Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Identification of the Lipid Antigens Recognized by rHIgM22, a Remyelination-promoting Antibody

Sara Grassi (sara.grassi@unimi.it)
University of Milan
Livia Cabitta
University of Milan
Simona Prioni
University of Milan
Laura Mauri
University of Milan
Maria Grazia Ciampa
University of Milan
Noriko Yokoyama
Juntendo University
Kazuhisa Iwabuchi
Juntendo University
Yana Zorina
Acorda Therapeutics, Inc
Alessandro Prinetti
University of Milan

Research Article

Keywords: rHIgM22, remyelination, multiple sclerosis, sulfatide, glycerophospholipids, sphingolipids

Posted Date: August 25th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1981498/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Neurochemical Research on January 25th, 2023. See the published version at https://doi.org/10.1007/s11064-023-03859-2.

// Abstract

Failure of the immune system to discriminate myelin components from foreign antigens plays a critical role in the pathophysiology of multiple sclerosis. In fact, the appearance of anti-myelin autoantibodies, targeting both proteins and glycolipids, is often responsible for functional alterations in myelin-producing cells in this disease. Nevertheless, some of these antibodies were reported to be beneficial for remyelination. Recombinant human IgM22 (rHIgM22) binds to myelin and to the surface of O4-positive oligodendrocytes, and promotes remyelination in mouse models of chronic demyelination. Interestingly, the identity of the antigen recognized by this antibody remains to be elucidated. The preferential binding of rHIgM22 to sulfatide-positive cells or tissues suggests that sulfatide might be part of the antigen pattern recognized by the antibody, however, cell populations lacking sulfatide expression are also responsive to rHIgM22. Thus, we assessed the binding of rHIgM22 *in vitro* to purified lipids and lipid extracts from various sources to identify the antigen(s) recognized by this antibody. Our results show that rHIgM22 is indeed able to bind both sulfatide and its deacylated form, whereas no significant binding for other myelin sphingolipids has been detected. Remarkably, binding of rHIgM22 to sulfatide in lipid monolayers can be positively or negatively regulated by the presence of other lipids. Moreover, rHIgM22 also binds to phosphatidylinositol, phosphatidylserine and phosphatidic acid, suggesting that not only sulfatide, but also other membrane lipids might play a role in the binding of rHIgM22 to oligodendrocytes and to other cell types not expressing sulfatide.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) whose hallmark is the development of focal plaques of demyelination, which in turn leads to diffuse neurodegeneration throughout the grey and white matter of the brain and spinal cord [1–3]. MS is the most common cause of non-traumatic disability in young people, with an onset usually between 20–40 years of age [4], affecting 2.5 million people throughout the world [5]. Despite its high prevalence, multiple sclerosis remains a challenging ailment to study. While genetic and environmental risk factors have been associated with the onset of the MS, the etiology is unknown, the pathophysiologic mechanisms are various, and the chronic and unpredictable course of the pathology represent a drawback when it comes to defining whether the positive effects of short-term treatment will be sustained [3]. Currently the most widely accepted hypothesis concerning MS pathogenesis is the autoimmune hypothesis, which proposes autoimmune inflammation as the cause of demyelination, and auto-reactive leukocytes as disease initiators [6–8]. A second hypothesis regarding MS onset is that the disease might be triggered by viral infection. This hypothesis does not exclude the autoimmune hypothesis, considering that virus could trigger the autoimmunity. Moreover, the viral infection might occur several years before the development of the MS lesions [9, 10]. Nevertheless, both hypothesis remain unproven. There is, however, a third hypothesis, defined as the oligodendrogliopathy hypothesis, based on neuropathological studies on MS, which suggests that oligodendrocyte death and microglial activation are the initial event in MS lesion formation, followed by immune responses to scavenge dead myelin. Contrary to the autoimmune

inflammation, these immune responses seem to be permissive for oligodendroglial regeneration and remyelination, consistently with the observation that removal of myelin debris is necessary for effective remyelination [11]. Whether the immune response is the cause of the pathogenesis or simply a consequence of the oligodendroglial cell death, the failure of the immune system to discriminate myelin components from foreign antigens plays a critical role in the pathophysiology of MS. Several CNS myelin proteins, including MBP, PLP, MAG and MOG, have been described as targets for autoantibodies in MS [12–18]. In addition, increased serum levels of glycolipids and of anti-glycolipid antibodies have also been reported in MS patients [19–23]. For example, it has been observed that some MS patients exhibit enhanced antibody response against sulfatides [24, 25]. Moreover, recent evidence has shown that increased levels of serum and cerebrospinal fluid sulfatides are found in MS patients and in their healthy siblings, with stage-specific accumulation of different molecular species [26–28], suggesting that the presence of sulfatide in these biological fluids could represent a risk/prognostic factor for the onset and progression of MS.

Studies have also shown how anti-sulfatide antibodies can interact with the surface of cultured oligodendrocytes and affect the lateral organization of sulfatide with myelin proteins with opposite consequences (demyelination *versus* stimulation of myelin formation), depending on the type of extracellular matrix protein prevalent in the culture environment [29]. Hence, these antibodies might play a role in the onset of the disease, but they might also represent an important immunological tool for the treatment of demyelinating diseases [30].

Recombinant human IgM22 (rHIgM22) is a remyelination promoting antibody, able to enter in the CNS, to accumulate in the demyelinated lesions, and to promote remyelination in mouse models of chronical demyelination [31, 32]. The antibody, currently being developed for MS treatment (ClinicalTrial.gov: NCT02398461; NCT01803867), was reported to bind selectively to myelin and to the surface of oligodendrocytes *in vitro* [30, 31]. However, the antigen recognized by rHIgM22 has yet to be identified. Nevertheless, *in vitro* studies show that rHIgM22 binds CNS tissue sections with a pattern similar to that of the anti-sulfatide antibody O4 [33], and that binding is abolished in CNS tissue sections from CST (-/-) mice [34], suggesting that sulfatide or a molecule in the sulfatide synthesis pathway might play a role in rHIgM22 antigen recognition. Indeed, in this paper we demonstrate that rHIgM22 binds to sulfatide *in vitro*; this binding is specific and can be modulated by the presence of other lipids. In addition, rHIgM22 binds to phosphatidic acid, phosphatidylserine and phosphatidylinositol, suggesting that not only sulfatide, but also other membrane lipids might play a role in the binding of rHIgM22 to oligodendrocytes.

Materials And Methods

Materials

HEPES, phosphate-buffered saline (PBS), Na_3VO_4, NaOH, $CHCl_3$, CH_3OH, polyisobuthylmethacrylate, *O*-phenylenediamine (OPD), H_2O_2, Citric Acid, HCl, sucrose, Tris-HCl, Na_2HPO_4, DOPC (1,2-Dioleoyl-sn-glycero-3-phosphocholine), n-octyl-β-D-glucoside, bovine serum albumin (BSA) were purchased from

Sigma Aldrich. DMEM:F12, StemPro Neural Supplement, Accutase were purchased from *Invitrogen.* EGF, PDGF-AA, FGF-2 were purchased from *Peprotech;* NH_4OH was purchased from Riedel-de Haën™; penicillin/streptomycin, DMEM High glucose, bovine fetal serum (FBS), glutamine were purchased from *Euroclone Spa*. $MgSO_4$, $CaCl_2$, 4-methoxybenzaldehyde (anisaldehyde), HPTLC were purchased from *Merck*. HPA sensor chips and HBS-N Buffer were purchased from *GE Healthcare Srl.* HRP-conjugated anti-Human IgM μ-chain antibody has been purchased from *Thermo Fisher Scientific, Inc*.

Pure galactosylceramide (GalCer), sulfatide, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were purchased from *Avanti Polar Lipids*; DOPC, sphingomyelin (SM), and phosphatidic acid (PA) were purchased from *Sigma Aldrich*. Lysosulfatide was purchased from *Matreya*. Gangliosides (GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b), glucosylceramide (GlcCer), glucosylsphingosine (GlcSph), and lactosylceramide (LacCer) were synthesized or extracted from natural sources and purified in our laboratories.

Chrompure Human IgM has been purchased from *Jackson Immuno Research, Inc.*, rHIgM22 antibody was provided by Acorda Therapeutics, Inc. (Ardsley, NY, USA).

