (ELISA). For the five specificities tested, lipids arrays were 5–25 times more sensitive than the conventional ELISA for detecting lipid-specific antibodies (**Table 1**).

Individuals with multiple sclerosis have antibodies to lipids

We applied lipid arrays to profile lipid-specific antibody responses in CSF derived from 16 individuals with multiple sclerosis (8 relapsing remitting; 8 secondary progressive) and 11 control individuals who had other neurological diseases.

We quantified lipid-array reactivity and used a statistical tool known as significance analysis of microarrays (SAM)[16] to identify lipids with statistically significant differences in array reactivity between multiple sclerosis and control samples. We ordered SAM-identified lipid features using a hierarchical cluster algorithm[17], and displayed cluster results as a heatmap using TreeView software[17]. The multiple sclerosis samples clustered and showed strong reactivity to lipids including sulfatide, 3β-hydroxy-5α-cholestan-15-one (an oxidized form of cholesterol), three separate forms of oxidized phosphatidylcholine, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine and sphingomyelin, and showed weaker reactivity to bacterial lipopolysaccharide (LPS) and the ganglioside asialo-GM1 (**Fig. 2a**). A sample from an OND individual (4117) who had spinal stenosis, which is not an inflammatory disorder but results from an anatomical narrowing of the spinal canal, clustered among the multiple sclerosis samples. When we compared only individuals with secondary progressive multiple sclerosis with the OND controls, SAM identified increased reactivity to two gangliosides, GM1 and asialo-GM1, in the individuals with secondary progressive multiple sclerosis (**Fig. 2b**).

Mice with acute EAE have lipid-specific antibodies

Based on our observations of lipid-specific autoantibodies in human multiple sclerosis (**Fig. 2**), we next determined whether autoantibodies directed against sulfatide and other lipids were present in mice with EAE. We probed lipid arrays with sera derived from SJL/J and C57BL/6 (B6) mice induced to develop EAE with one of two nonlipidated myelin peptides, either $PLP_{139–151}$ or $MOG_{35–55}$, respectively, emulsified in complete Freund adjuvant (CFA). Lipid arrays identified autoantibodies directed against sulfatide, asialo-GM1, cerebroside and other lipids in serum derived from mice with EAE (**Fig. 3**). In SJL/J mice, lipid-specific antibody reactivities were very low before induction (day –4) and immediately after induction of EAE (day 3), whereas substantial reactivity was detected and persisted after development of paralysis that characterizes clinical EAE (**Fig. 3a**). We also observed

Table 1 Comparison of lipid arrays with ELISA

	α-GM1		α-asialo GM1		α-GM2		α-GD3		α-cardiolipin	
Dilution	Array	ELISA	Array	ELISA	Array	ELISA	Array	ELISA	Array	ELISA
1:200	Max	1.855	Max	1.351	Max	0.147	7453	0.121	5654	0.874
1:1,000	Max	1.369	Max	1.364	12850	0.042	1284	0	3598	0.266
1:5,000	Max	0.518	Max	0.901	10279	0.011	0	0	1552	0.065
1:25,000	21844	0.151	21330	0.349	1542	0	0	0	0	0
1:125,000	3084	0.027	17475	0.081	0	0	0	0	0	0
1:625,000	514	0	4864	0		0	0	0	0	0
1:3,125,000	0	0	1542	0		0	0	0	0	0

Identical samples of diluted sera containing antibodies specific for five different lipids were assayed using lipid arrays and ELISA. The minimum limit for positive reactivity was set at 0.100 for ELISA and 1,000 digital chemiluminescence units for the lipid array results.