Oligodendrocytes isolation

MGC cultures were prepared according to Watzlawik et al [35]. Oligodendrocytes were harvested, through shaking procedure, from 8–10 days old mixed glial cell cultures, when the cell were mostly immature, containing progenitor cells and immature oligodendrocytes. Briefly, microglia and dead cells are removed with a 30-minute shake at 37°C, 150 rpm in Corning® LSE™ Benchtop Shaking Incubator. After an 18–20 hour shaking to detach oligodendrocytes, the cell suspension was plated twice on untreated, non-TC, dishes to further remove microglia and astrocytes, before being centrifuged for 10 minutes at 8°C, 850 rpm. The supernatant was discarded, and the pellet suspended in oligodendrocyte precursor cells (OPC) proliferative medium (DMEM:F12 1:1 containing 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 2% StemPro Neural Supplement, 10 ng/mL EGF, 10 ng/mL PDGF-AA, 10 ng/mL FGF-2). Media was replaced every 3/4 days and cells were carried using Accutase (Invitrogen). The cells obtained with this method are mostly immature oligodendrocytes. To obtain mature oligodendrocytes, differentiation medium containing 40 mM T3 was added to the cultured OPCs. The collected cells were then stored at -80°C, before being lyophilized and subjected to lipid extraction.

Myelin isolation

Purification of myelin from mouse brain was performed using an optimized version of the protocol described in [36]. Frozen brains from C57BL/6N wild type mice ranging from 2 to 4 months of age were thawed at room temperature (RT) before removing the cerebellum and the meningeal membranes. Fifty mg of tissue were suspended in 500 µL of 0.25 M sucrose in 10 mM Tris-HCl, pH 7.4 and Dounce homogenized (10 strokes, tight) before being centrifuged at 500 g, 4°C for 10 minutes. The supernatant was collected and further centrifuged at 21000 g, 4°C for 10 minutes with Ultra-centrifuge Beckman TL-100. The supernatant was discarded, and the pellet was suspended in 500 µL of 0.25 M sucrose in 10

mM Tris-HCl, pH 7.4 and then layered on top of 1250 µL of 0.88 M sucrose in 10 mM Tris-HCl, pH 7.4. After this, the samples were centrifuged at 21000 g, 4°C for 10 minutes to separate the mitochondria from the myelin vesicles. In fact, myelin will float on the surface whereas the denser mitochondria will pellet at the bottom of the tube. The myelin vesicle layer was carefully recovered, suspended in an equal volume of ice-cold water and then centrifuged at 21000 g, 4°C for 10 minutes. After discarding the supernatant, the pellet was suspended in 500 µL of 0.25 M sucrose in 10 mM Tris- HCl, pH 7.4, layered on top of 1250 µL of 0.88 M sucrose in 10 mM Tris-HCl, pH 7.4 and centrifuged at 21000 g, 4°C for 10 minutes in order to separate myelin from membrane debris. The myelin layer was carefully recovered, suspended in an equal volume of ice-cold water and then centrifuged at 21000 g, 4°C for 10 minutes. The supernatant was discarded, and the pellet was suspended in 120 µL of ice-cold water. Protein content was determined with DC protein assays (Bio-Rad).

Sample preparation

Frozen brains from wild type (WT) C57BL/6N mice and acid sphingomyelinase knock-out (ASM(-/-)) mutant C57BL/6N mice were thawed at room temperature (RT). Meninges were removed, the brains were minced with a surgical blade, suspended in ice-cold water and subjected to sonication. The samples were then Dounce homogenized (10 strokes, tight) before being snap frozen and subsequently lyophilized.

Frozen rHIgM22-positive rat oligodendrocytes from Acorda were thawed at RT, suspended in ice-cold water, snap frozen and then lyophilized. Cultured oligodendrocytes were collected after washing the flasks and/or petri dishes twice with PBS containing 1 mM Na_3VO_4. The cells were scraped twice in PBS containing 1 mM Na_3VO_4 and centrifuged at 3000 rpm, 4°C for 5 minutes. The supernatant was discarded, suspended in ice-cold water, snap frozen and lyophilized.

Myelin, prepared from mouse brain following the procedure described in the previous paragraph, was snap frozen and lyophilized.

Total lipid extraction, phase partitioning and alkali treatment

Lipids from the lyophilized samples were extracted with $CHCl_3$:CH_3OH:H_2O 20:10:1 (v/v/v) and subjected to a modified two-phase Folch's partitioning to obtain the aqueous (Aq. Ph.) and the organic phases (Or. Ph.) [37]. Briefly, 1550 µL of the solvent system were added to the lyophilized samples. The samples were then mixed at 1100 rpm, RT for 15 minutes and centrifuged at 13200 rpm, RT for 15 minutes. The supernatant was collected as Total lipid extract (TLE) and the extraction was repeated again twice by adding the 1550 µL of the solvent system to the pellets. The pellets were air dried and suspended in 1M NaOH and incubated overnight at RT before being with water to 0.05M NaOH to allow the determination of the protein content with DC assay. Aliquots of the TLE were then subjected to phase partitioning adding either 20% of water by volume. The samples were then mixed at 1100 rpm, RT for 15 minutes and centrifuged at 13200 rpm, RT for 15 minutes. The Aq. Ph. were recovered, and CH_3OH:H_2O 1:1 (v/v) were added to the organic phase before mixing the samples at 1100 rpm, RT for 15 minutes and centrifuging

at 13200 rpm, RT for 15 minutes. The new aqueous phases were recovered and united to the ones previously collected. The organic phases were dried under N_2 flux and suspended in a known volume of $CHCl_3$:CH_3OH 2:1 (v/v). Aliquots of the organic phases were then subjected to alkali treatment to remove glycerophospholipids [37].

Thin Layer Chromatography (TLC)

Lipids and samples were separated using HPTLC plate (silica gel 60 HPTLC, Merck) with the following solvent systems: $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) for sulfatide, lysosulfatide, neutral glycolipids, methanolized organic phases and phospholipids; $CHCl_3$:CH_3OH:$CaCl_2$ 0,2% 50:42:11 (v/v/v) for S1P and gangliosides. After separation, lipids were detected either by spraying the TLC plates with the colorimetric reagent anisaldehyde or by TLC immunostaining.

TLC immunostaining

After chromatographic separation, the TLC plates were coated three times with a polyisobuthylmethacrylate solution [37], and air dried for 1 hour at room temperature (RT) before being immersed in blocking solution (3% BSA in PBS) for 1 hour, RT. After washing thrice (1 min each) with PBS, the plates were incubated with rHIgM22 or isotype human IgM (Chrompure Human IgM; Jackson Immunoresearch; negative control) at 0.5, 2.5 or 5.0 µg/mL in 1% BSA in PBS, overnight at 4°C. After the incubation with the primary antibody, the plates were washed three times with PBS, incubated with an HRP-conjugated anti-Human IgM µ-chain antibody for 1 hour, RT, and developed using *O*-phenylenediamine (OPD)/H_2O_2 in 0.05 M citrate-phosphate buffer pH 5.0.

Surface plasmon resonance

SPR experiments were performed using a BIAcore 3000 analytical system (GE Healthcare, Uppsala, Sweden) with HPA sensor chips. Different amounts of sulfatide ($1–1x10^{-1}$ – $1x10^{-2}$ – $1x10^{-3}$ – $5x10^{-4}$ µmol) or of lysosulfatide ($1x10^{-1}$ – $5x10^{-1}$ µmol) in $CHCl_3$:CH_3OH 2:1 (v/v) were mixed with 1 µmol of DOPC, either alone or in presence of different amounts of a second lipid (cholesterol, GalCer, SM), and dried under N_2 flux. The residue was then suspended in 200 µL HBS-N buffer (20 mM 4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid (HEPES) pH 7.4, 150 mM NaCl), mixed with a vortex mixer and sonicated for 15 minutes with a water bath sonicator. The solutions were then filtered using a 0.22 µm polyvinylidene difluoride (PVDF) syringe-driven filter unit (Merck). The sensor chip was pretreated with 40 mM n-octyl-β-D-glucoside and the liposome solutions were immobilized on the chip for 30 min at a flow rate of 2 µL/min, using HBS-N buffer as running buffer. The chip surface was then washed with 50 mM NaOH for 1 min at 5 µL/min, and blocked with 100 µg/ml bovine serum albumin (BSA) before proceeding with the analysis. For analysis, 5 µg/mL of rHIgM22 or control IgM were injected at a flow rate of 10 µL/min. Signals generated in a negative control cell without target lipid have been subtracted from the experimental values. Quantitative evaluation of the binding and dissociation reactions were performed using the software BIAevaluation version 3.1 [38].

Other Experimental Procedures

The protein content was determined by the Bio-Rad DC assay kit using BSA as the reference standard.

Statistical analysis

Experiments were run in triplicate, unless otherwise stated. Data are expressed as mean value ± SD and were analyzed by one-way analysis of variance followed by Student-Neuman-Keul's test. *p*-values are indicated in the legend of each figure.

Results

Binding to sulfatide

The myelin membrane has a high lipid content and is enriched in glycolipids [39, 40]. In particular, galactosylceramide (GalCer) and sulfatide account, respectively, for about 20 and 5% of myelin lipids [41, 42]. rHIgM22 binds to myelin and to the surface of oligodendrocytes *in vitro* [31, 34]. Moreover it binds to CNS tissue sections with a pattern similar to that of O4, an anti-sulfatide antibody [33], suggesting that sulfatide could be one of the antigens recognized by this remyelination-promoting antibody.