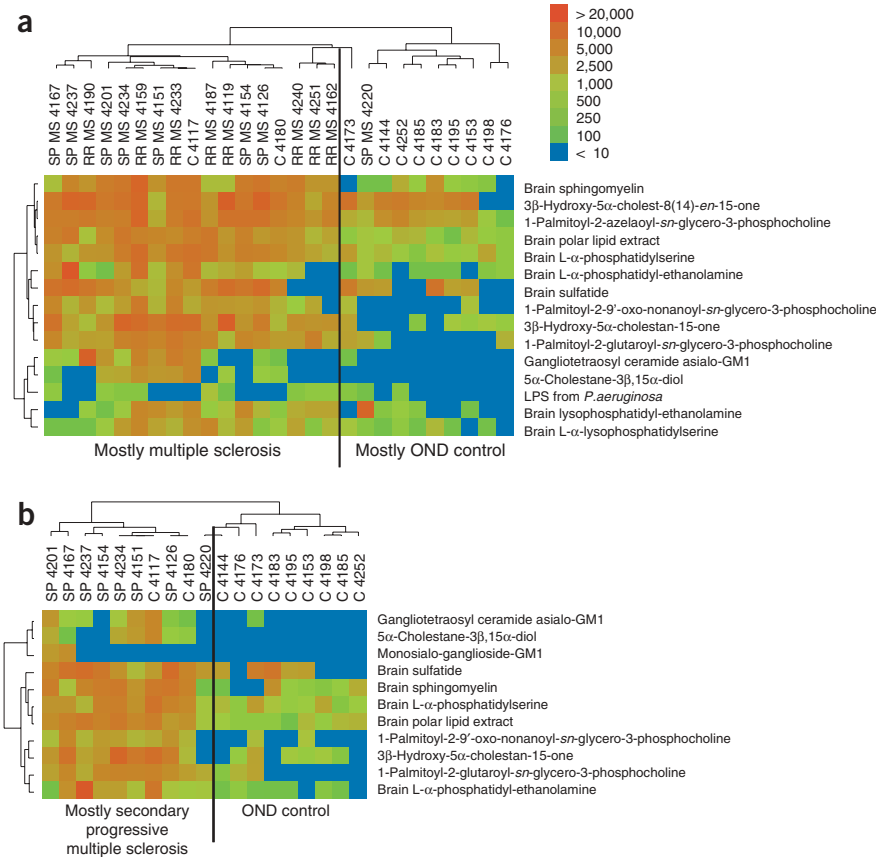


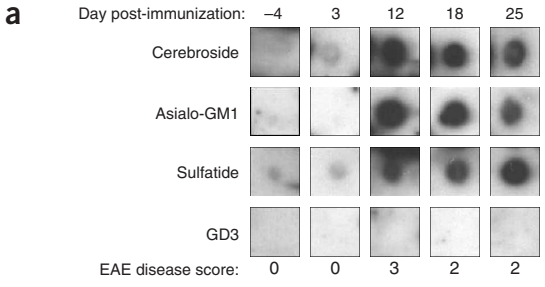
Figure 2 Individuals with multiple sclerosis have increased lipid-specific antibodies. (**a**) Lipid-array profiling of antibody reactivity in CSF samples from 16 subjects with multiple sclerosis and 11 OND controls. Dendrograms depicting the cluster relationships between subjects are shown above and between lipid antigens to the left. After clustering, labels were added at the base of the heatmap to indicate the general location of the clusters of individuals with multiple sclerosis and OND controls. (**b**) Individuals with secondary progressive multiple sclerosis possess increased ganglioside-specific antibodies compared to OND controls.

increased antibodies binding brain polar lipid extract, oxidized lipids and sulfatide in samples from B6 mice with established EAE as compared to preinduction samples (**Fig. 3b**).

Coimmunization with sulfatide worsens EAE

We next determined whether the array-identified lipid targets could alter the disease course of EAE when used as immunogens delivered with myelin peptides emulsified in CFA. We performed experiments using sulfatide and cerebroside, as both lipids are components of the myelin sheath and antibodies against them are present in mice with acute EAE (**Fig. 3**). We mixed 6 μg of sulfatide or cerebroside with $PLP_{139–151}$ and emulsified the lipids with CFA for immunization of SJL/J. We intraperitoneally reimmunized the mice with 6 μg lipid or vehicle at day 4 and day 7 after initial immunization. Coimmunization of SJL/J mice with sulfatide (**Fig. 4a**), and to a lesser degree cerebroside (data not shown), induced a more severe EAE disease course ($P < 0.05$). This is notable because we identified antibodies to sulfatide, but not cerebroside, through our array analysis (**Fig. 2**) as being present in CSF derived from individuals with multiple sclerosis.