Binding of rHIgM22 to different amounts (from 0.05 to 10.00 nmol) of purified sulfatide from commercial sources was assessed through TLC immunostaining (Fig. 1). The assays were performed using different concentrations (0.5–2.5–5.0 µg/mL) of rHIgM22 or of a non-immunogenic human IgM (negative control), as detailed in Materials and Methods. rHIgM22 was able to bind pure sulfatide with a signal detectable from 0.5 nmol of the target lipid and proportional to its amount. The binding decreased if the concentration of the antibody was lowered, however the intensity of the signals was not quantitatively proportional to rHIgM22 concentration, at least below 2.5 µg/mL. A cross-reactivity of the control human IgM was also observed, however it was significantly lower than that of rHIgM22 for all experimental points, except for the IgM concentration of 0.5 µg/mL.

Thin layer chromatographic of different amounts of purified sulfatide (from 0.05 to 10.00 nmol) was performed using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system. Following chromatography, the TLC plates were subjected either to colorimetric detection with anisaldehyde reagent (A) or to TLC immunostaining with different concentrations (0.5, 2.5 and 5.0 µg/mL) of rHIgM22 or control Human IgM (B), as described in "Methods". Optical density (OD) of each band was calculated by densitometry; the data are expressed as mean ± SD of three independent experiments (C).

On the basis of these results, surface plasmon resonance experiments were set up with the purposes 1) to verify the specificity of rHIgM22 binding to sulfatide; 2) to allow a more quantitative analysis of the binding; 3) to perform experiments under conditions allowing a more physiological presentation of the antigen. Binding of rHIgM22 to different amounts of sulfatide (from $5x10^{-4}$ to 1 µmol), in DOPC monolayers, was analyzed using sensor chips HPA.As shown in Fig. 2, rHIgM22 binding was significantly higher than the binding of the control IgM for all experimental points. Furthermore, the shape of the sensorgram was consistent with a specific binding for rHIgM22, and the binding response increased with

an increase of the amount of sulfatide present in the monolayers. Taking into account the values of maximum response and the prompt return to the baseline upon antibody removal, the DOPC:sulfatide 1:1x10^{-2} molar ratio was selected for further experiments aimed to analyze the binding response of rHIgM22 for monolayers containing different lipids. Under these experimental conditions, the binding response of rHIgM22 to sulfatide containing monolayers was consistent and highly reproducible (Fig. 2C).

DOPC (1 μmol) was mixed with different amounts of sulfatide (from 5×10^{-4} μmol to 1 μmol) and dried. The lipid mixture was suspended in HBS-N buffer, mixed vigorously and sonicated. The resulting liposomes were immobilized on a HPA sensor chip. The surface was washed briefly with 50mM NaOH and blocked with 100 μg/mL BSA. For analysis, 5 μg/mL rHIgM22 or Human IgM were injected. The binding of the two antibodies is represented as a sensorgram (A) and also as maximum response (B). Maximum response is expressed as mean ± SD of three independent experiments; *, $p < 0.05$ versus control. Reproducibility of the binding response under the experimental conditions DOPC:sulfatide 1: 1x10^{-2} molar ratio is shown in (C).

Effect of different lipids on the binding of rHIgM22 to sulfatide containing monolayers

Previous studies, performed using model membranes, have shown that antibody recognition of sulfatide is affected by the membrane lipid microenvironment. In fact, it has been seen that in a polyclonal anti-sulfatide serum fewer antibodies were able to recognize their own target in a sphingomyelin/cholesterol environment than in a phosphatidylcholine/cholesterol environment. Moreover, length and hydroxylation of fatty acid chain of PC or of SM seemed to restrict the recognition to higher affinity antibodies [43, 44].

Therefore, to verify whether the binding of rHIgM22 could be affected by lipid microenvironment, antibody binding to sulfatide containing monolayers was assessed through surface plasmon resonance experiments. In these experiments, monolayers were prepared by mixing a fixed amount of DOPC and of sulfatide, respectively 1 μmol and 1x10^{-2} μmol, with a third lipid using either molar ratios corresponding to those found in the myelin membrane or a 10-fold higher amount of the third lipid, possibly reflecting the molar ratio expected for a lipid raft-like microenvironment. Binding analysis revealed that the binding response of rHIgM22 to sulfatide containing monolayers was reduced in the presence of either GalCer (6.6×10^{-1} or 6.6×10^{-2} μmol) or cholesterol (1×10^{-1} μmol), while the addition of SM (1.2×10^{-2} μmol) to the monolayers resulted in a higher binding (Fig. 3). These finding suggest that the presence of different lipids, at a certain density might be required to allow an optimal recognition of the antigen by rHIgM22.

Liposomes composed of DOPC (1 μmol), a fixed amount of sulfatide (1×10^{-2} μmol) and different amounts of GalCer (6.6×10^{-1} μmol or 6.6×10^{-2} μmol), Cholesterol (1×10^{-1} μmol), or SM (1.2×10^{-1} μmol) were prepared as described in “Methods”. The liposomes were immobilized on an HPA sensor chip. For analysis, 5 μg/mL rHIgM22 or human IgM were injected. The binding of the two antibodies is represented as maximum response. Data are expressed as mean ± SD of three independent experiments.

Binding to lysosulfatide

The deacylated form of sulfatide, lysosulfatide, is present as a minor component in the normal CNS but its levels can be increased as a consequence of some pathological conditions, such as metachromatic leukodystrophy [45, 46]. Considering that sulfatide and lysosulfatide bear the same 3-*O*-sulfo-galactose head group, the binding of rHIgM22 to lysosulfatide was analyzed. The binding to lysosulfatide was assessed using both TLC immunostaining and SPR experiments and, in both experimental settings, the antibody resulted able to bind the lipid. In TLC immunostaining assay (Fig. 4A), rHIgM22 was able to recognize lysosulfatide, even though the binding resulted significantly lower than that to sulfatide for equimolar amount of the two lipids. Nevertheless, surface plasmon resonance experiments confirmed that the binding of rHIgM22 to the lysolipid was specific (Fig. 4B).

Thin layer chromatographic of different amounts of purified lysosulfatide (from 0.05 to 10.00 nmol) was performed using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system. Following separation, the TLC plates were subjected either to colorimetric detection with anisaldehyde reagent, or to TLC immunostaining with rHIgM22 or human IgM, as described in “Methods”. Optical density (OD) of each band was calculated by densitometry; the data are expressed as mean ± SD of three independent experiments (A). Liposomes composed of DOPC (1 μmol) and different amounts of lysosulfatide ($1{\times}10^{-1}$ μmol or $5{\times}10^{-1}$ μmol), prepared as described in “Methods”, were immobilized on an HPA sensor chip. For analysis, 5 μg/mL rHIgM22 or human IgM were injected. The binding of the two antibodies is represented as maximum response. Data are expressed as mean ± SD of three independent experiments; *, $p < 0.05$ versus control (B).

Binding to glycolipids and sphingosine 1-phosphate

As mentioned in previous paragraphs, the myelin membrane has a high lipid content and is enriched in glycolipids (31% vs. 7% for liver cell plasma membranes) [47], in particular galactosylceramide [48]. Another major component of the myelin membrane is sphingomyelin (~ 5% of total lipids). On the other hand, gangliosides, the most abundant glycosphingolipids in the nervous system, are a minor components of the myelin membrane (< 1% of total lipids) [49]. Binding of rHIgM22 to different sphingolipids was assessed using TLC immunostaining. rHIgM22, at 5.0 μg/mL, showed no significant binding to GalCer, LacCer, SM, nor to any of the mono- (GM3, GM2, GM1) and polysialogangliosides (GD3, GD1a and GD1b) analyzed (Fig. 5A, C). As a recent paper from Nair S. et al [50], reported that immunoglobulins present in the sera of patients with monoclonal gammopathies are reactive not only against lyso-PC, but also against glucosylsphingosine (GlcSph, lyso-GlcCer), so binding to glucosylsphingosine was assessed. Due to its role as a precursor for the synthesis of GlcSph, the binding to glucosylceramide (GlcCer) was also analyzed.

Binding of rHIgM22 to sphingosine 1-phosphate was also assessed using TLC immunostaining. Neither rHIgM22, nor control IgM, however, showed a significant binding to GlcSph, GlcCer or sphingosine 1-phosphate (Fig. 5B).