To determine whether the mice coimmunized with sulfatide had increased antibodies to sulfatide and other lipids, we analyzed their sera on the lipid arrays. SAM and hierarchical cluster analysis showed statistically increased sulfatide-specific and multiple other



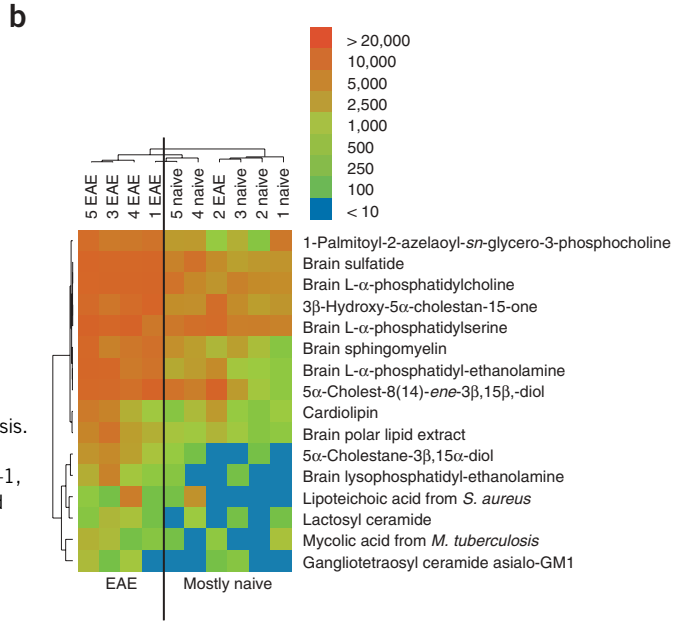


Figure 3 Mice with EAE have increased lipid-specific antibodies. (**a**) Serial serum samples derived from SJL/J mice induced to develop EAE with $PLP_{139-151}$ emulsified in CFA were analyzed on lipid microarrays. Antigen features representing the cerebroside, asialo-GM1 and sulfatide lipids from individual arrays were cut and pasted into columns to facilitate visual analysis. Ganglioside GD3 features provide an example of a lipid with no increase in lipid-specific antibodies over disease course of EAE. (**b**) Preinduction (day –1, naive) and acute EAE (day 20) serum samples from C57BL/6 mice induced for EAE with MOG_{35-55} were analyzed on lipid arrays. The list of lipids determined to have statistically significant differences in reactivity between the naive and acute EAE groups is shown to the right of the heatmap.

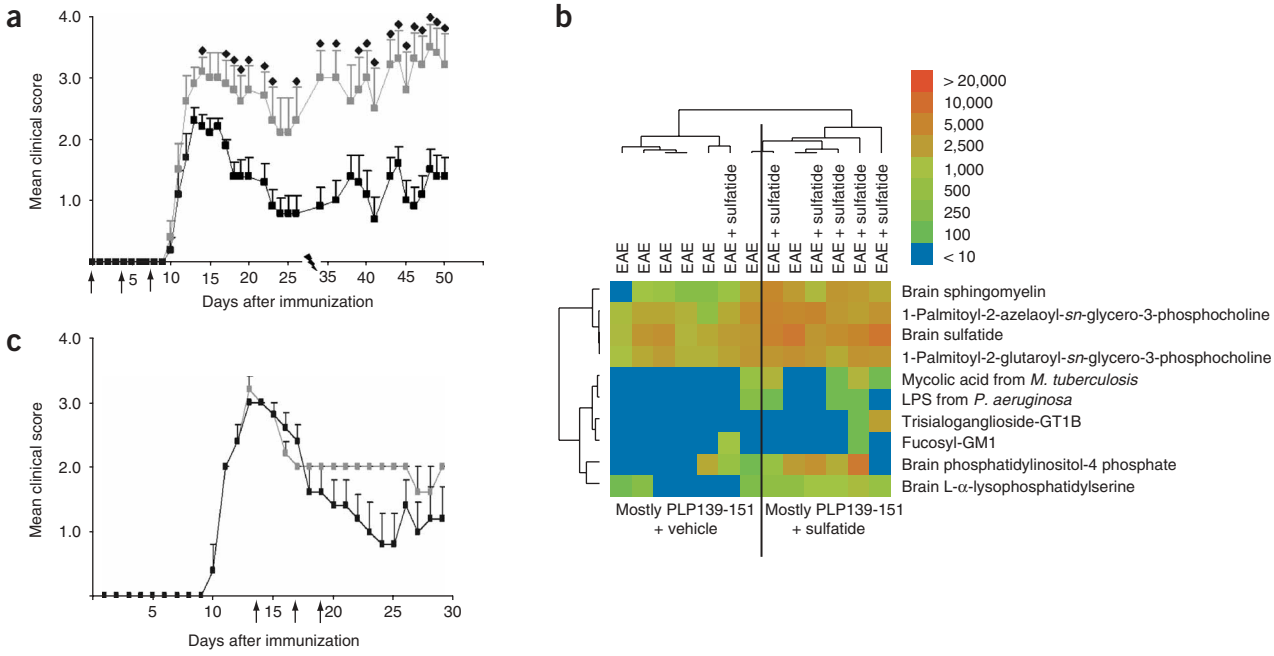


Figure 4 Immunization with sulfatide plus myelin peptide results in a more severe disease course of EAE. (**a**) Coimmunization of SJL/J mice with sulfatide (6 μg/mouse) and $PLP_{139–151}$ (100 μg/mouse) emulsified in CFA (gray box, sulfatide, $n = 10$; black box, vehicle control, $n = 10$). An additional immunization of sulfatide (6 μg/mouse) or vehicle was given intraperitoneally on day 4 and day 7 after immunization. Each point represents the mean + s.e.m. ($^*P < 0.05$; Student *t*-test). (**b**) Lipid array analysis shows increased lipid-specific antibodies in serum samples derived from SJL/J mice immunized with $PLP_{139–151}$ plus sulfatide to develop EAE. The heatmap and dendrograms represent the SAM and hierarchical cluster analysis of lipid-array results. (**c**) Modulation of EAE disease course after intravenous injection of O4 sulfatide-specific antibody versus IgM isotype control (gray box, O4, $n = 5$; black box, IgM control, $n = 5$). Antibody was given on days 14, 17 and 19 after immunization. Mice that received O4 antibody had a worse cumulative disease course compared to IgM controls after the final antibody injection ($P = 0.036$; Student *t*-test).

lipid-specific antibody reactivities in the mice coimmunized with sulfatide as compared to the control group with EAE (**Fig. 4b**).

Transfer of sulfatide-specific antibody worsens EAE

To determine whether the antibodies against sulfatide were pathogenic, we injected into mice with acute EAE the sulfatide-specific antibody O4 or IgM isotype control antibody through the tail vein. We administered antibody (100 μg) on day 14, just after the peak of disease in acute EAE, followed by 50 μg each on days 17 and 19. Transfer of O4 antibody caused more severe EAE (**Fig. 4c**). In contrast, transfer of sulfatide-specific T cells into mice with EAE on days 14 and 28 after immunization did not alter the clinical course of EAE (data not shown).

DISCUSSION

Multiple sclerosis is presumed to be an autoimmune disease directed against the lipid-rich myelin sheath. We developed lipid arrays to profile lipid-specific antibody responses in CSF samples from individuals with multiple sclerosis and controls. Statistical analysis showed increased lipid-specific antibody reactivity against myelin lipids including sulfatide and sphingomyelin, against oxidized lipids including 3β-hydroxy-5α-cholestan-15-one and 1-palmitoyl-2-(9′-oxo-nonanoyl)-*sn*-glycero-3-phosphocholine, and against microbial lipids including LPS in individuals with multiple sclerosis.

Antibody reactivity to oxidized lipids has not previously been described in multiple sclerosis. In inflamed brain plaques from individuals with multiple sclerosis, there are reactive oxygen species such as nitric oxide, which could oxidize lipids to create neoantigens that become targets of an autoimmune response. In fact, the brain and myelin lipid compositions differ between individuals with multiple sclerosis and healthy individuals[18,19]. Individuals with multiple sclerosis have increased cholesterol esters compared to controls[20]. Therefore, individuals with multiple sclerosis may develop immune responses to lipids that are altered by oxidative processes.

The reactivity to the microbial lipid LPS suggests that microbial lipids may have a role in the immune response in multiple sclerosis. LPS binds Toll-like receptor 4 to promote the innate immune response. Nevertheless, the adaptive immune response can recognize LPS as well. In fact, the LPS component of *Campylobacter jejuni* resembles self-gangliosides and can trigger an autoimmune response that results in Guillain-Barré syndrome[10].