Binding of rHIgM22 to different neutral glycolipids, S1P and gangliosides was assessed using TLC immunostaining. Different amounts of purified GalCer, LacCer and SM (from 0.05 to 10.00 nmol) or GlcSph, GlcCer (from 2.00 to 8.00 nmol) were separated on TLC plates using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system; while for separation of S1P (from 1.00 to 4.00 nmol) and gangliosides (4.00 nmol), $CHCl_3$:CH_3OH:$CaCl_2$ 0,2% 50:42:11 (v/v/v) was used as solvent system. Following separation, the TLC plates were subjected either to colorimetric detection with anisaldehyde reagent (A) or to TLC immunostaining with 5.0 µg/mL of rHIgM22 or Human IgM (B), as described in “Methods”.

Binding of rHIgM22 to lipid extracts from biological samples

The results obtained from the experiments on pure lipids suggest that rHIgM22 binds to pure sulfatide and, with a lesser affinity, to lysosulfatide, whereas it does not show any significant binding to any other sphingolipid. The experiments that will be described in this section were aimed to analyze the binding of rHIgM22 to lipid mixtures obtained from a variety of relevant biological samples. Using standard lipid extraction and purification procedures, we have prepared a total lipid extract that has been further fractionated as described in Materials and Methods. Total lipid extracts and organic phases from different CNS cells and tissues (mixed glial cells, mice brain, oligodendrocytes, and myelin) were analyzed (data not shown) however, the presence of glycerophospholipids in these samples strongly affected the binding of both rHIgM22 and control IgM. Both the antibodies, in fact, showed a binding to multiple bands with an intensity that was not proportional to the amount of the lipid samples. Therefore, even if the amount of sulfatide theoretically present in the samples was comparable to the amount detectable using pure sulfatide, the identification of signals corresponding to sulfatide was impossible. The removal of glycerophospholipids through alkali treatment of the organic phases proved to be a useful tool for the analysis of the binding of rHIgM22. When the binding of rHIgM22 to the methanolyzed organic phases, devoid of glycerophospholipids, was assessed through TLC immunostaining, a double band co-migrating with the sulfatide standard was detectable in all the samples analyzed (Fig. 6). The control IgM also shows a weak reactivity for the same antigen however the binding of control IgM is significantly lower than that of rHIgM22 in all the analyzed samples.

Methanolyzed organic phases (Or.Ph.Met) (amounts equivalent to 150 µg protein) obtained from wild type mouse brain, ASMKO mouse brain, rHIgM22-positive OPC and mouse myelin, as described in “Methods”, were separated on TLC plates using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system. After chromatographic separation, the plates were subjected to TLC immunostaining with 5.0 µg/mL of rHIgM22 or human IgM (B), as described in “Methods”.

Binding to glycerophospholipids

From a quantitative point of view, the most significant lipids present in the myelin membrane, comprising 65% of the total lipid dry weight, are cholesterol, GalCer and phosphatidylethanolamine (PE), the latter consisting in large part of plasmalogens [51]. This, coupled with the observation of a binding of both rHIgM22 and control IgM to glycerophospholipids in total lipid extracts, lead us to assess the binding of

the two antibodies to purified glycerophospholipids through TLC immunostaining. Under experimental conditions similar to those used to assess binding to sulfatide, rHIgM22 showed no significant binding to PC. Both rHIgM22 and control IgM bind PE, thus we regarded this binding as non-specific rHIgM22, on the other hand, showed a significant binding to phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositol (PI). In these cases, the control IgM gave no significant binding, suggesting that binding of rHIgM22 to these lipids might be specific (Fig. 7).

Binding of rHIgM22 to different phospholipids was assessed using TLC immunostaining. Different amounts (from 0.10 to 10.00 nmol) of purified phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylinositols (PI) were subjected to chromatography on TLC plates using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system. After chromatography, the TLC plates were subjected either to colorimetric detection with anisaldehyde reagent (A) or to TLC immunostaining with 5.0 μg/mL of rHIgM22 or Human IgM (B), as described in “Methods”.

Discussion

MS is characterized by the development of focal areas of demyelination on a background of inflammation. As of now there is no actual cure for MS, and the current FDA approved therapies mainly target the inflammatory component, in order to contain myelin damage. Some of these strategies however, for example the use of Fingolimod, are also able to increase the efficiency of the remyelination process [52–55]. In most mouse models, remyelination is a spontaneous process, occurring in response to demyelination, and leads to functional recovery. In MS in humans, however, this process is poorly efficient and its failure ultimately results in axonal dysfunction, degeneration, and loss of sensory and motor function [56]. For this reason, therapies that increase the chances of the regenerative outcome of demyelination have been getting more and more attention recently. The strategies that are currently being developed to increase efficiency of remyelination can be grouped as follows: 1) cell transplant, involving the transplantation of myelination-competent cells directly into lesion sites [57, 58]; 2) promotion of repair by the resident CNS stem- and precursor-cell populations, through the administration of growth, trophic, and neuroprotective factors [59]; 3) use of CNS reactive antibodies to induce remyelination [60]. Two of these remyelination promoting antibodies, BIIB033 and rHIgM22, are currently in clinical trial for MS treatment. The first is an anti-LINGO-1 IgG acting on LINGO-1, a protein known to inhibit remyelination via RhoA activation, and is currently being tested in a Phase II study in relapsing-remitting MS (ClinicalTrial.gov: NCT01864148). The second antibody, instead, is an IgM sharing several features with naturally occurring antibodies and is currently undergoing a phase I clinical trial aimed to evaluate safety and tolerability in relapsing MS patients (ClinicalTrial.gov: NCT02398461), after the first phase I clinical trial in MS patients was completed successfully (ClinicalTrial.gov: NCT01803867). This antibody, rHIgM22, first identified from a patient with Waldenström macroglobulinemia, has been shown to bind selectively to myelin and to the surface of oligodendrocytes *in vitro* [30, 31]. Moreover, there is evidence showing that this antibody is able to enter the central nervous system, accumulate in the demyelinated lesions, and promote remyelination in mouse models of chronical demyelination [31, 32]. The signaling

mechanisms through which this antibody exerts its function is still unclear, even if recent evidence suggests the involvement of a pathway involving Lyn and ERK cascade, which leads to inhibition of the apoptotic pathway and also to inhibition of OPCs differentiation and promotion of OPCs proliferation [35, 61]. Lyn activation seems to be subsequent to an rHIgM22 mediated reorganization of a signaling complex which includes Lyn itself, integrin αvβ3 and PDGFαR [34, 62]. Nevertheless, the actual binding target of rHIgM22 has yet to be identified. Evidence suggests that the antigen recognized by rHIgM22 could be associated with plasma membrane lipid rafts and that this target could be represented either by a sulfated glycolipid or by a multimolecular complex including a sulfated antigen. rHIgM22, in fact, binds the CNS tissues with a pattern similar to that of O4, an anti-sulfatide antibody (even if probably other molecules are as well recognized by O4 [63, 64]. Moreover, binding of rHIgM22 is abolished in CNS tissues from CST (-/-) mice, suggesting that the antigen recognized by rHIgM22 could be one or more CST-sulfated antigens present in myelin and on the surface of oligodendrocyte [33, 34]. On the other hand, it is known that the expression of several myelin proteins is deeply altered in CST (-/-) mice [65].

The analysis of the binding of rHIgM22 to different amounts of sulfatide, tested with different techniques (TLC immunostaining and surface plasmon resonance assays), revealed that rHIgM22 is indeed able to recognize sulfatide *in vitro*. Moreover, SPR experiments, where antigen presentation is closer to the one happening in a biological membrane respect to TLC immunostaining, showed that the binding of this remyelination promoting antibody to sulfatide is specific, suggesting that sulfatide could actually be one of the molecular targets of rHIgM22. Interestingly, rHIgM22 is also able to recognize lysosulfatide, the deacylated form of sulfatide, usually present in the normal CNS as a minor component, but whose levels can be increased in some pathological conditions, such as metachromatic leukodystrophy, a demyelinating disease [45, 46, 66]. Moreover, the binding of rHIgM22 to lysosulfatide is specific. In both cases, however, the affinity resulted quite low if compared to that of other known anti-glycolipid antibodies, such as the anti-lactosylceramide T5A7, for their own target [38]. Evidence reported in the literature, on the other hand, shows that, in model membranes, antibody recognition of sulfatide is affected by the membrane lipid microenvironment. This evidence suggests that the lipid environment might play a role in the determination of the surface topology of sulfatide. Distinct populations of anti-sulfatide antibodies show a different reactivity to sulfatide in a dipalmitoyl-PC/cholesterol environment or in a sphingomyelin/cholesterol environment. Moreover, length and hydroxylation of fatty acid chain of PC or of SM seem to restrict the recognition to higher affinity antibodies [43, 44]. Interestingly, our data obtained using SPR assays suggests that the binding of rHIgM22 to sulfatide might be affected by the composition of the lipid microenvironment. In particular, the presence of either GalCer or cholesterol lead to a reduction of the binding of rHIgM22 to sulfatide-containing monolayers, whereas SM has the opposite effect, suggesting that that the presence of different lipids, at a certain density, might be required to allow an optimal recognition of the antigen by rHIgM22. Sulfatide topology, distribution and dynamics in phospholipid bilayers, however, is also affected by the presence of proteins that are supposed to be physiologically relevant partners of sulfatide, such as myelin basic protein (MBP) [67, 68]. Furthermore, several studies highlighted a role of external factors, like the presence of soluble sulfatide binding proteins [69], pH [70, 71], and the presence of cations [72, 73] in the dynamics and distribution of

sulfatide in phospholipid bilayers. This suggests that the binding of rHIgM22 to sulfatide in oligodendrocytes and in myelin could be affected by a plethora of factors, and could actually be different than the one observed in the *in vitro* experiments.

The analysis regarding the binding of rHIgM22 was not limited to sulfatide and its deacylated form. Considering that glycerophospholipids in myelin represent approximately 43% of the total dry weight [49], and that we observed a diffuse binding of both rHIgM22 and control IgM to glycerophospholipids in total lipid extracts, the binding of rHIgM22 to several pure glycerophospholipids was assessed through TLC immunostaining. This set of experiments revealed no significant binding of rHIgM22 to PC, the most abundant phospholipid in any biological membrane, or to its deacylated form, lyso-PC, whereas a non-specific weak binding to PE, and a specific binding to PA, PS and PI was observed under experimental conditions similar to those used to assess binding to sulfatide. These lipids are usually enriched in the cytoplasmic leaflet of the plasma membrane, nevertheless the start point of several important biological processes causes a redistribution of PS from the inner to the outer, surface of the plasma membrane. For example, during the blood-clotting cascade, the transbilayer asymmetry of PS in the plasma membrane of activated platelets is markedly altered so that PS becomes exposed on the cell surface [74, 75]. During sperm maturation, the asymmetric distribution of PS in the plasma membrane changes and PS becomes exposed on the surface of the sperm [76]. The well-characterized process where there is a transbilayer movement of PS from the inner to the outer leaflet is during the early stages of apoptosis: the exposure of PS on the surface of apoptotic cells ("eat me" signal) has been identified as both an early event in apoptosis and a prerequisite for engulfment of these cells by phagocytic cells [77–80]. In addition, the exposure of PS on the surface of red blood cells serves as a signal for eryptosis [81]. Furthermore, lyso-PS (PS hydrolysis product) is exposed on the surface of activated and dying neutrophils thus initiating the clearance of these cells during acute inflammation [82]. PS has not only extracellular functions, but it has been demonstrated also its participation in many intracellular processes. For example, PS is the precursor of PE via the mitochondrial enzyme PS decarboxylase [83]. Although PS represents a minor phospholipid in mammalian cells, it is required for many fundamental cellular processes. The essential role of PS in mammalian cells was highlighted by the observation that mice in which PS synthesis was completely eliminated did not survive [84].

The binding of rHIgM22 to other myelin glycolipids, including GalCer, GlcCer, LacCer, SM, and several gangliosides, was also assessed, however the antibody did not show a significant binding to any of the aforementioned lipids.

Since it has been demonstrated that in mixed glial cell cultures rHIgM22 induces an increased production and release to the extracellular environment of sphingosine 1-phosphate [85], we also assessed the binding of rHIgM22 to this lipid. However, no significant binding was observed.

The binding of rHIgM22 was also observed in lipid mixtures obtained from a variety of relevant biological samples, including wild type, ASM (-/-), CST (+/-) and CST (-/-) mice brains, mouse mixed glial cells (MGC), mouse astrocytes, rat rHIgM22^{+} oligodendrocytes, rat microglia, and mouse myelin. The analysis

of these samples not only showed the presence of a double band co-migrating with the sulfatide standard, thus confirming the data obtained using pure sulfatide from a commercial source.

Summarizing, the data so far collected demonstrate that rHIgM22 binds to sulfatide and, to a lesser extent, to lysosulfatide *in vitro*, which is in agreement with the observation that rHIgM22 is able to bind to myelin and to oligodendrocytes, and that its binding is abolished in CNS tissue from CST (-/-) mice. Moreover, the binding affinity for both sulfatide and its deacylated derivate is low, even if the binding is specific. However, our data shows that the binding affinity of rHIgM22 for sulfatide can be modulated by the presence of other lipids suggesting a possible role of the membrane microenvironment in the recognition of the antigen by rHIgM22. In addition, rHIgM22 also reacts with phosphatidic acid, phosphatidylserine and phosphatidylinositol. This suggests that not only sulfatide, but also other membrane lipids might play a role in the binding of rHIgM22 to oligodendrocytes and other cell types. Indeed, this observation could explain why rHIgM22 is able to elicit biological responses in cell types (including astrocytes [86] and microglia [87]) Moreover, binding of rHIgM22 to intact cells might require a complex molecular arrangement and or a peculiar cell surface recognition pattern (especially considering the multivalent nature of IgMs). In cell types expressing significant sulfatide levels, sulfatide might be the key actor in the functional rHIgM22 antigen localized at the cell surface, thus explaining the strong surface labeling observed in oligodendrocytes. However, in cell types with low or absent sulfatide expression, other lipids might contribute in the assembly of a surface recognition patterns, still able to elicit cellular responses. Understanding whether rHIgM22 effect on remyelination involves a lipid-organized membrane complex, and the exact identity of the antigen involved and their organization in this complex is of great importance. The identification of the binding targets of this antibody, able to promote remyelination in validated mouse models of MS, and the characterization of their membrane microenvironment could significantly contribute to the reveal the signaling mechanisms underlying the biological activity of rHIgM22. This, in turn, would allow to obtain a better comprehension of the process of (re)myelination, and of the molecular mechanism involved in the pathophysiology of multiple sclerosis, thus allowing to define new potential therapeutic targets.

Abbreviations

ASM, acid sphingomyelinase; CNS, central nervous system; CST, cerebroside sulfotransferase; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; GalCer, galactosylceramide; GlcCer, glucosylceramide; GlcSph, glucosylsphingosyne; LacCer, lactosylceramide; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MGC, mixed glial cells; MOG, myelin oligodendrocyte protein; MS, multiple sclerosis; OPC, oligodendrocyte precursor cells; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLP, myelin proteolipid protein; PS, phosphatidylserine; rHIgM22, recombinant human IgM22; SM, sphingomyelin; SPR, surface plasmon resonance; TLC, thin layer chromatography

Declarations

Funding

rHIgM22 and funding for this work was provided by Acorda Therapeutics, Inc., under a Research Agreement between University of Milano and Acorda Therapeutics (A.P. as the Principal Investigator). The funders had no role in study design, data collection, analysis and interpretation, decision to publish, or preparation of the manuscript and in the decision to submit the article for publication.

Competing interests

AP is an Editor for Neurochemical Research.

Author Contributions

S.G. and L.C. wrote the main manuscript and prepared the figures. S.G., L.C. and S.P. performed the sample preparation, lipid extraction and TLC immunostaining experiments with the assistance of L.M. and M.G.C. . Y.Z. performed the OPC isolation. S.G. perfomed the SPR experiments with the assistance of N.Y. and K.I. . A.P. conceived and designed the experiments. All authors reviewed the manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

The Authors thank Professor Sandro Sonnino for his valuable and constructive suggestions during the planning and development of this research work.

References

1. Lassmann H, Bruck W, Lucchinetti CF (2007) The immunopathology of multiple sclerosis: an overview. Brain Pathol 17(2):210–218. doi:10.1111/j.1750-3639.2007.00064.x
2. Frohman EM, Racke MK, Raine CS (2006) Multiple sclerosis–the plaque and its pathogenesis. N Engl J Med 354(9):942–955. doi:10.1056/NEJMra052130
3. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG (2000) Multiple sclerosis. N Engl J Med 343(13):938–952. doi:10.1056/NEJM200009283431307
4. Weinshenker BG (1998) The natural history of multiple sclerosis: update 1998. Semin Neurol 18(3):301–307. doi:10.1055/s-2008-1040881
5. Pugliatti M, Sotgiu S, Rosati G (2002) The worldwide prevalence of multiple sclerosis. Clin Neurol Neurosurg 104(3):182–191
6. Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, Fontana A (1987) Antigen presentation and tumor cytotoxicity by interferon-gamma-treated microglial cells. Eur J Immunol 17(9):1271–1278.