Individuals with multiple sclerosis also showed increased antibody reactivity to one ganglioside, asialo-GM1. Notably, when we compared individuals with secondary progressive multiple sclerosis to OND controls, the individuals with secondary progressive multiple sclerosis showed increased reactivity to an additional ganglioside, GM1. Gangliosides are quantitatively enriched in the outer leaflet of the plasma membranes of neuronal cells, and the ganglioside-specific antibodies observed in these individuals may facilitate the destruction of neurons which is associated with secondary progressive multiple sclerosis[21–24].

Lipid-array technology also identified possible lipid antigens in mouse EAE. These data suggest that autoreactive B-cell responses in EAE, in addition to expanding to target additional polypeptide epitopes[25], also undergo intermolecular epitope spreading to target lipid components of the myelin sheath. Compilation of results from lipid-array analyses identified a panel of lipids targeted by antibodies in both mouse EAE and human multiple sclerosis, including sulfatide, oxidized phosphocholine, oxidized cholesterol, sphingomyelin and asialo-GM1.

We further investigated the potential role of lipid-specific T cells and antibodies in autoimmune demyelination. When we immunized EAE-susceptible mice with sulfatide in combination with myelin peptide emulsified in CFA, the mice developed a more severe disease course. Sulfatide coimmunization induced increased sulfatide-specific antibody responses and administration of exogenous sulfatide-specific antibodies worsened the disease severity of EAE. Similarly, it has been shown that guinea pigs induced to develop EAE with sulfatide[26] in conjunction with MBP showed increased demyelination. Implantation in the spinal cord of a hybridoma secreting sulfatide-specific antibody has been shown to cause demyelination of the CNS in rats[27]. Together, these observations suggest that autoimmune responses directed against sulfatide and other lipids contribute to the pathogenesis of autoimmune demyelinating disease.

Given that lipid-specific autoimmunity is present in individuals with multiple sclerosis and is associated with more severe autoimmune demyelination in EAE, future development of tolerizing therapies to attenuate autoimmune responses against lipids could provide benefit to individuals with multiple sclerosis. Coimmunizing with self-lipid emulsified in CFA in our study and others[28] worsened EAE. Conversely, another group showed that intraperitoneal administration of a larger dose (20 μg) of sulfatide could alleviate signs of EAE[29]. These data suggest that the timing and route of lipid administration are important factors in the outcome of lipid-based immunomodulatory agents.

Lipid arrays enable simple, chemiluminescence-based, multiplex analysis of lipid-specific antibody responses. We believe that the hydrophobic part of the lipid anchors the lipid molecule to the hydrophobic PVDF array surface. The lipid molecules are thereby oriented so that the polar regions, such as the sulfate group or glycan molecule, are accessible for antibody binding. In addition to its use for studying autoimmune responses, the lipid microarray could be applied to profile immune responses against microbial lipids, such as those targeted by protective immune responses against *Mycobacterium tuberculosis*.

Identification of autoantigen targets of B and T lymphocytes is essential for understanding the etiology and pathogenesis of multiple sclerosis and other autoimmune diseases. Our data suggest that sulfatide-specific and other lipid-specific responses can contribute to the pathogenesis of autoimmune demyelinating disease.

METHODS

Lipids. We obtained lipids from Matreya, Avanti Polar Lipids, Calbiochem, Sigma Chemicals, Biodesign International and Accurate Chemical (**Supplementary Table 1** online) and dissolved them in mixtures of chloroform, methanol and water to a final concentration of 1 mg/ml, except for gangliosides which we diluted to 0.1 mg/ml, and LPS and teichoic acid which we diluted to 0.01 mg/ml.

Antibodies. We purchased antibodies against GM1, GM2, asialo-GM1, GD3 and GM4 from Matreya and Calbiochem. We purchased cardiolipin-specific serum from individuals with lupus from Immunovision and Louisville APL Diagnostics. We obtained sulfatide-specific and cerebroside-specific antibodies from the O4 and O1 hybridomas[27,30]. We also purchased purified O4 antibody from R&D Systems. We purchased IgM isotype control from Chemicon International. Samples of human CSF were provided by I.C. and K.G.W.