doi:10.1002/eji.1830170909

7. Pang Y, Campbell L, Zheng B, Fan L, Cai Z, Rhodes P (2010) Lipopolysaccharide-activated microglia induce death of oligodendrocyte progenitor cells and impede their development. Neuroscience 166(2):464–475. doi:10.1016/j.neuroscience.2009.12.040
8. Nakahara J, Maeda M, Aiso S, Suzuki N (2012) Current concepts in multiple sclerosis: autoimmunity versus oligodendrogliopathy. Clin Rev Allergy Immunol 42(1):26–34. doi:10.1007/s12016-011-8287-6
9. Alter M, Zhen-xin Z, Davanipour Z, Sobel E, Min Lai S, LaRue L (1987) Does delay in acquiring childhood infection increase risk of multiple sclerosis? Italian J Neurol Sci Suppl 6:11–16
10. Rodriguez M (2007) Effectors of demyelination and remyelination in the CNS: implications for multiple sclerosis. Brain Pathol 17(2):219–229. doi:10.1111/j.1750-3639.2007.00065.x
11. Kotter MR, Li WW, Zhao C, Franklin RJ (2006) Myelin impairs CNS remyelination by inhibiting oligodendrocyte precursor cell differentiation. J neuroscience: official J Soc Neurosci 26(1):328–332. doi:10.1523/JNEUROSCI.2615-05.2006
12. Zamvil SS, Steinman L (1990) The T lymphocyte in experimental allergic encephalomyelitis. Annu Rev Immunol 8:579–621. doi:10.1146/annurev.iy.08.040190.003051
13. Steinman L (1993) Connections between the immune system and the nervous system. Proc Natl Acad Sci USA 90(17):7912–7914
14. Steinman L, Lindsey JW, Alters S, Hodgkinson S (1993) From treatment of experimental allergic encephalomyelitis to clinical trials in multiple sclerosis. Immunol Ser 59:253–260
15. Steinman L (1995) Multiple sclerosis. Presenting an odd autoantigen. Nature 375(6534):739–740. doi:10.1038/375739b0
16. Kerlero de Rosbo N, Milo R, Lees MB, Burger D, Bernard CC, Ben-Nun A (1993) Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein. J Clin Investig 92(6):2602–2608. doi:10.1172/JCI116875
17. Jaskiewicz E (2004) [Epitopes on myelin proteins recognized by autoantibodies present in multiple sclerosis patients]. Postepy Hig Med Dosw (Online) 58:472–482
18. Meinl E, Hohlfeld R (2002) Immunopathogenesis of multiple sclerosis: MBP and beyond. Clin Exp Immunol 128(3):395–397
19. Sela BA, Konat G, Offner H (1982) Elevated ganglioside concentration in serum and peripheral blood lymphocytes from multiple sclerosis patients in remission. J Neurol Sci 54(1):143–148
20. Lubetzki C, Thuillier Y, Galli A, Lyon-Caen O, Lhermitte F, Zalc B (1989) Galactosylceramide: a reliable serum index of demyelination in multiple sclerosis. Ann Neurol 26(3):407–409. doi:10.1002/ana.410260319
21. Bansal AS, Abdul-Karim B, Malik RA, Goulding P, Pumphrey RS, Boulton AJ, Holt PL, Wilson PB (1994) IgM ganglioside GM1 antibodies in patients with autoimmune disease or neuropathy, and controls. J Clin Pathol 47(4):300–302

22. Endo T, Scott DD, Stewart SS, Kundu SK, Marcus DM (1984) Antibodies to glycosphingolipids in patients with multiple sclerosis and SLE. Adv Exp Med Biol 174:455–461
23. Ilyas AA, Chen ZW, Cook SD (2003) Antibodies to sulfatide in cerebrospinal fluid of patients with multiple sclerosis. J Neuroimmunol 139(1–2):76–80
24. Kanter JL, Narayana S, Ho PP, Catz I, Warren KG, Sobel RA, Steinman L, Robinson WH (2006) Lipid microarrays identify key mediators of autoimmune brain inflammation. Nat Med 12(1):138–143. doi:10.1038/nm1344
25. Quintana FJ, Farez MF, Viglietta V, Iglesias AH, Merbl Y, Izquierdo G, Lucas M, Basso AS, Khoury SJ, Lucchinetti CF, Cohen IR, Weiner HL (2008) Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis. Proc Natl Acad Sci USA 105(48):18889–18894. doi:10.1073/pnas.0806310105
26. Moyano AL, Pituch K, Li G, van Breemen R, Mansson JE, Givogri MI (2013) Levels of plasma sulfatides C18: 0 and C24 : 1 correlate with disease status in relapsing-remitting multiple sclerosis. J Neurochem 127(5):600–604. doi:10.1111/jnc.12341
27. Haghighi S, Lekman A, Nilsson S, Blomqvist M, Andersen O (2012) Myelin glycosphingolipid immunoreactivity and CSF levels in multiple sclerosis. Acta Neurol Scand 125(1):64–70. doi:10.1111/j.1600-0404.2011.01554.x
28. Haghighi S, Lekman A, Nilsson S, Blomqvist M, Andersen O (2013) Increased CSF sulfatide levels and serum glycosphingolipid antibody levels in healthy siblings of multiple sclerosis patients. J Neurol Sci 326(1–2):35–39. doi:10.1016/j.jns.2013.01.007
29. Ozgen H, Schrimpf W, Hendrix J, de Jonge JC, Lamb DC, Hoekstra D, Kahya N, Baron W (2014) The lateral membrane organization and dynamics of myelin proteins PLP and MBP are dictated by distinct galactolipids and the extracellular matrix. PLoS ONE 9(7):e101834. doi:10.1371/journal.pone.0101834
30. Rodriguez M, Warrington AE, Pease LR (2009) Invited article: human natural autoantibodies in the treatment of neurologic disease. Neurology 72(14):1269–1276. doi:10.1212/01.wnl.0000345662.05861.e4
31. Warrington AE, Asakura K, Bieber AJ, Ciric B, Van Keulen V, Kaveri SV, Kyle RA, Pease LR, Rodriguez M (2000) Human monoclonal antibodies reactive to oligodendrocytes promote remyelination in a model of multiple sclerosis. Proc Natl Acad Sci U S A 97(12):6820–6825. doi:10.1073/pnas.97.12.6820
32. Pirko I, Ciric B, Gamez J, Bieber AJ, Warrington AE, Johnson AJ, Hanson DP, Pease LR, Macura SI, Rodriguez M (2004) A human antibody that promotes remyelination enters the CNS and decreases lesion load as detected by T2-weighted spinal cord MRI in a virus-induced murine model of MS. FASEB J 18(13):1577–1579. doi:10.1096/fj.04-2026fje
33. Howe CL, Bieber AJ, Warrington AE, Pease LR, Rodriguez M (2004) Antiapoptotic signaling by a remyelination-promoting human antimyelin antibody. Neurobiol Dis 15(1):120–131

34. Wright BR, Warrington AE, Edberg DD, Rodriguez M (2009) Cellular mechanisms of central nervous system repair by natural autoreactive monoclonal antibodies. Arch Neurol 66(12):1456–1459. doi:10.1001/archneurol.2009.262
35. Watzlawik JO, Warrington AE, Rodriguez M (2013) PDGF is required for remyelination-promoting IgM stimulation of oligodendrocyte progenitor cell proliferation. PLoS ONE 8(2):e55149. doi:10.1371/journal.pone.0055149
36. Norton WT, Poduslo SE (1973) Myelination in rat brain: method of myelin isolation. J Neurochem 21(4):749–757. doi:10.1111/j.1471-4159.1973.tb07519.x
37. Scandroglio F, Loberto N, Valsecchi M, Chigorno V, Prinetti A, Sonnino S (2009) Thin layer chromatography of gangliosides. Glycoconj J 26(8):961–973. doi:10.1007/s10719-008-9145-5
38. Iwabuchi K, Masuda H, Kaga N, Nakayama H, Matsumoto R, Iwahara C, Yoshizaki F, Tamaki Y, Kobayashi T, Hayakawa T, Ishii K, Yanagida M, Ogawa H, Takamori K (2015) Properties and functions of lactosylceramide from mouse neutrophils. Glycobiology 25(6):655–668. doi:10.1093/glycob/cwv008
39. Jackman N, Ishii A, Bansal R (2009) Oligodendrocyte development and myelin biogenesis: parsing out the roles of glycosphingolipids. Physiol (Bethesda) 24:290–297. doi:10.1152/physiol.00016.2009
40. Taylor CM, Marta CB, Claycomb RJ, Han DK, Rasband MN, Coetzee T, Pfeiffer SE (2004) Proteomic mapping provides powerful insights into functional myelin biology. Proc Natl Acad Sci USA 101(13):4643–4648. doi:10.1073/pnas.0400922101
41. Eckhardt M (2008) The role and metabolism of sulfatide in the nervous system. Mol Neurobiol 37(2–3):93–103. doi:10.1007/s12035-008-8022-3
42. Stoffel W, Bosio A (1997) Myelin glycolipids and their functions. Curr Opin Neurobiol 7(5):654–661
43. Bjorkqvist YJ, Nybond S, Nyholm TK, Slotte JP, Ramstedt B (2008) N-palmitoyl-sulfatide participates in lateral domain formation in complex lipid bilayers. Biochim Biophys Acta 1778(4):954–962. doi:10.1016/j.bbamem.2007.12.016
44. Stewart RJ, Boggs JM (1990) Dependence of the surface expression of the glycolipid cerebroside sulfate on its lipid environment: comparison of sphingomyelin and phosphatidylcholine. Biochemistry 29(15):3644–3653. doi:10.1021/bi00467a009
45. Blomqvist M, Gieselmann V, Mansson JE (2011) Accumulation of lysosulfatide in the brain of arylsulfatase A-deficient mice. Lipids Health Dis 10:28. doi:10.1186/1476-511X-10-28
46. Toda K, Kobayashi T, Goto I, Ohno K, Eto Y, Inui K, Okada S (1990) Lysosulfatide (sulfogalactosylsphingosine) accumulation in tissues from patients with metachromatic leukodystrophy. J Neurochem 55(5):1585–1591. doi:10.1111/j.1471-4159.1990.tb04942.x
47. Chrast R, Saher G, Nave KA, Verheijen MH (2011) Lipid metabolism in myelinating glial cells: lessons from human inherited disorders and mouse models. J Lipid Res 52(3):419–434. doi:10.1194/jlr.R009761