Subject samples. All human samples were collected and used under protocols approved by the Institutional Review Boards of the University of Alberta and Stanford University. All individuals with multiple sclerosis except subject 4154 were undergoing a relapse at the time of lumbar puncture. All individuals with multiple sclerosis except subject 4251 possessed oligoclonal bands in their CSF, whereas all OND controls were negative. OND controls included individuals with motor neuron disease (subject 4252), vascular leukoencephalopathy (subject 4173), spinal stenosis (subject 4117), clipped cerebral aneurysm with functional neurological symptoms (subject 4185), an individual undergoing breast implant removal who was having functional neurological symptoms (subject 4198) and other individuals having a functional neurological symptom for whom multiple sclerosis was ruled out.

Lipid array production. A Camag Automatic TLC Sampler 4 (ATS4) robot was adapted to print lipids in ordered arrays on PVDF membranes affixed to the surface of microscope slides using double-sided Scotch tape (3M). The ATS4 sprays 200 nl containing 10 to 100 pmol of lipids solubilized in chloroform-methanol-water mixtures under nitrogen gas to form individual features. At our current printing density, the ATS4 can print arrays containing up to 200 individual features. Twelve slides are printed in each 'print run'. Printed membranes are stored dry and retained reactivity for several months.

Probing lipid arrays. We blocked arrays overnight at 4 °C with 1% fatty acid–free BSA (Sigma) in PBS (Gibco BRL). We then probed arrays with 1:10 dilutions of CSF, or 1:200 dilutions of sera for 2 h at 4 °C. After washing with blocking solution, we added a secondary antibody (human IgG+IgM, Jackson Immunoresearch; human IgG–specific, Jackson Immunoresearch; rabbit IgG–specific, Amersham; or mouse IgG, Amersham) conjugated to horseradish peroxidase (HRP) in blocking solution, and visualized bound secondary antibodies using chemiluminescence (ECL Plus, Amersham) and autoradiography.

Analysis of lipid array data. We used GenePix Pro 5.0 software (Molecular Devices) to extract the net median pixel intensities for individual features from the digital images produced by scanning array autoradiographs. We generated mean net digital chemiluminescence units from the mean values from two identical lipid antigen features on each array. We applied the SAM[16] algorithm (version 1.2) to identify lipids with statistically significant differences in array reactivity between groups of humans or mice. Log base 2 values of mean net digital chemiluminescence units (for analyses involving human samples, mean background reactivity of secondary antibody alone was subtracted; for all analyses, values less than 10 were set to 10 and resulting values divided by 300) were input into SAM and in each panel the list of antigens receiving the lowest q value (representing the false discovery rate) and having SAM scores of >1.1 are reported. This lowest q value was 0.039 for **Figure 2a**, 0.063 for **Figure 2b**, 0.026 for **Figure 3b** and 0.36 for **Figure 4b**. We arranged SAM results into relationships using Cluster software and displayed using TreeView software[17]. For the analyses involving human samples (**Figure 2a**,**b**), to enhance visual differences 5,000 units was subtracted from the mean sulfatide reactivity before generating log base 2 values for Cluster analysis and TreeView display. For **Figures 3b** and **4b**, the log base 2 values were input directly into Cluster and TreeView.

Lipid ELISA. We coated Costar enzyme immunoassay plates with gangliosides (1 μg/well) and cardiolipin (2 μg/well), blocked them with Assay Diluent (Pharmingen) and detected primary antibody binding with HRP-conjugated human IgG–specific or rabbit IgG–specific antibody.

EAE induction and lipid coimmunization. For induction of EAE in C57BL/6 mice (Jackson Laboratories), we induced disease in 8–10-week-old female mice by subcutaneous immunization with 100 μg of $MOG_{35–55}$ emulsified in CFA (Difco Laboratories), accompanied by 300 ng of pertussis toxin (Life Technologies) intraperitoneally on days 0 and 2. For induction of EAE in SJL mice (Jackson Laboratories), we subcutaneously immunized 8–10-week-old female mice with 100 μg of $PLP_{139–151}$ emulsified in CFA. We administered three injections of sulfatide (6 μg/mouse/injection) or vehicle (0.025% Tween-20 in PBS) on days 0, 4 and 7 after immunization with CNS antigens. On day 0 we emulsified the sulfatide or vehicle together with $PLP_{139–151}$ in CFA and administered the mixture by subcutaneous injection. For subsequent time points, we intraperitoneally injected sulfatide or vehicle as previously described[28]. We monitored clinical disease daily using the following scoring system: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, death. Animal experiments were approved by and performed in compliance with the guidelines of the Institutional Animal Care and Use Committee.

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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