48. Grassi S, Prioni S, Cabitta L, Aureli M, Sonnino S, Prinetti A (2016) The Role of 3-O-Sulfogalactosylceramide, Sulfatide, in the Lateral Organization of Myelin Membrane. Neurochem Res 41(1–2):130–143. doi:10.1007/s11064-015-1747-2
49. O'Brien JS, Sampson EL (1965) Lipid composition of the normal human brain: gray matter, white matter, and myelin. J Lipid Res 6(4):537–544
50. Nair S, Branagan AR, Liu J, Boddupalli CS, Mistry PK, Dhodapkar MV (2016) Clonal Immunoglobulin against Lysolipids in the Origin of Myeloma. N Engl J Med 374(6):555–561. doi:10.1056/NEJMoa1508808
51. O'Brien JS, Sampson EL (1965) Myelin Membrane: a molecular abnormality. Science 150(3703):1613–1614
52. Healy LM, Michell-Robinson MA, Antel JP (2015) Regulation of human glia by multiple sclerosis disease modifying therapies. Semin Immunopathol 37(6):639–649. doi:10.1007/s00281-015-0514-4
53. Choi JW, Gardell SE, Herr DR, Rivera R, Lee CW, Noguchi K, Teo ST, Yung YC, Lu M, Kennedy G, Chun J (2011) FTY720 (fingolimod) efficacy in an animal model of multiple sclerosis requires astrocyte sphingosine 1-phosphate receptor 1 (S1P1) modulation. Proc Natl Acad Sci USA 108(2):751–756. doi:10.1073/pnas.1014154108
54. Miron VE, Jung CG, Kim HJ, Kennedy TE, Soliven B, Antel JP (2008) FTY720 modulates human oligodendrocyte progenitor process extension and survival. Ann Neurol 63(1):61–71. doi:10.1002/ana.21227
55. Miron VE, Ludwin SK, Darlington PJ, Jarjour AA, Soliven B, Kennedy TE, Antel JP (2010) Fingolimod (FTY720) enhances remyelination following demyelination of organotypic cerebellar slices. Am J Pathol 176(6):2682–2694. doi:10.2353/ajpath.2010.091234
56. Alizadeh A, Dyck SM, Karimi-Abdolrezaee S (2015) Myelin damage and repair in pathologic CNS: challenges and prospects. Front Mol Neurosci 8:35. doi:10.3389/fnmol.2015.00035
57. Duncan ID, Aguayo AJ, Bunge RP, Wood PM (1981) Transplantation of rat Schwann cells grown in tissue culture into the mouse spinal cord. J Neurol Sci 49(2):241–252
58. Blakemore WF, Crang AJ (1985) The use of cultured autologous Schwann cells to remyelinate areas of persistent demyelination in the central nervous system. J Neurol Sci 70(2):207–223
59. Ben-Hur T, Goldman SA (2008) Prospects of cell therapy for disorders of myelin. Ann N Y Acad Sci 1142:218–249. doi:10.1196/annals.1444.014
60. Warrington AE, Bieber AJ, Ciric B, Van Keulen V, Pease LR, Mitsunaga Y, Paz Soldan MM, Rodriguez M (2001) Immunoglobulin-mediated CNS repair. J Allergy Clin Immunol 108(4 Suppl):S121–125
61. Watzlawik JO, Wootla B, Painter MM, Warrington AE, Rodriguez M (2013) Cellular targets and mechanistic strategies of remyelination-promoting IgMs as part of the naturally occurring autoantibody repertoire. Expert Rev Neurother 13(9):1017–1029. doi:10.1586/14737175.2013.835601
62. Watzlawik J, Holicky E, Edberg DD, Marks DL, Warrington AE, Wright BR, Pagano RE, Rodriguez M (2010) Human remyelination promoting antibody inhibits apoptotic signaling and differentiation

through Lyn kinase in primary rat oligodendrocytes. Glia 58(15):1782–1793. doi:10.1002/glia.21048

63. Bansal R, Stefansson K, Pfeiffer SE (1992) Proligodendroblast antigen (POA), a developmental antigen expressed by A007/O4-positive oligodendrocyte progenitors prior to the appearance of sulfatide and galactocerebroside. J Neurochem 58(6):2221–2229. doi:10.1111/j.1471-4159.1992.tb10967.x
64. Bansal R, Warrington AE, Gard AL, Ranscht B, Pfeiffer SE (1989) Multiple and novel specificities of monoclonal antibodies O1, O4, and R-mAb used in the analysis of oligodendrocyte development. J Neurosci Res 24(4):548–557. doi:10.1002/jnr.490240413
65. Fewou SN, Fernandes A, Stockdale K, Francone VP, Dupree JL, Rosenbluth J, Pfeiffer SE, Bansal R (2010) Myelin protein composition is altered in mice lacking either sulfated or both sulfated and non-sulfated galactolipids. J Neurochem 112(3):599–610. doi:10.1111/j.1471-4159.2009.06464.x
66. Rosengren B, Fredman P, Mansson JE, Svennerholm L (1989) Lysosulfatide (galactosylsphingosine-3-O-sulfate) from metachromatic leukodystrophy and normal human brain. J Neurochem 52(4):1035–1041
67. Maggio B (1997) Molecular interactions of the major myelin glycosphingolipids and myelin basic protein in model membranes. Neurochem Res 22(4):475–481
68. Maggio B, Sturtevant JM, Yu RK (1987) Effect of myelin basic protein on the thermotropic behavior of aqueous dispersions of neutral and anionic glycosphingolipids and their mixtures with dipalmitoylphosphatidylcholine. J Biol Chem 262(6):2652–2659
69. Viani P, Cervato G, Gatti P, Cestaro B (1992) Calcitonin-induced changes in the organization of sulfatide-containing membranes. Biochim Biophys Acta 1106(1):77–84
70. Wu X, Li QT (1999) Hydration and stability of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles. Biochim Biophys Acta 1416(1–2):285–294
71. Wu X, Lee KH, Li QT (1996) Stability and pH sensitivity of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles. Biochim Biophys Acta 1284(1):13–19
72. Wu X, Li QT (1999) Ca2+-induced fusion of sulfatide-containing phosphatidylethanolamine small unilamellar vesicles. J Lipid Res 40(7):1254–1262
73. Rintoul DA, Welti R (1989) Thermotropic behavior of mixtures of glycosphingolipids and phosphatidylcholine: effect of monovalent cations on sulfatide and galactosylceramide. Biochemistry 28(1):26–31
74. Zachowski A (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J 294(Pt 1):1–14. doi:10.1042/bj2940001
75. Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF (1982) Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. Eur J Biochem 122(2):429–436. doi:10.1111/j.1432-1033.1982.tb05898.x
76. Gadella BM, Harrison RA (2000) The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. Development 127(11):2407–2420

77. Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 148(7):2207–2216
78. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL (2001) Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. J Biol Chem 276(2):1071–1077. doi:10.1074/jbc.M003649200
79. Park D, Hochreiter-Hufford A, Ravichandran KS (2009) The phosphatidylserine receptor TIM-4 does not mediate direct signaling. Curr Biol 19(4):346–351. doi:10.1016/j.cub.2009.01.042
80. Li W (2012) Eat-me signals: keys to molecular phagocyte biology and "appetite" control. J Cell Physiol 227(4):1291–1297. doi:10.1002/jcp.22815
81. Zelenak C, Pasham V, Jilani K, Tripodi PM, Rosaclerio L, Pathare G, Lupescu A, Faggio C, Qadri SM, Lang F (2012) Tanshinone IIA stimulates erythrocyte phosphatidylserine exposure. Cell Physiol Biochem 30(1):282–294. doi:10.1159/000339064
82. Frasch SC, Fernandez-Boyanapalli RF, Berry KZ, Leslie CC, Bonventre JV, Murphy RC, Henson PM, Bratton DL (2011) Signaling via macrophage G2A enhances efferocytosis of dying neutrophils by augmentation of Rac activity. J Biol Chem 286(14):12108–12122. doi:10.1074/jbc.M110.181800
83. Vance JE, Tasseva G (2013) Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. Biochim Biophys Acta 1831(3):543–554. doi:10.1016/j.bbalip.2012.08.016
84. Arikketh D, Nelson R, Vance JE (2008) Defining the importance of phosphatidylserine synthase-1 (PSS1): unexpected viability of PSS1-deficient mice. J Biol Chem 283(19):12888–12897. doi:10.1074/jbc.M800714200
85. Grassi S, Giussani P, Prioni S, Button D, Cao J, Hakimi I, Sarmiere P, Srinivas M, Cabitta L, Sonnino S, Prinetti A (2019) Human Remyelination Promoting Antibody Stimulates Astrocytes Proliferation Through Modulation of the Sphingolipid Rheostat in Primary Rat Mixed Glial Cultures. Neurochem Res 44(6):1460–1474. doi:10.1007/s11064-018-2701-x
86. Paz Soldan MM, Warrington AE, Bieber AJ, Ciric B, Van Keulen V, Pease LR, Rodriguez M (2003) Remyelination-promoting antibodies activate distinct Ca2 + influx pathways in astrocytes and oligodendrocytes: relationship to the mechanism of myelin repair. Mol Cell Neurosci 22(1):14–24. doi:10.1016/s1044-7431(02)00018-0
87. Zorina Y, Stricker J, Caggiano AO, Button DC (2018) Human IgM antibody rHIgM22 promotes phagocytic clearance of myelin debris by microglia. Sci Rep 8(1):9392. doi:10.1038/s41598-018-27559-yStatements & Declarations

Figures

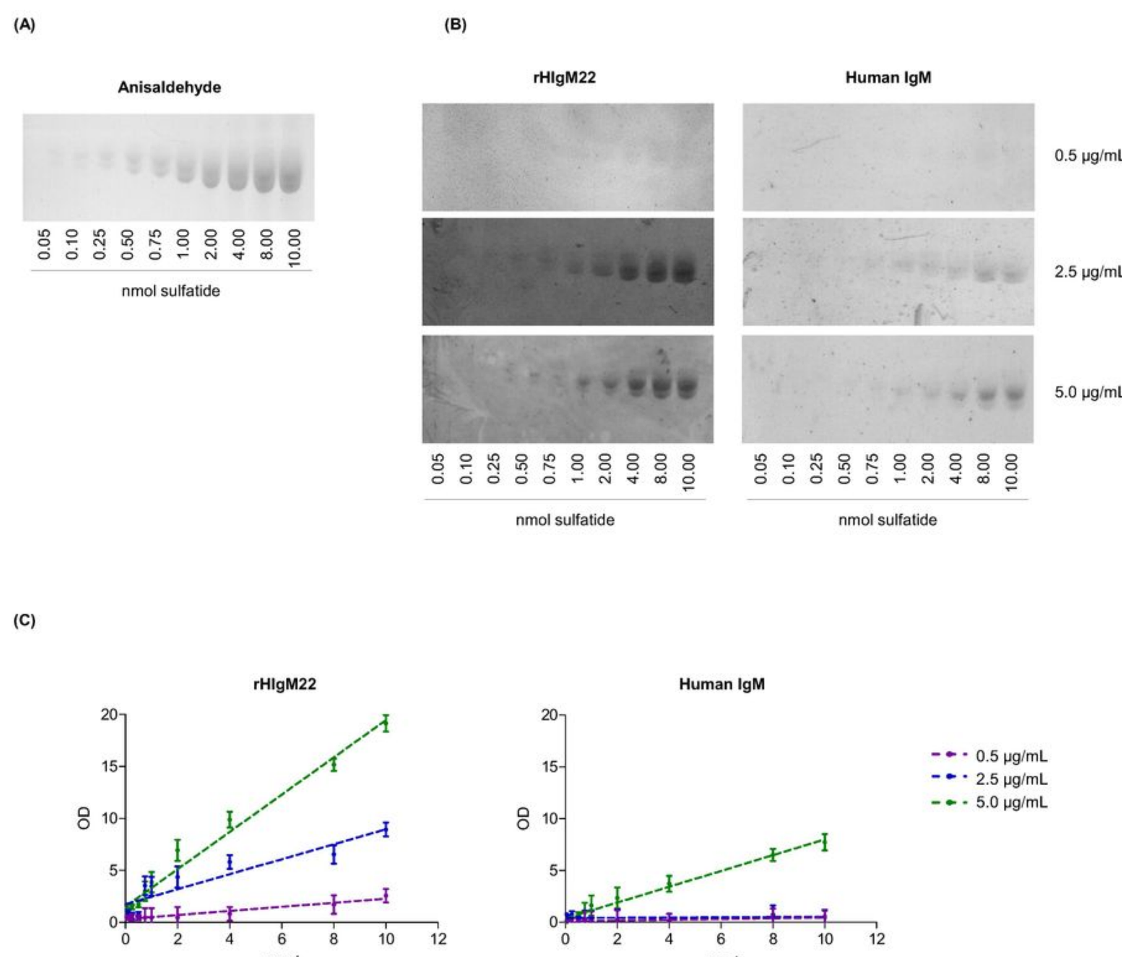


Figure 1

Binding of rHIgM22 to sulfatide on thin layer chromatographic support.

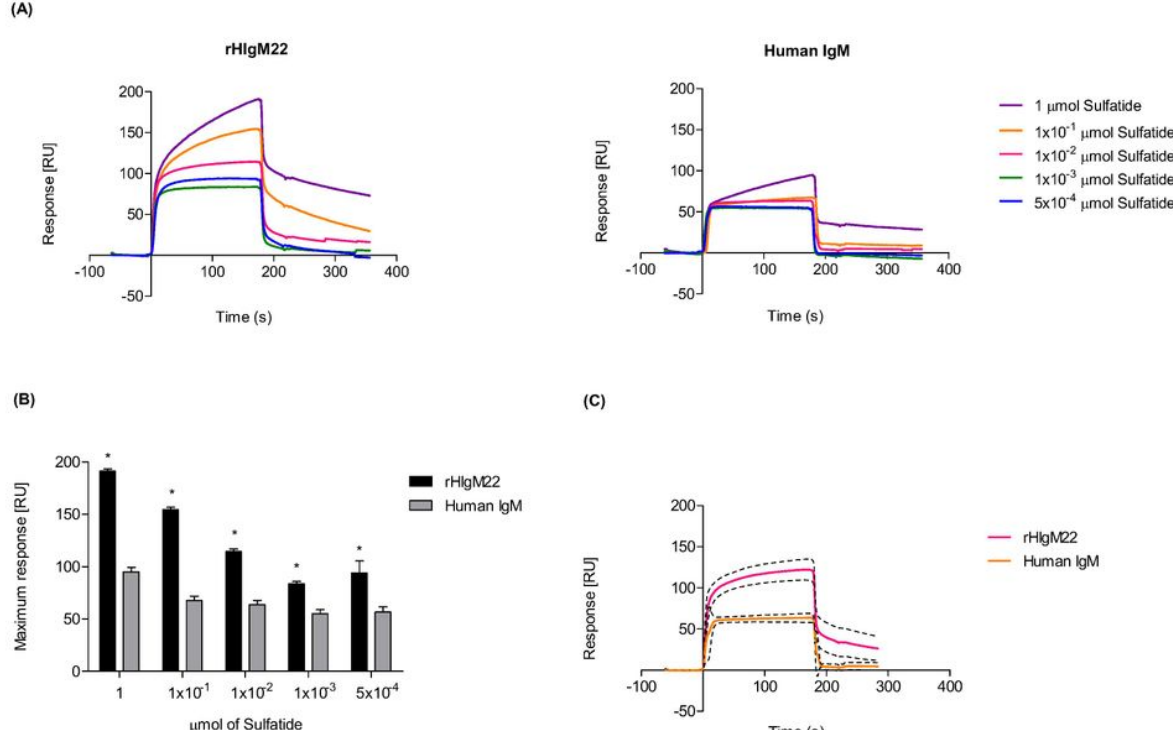


Figure 2

Binding of rHIgM22 to sulfatide in DOPC monolayers.

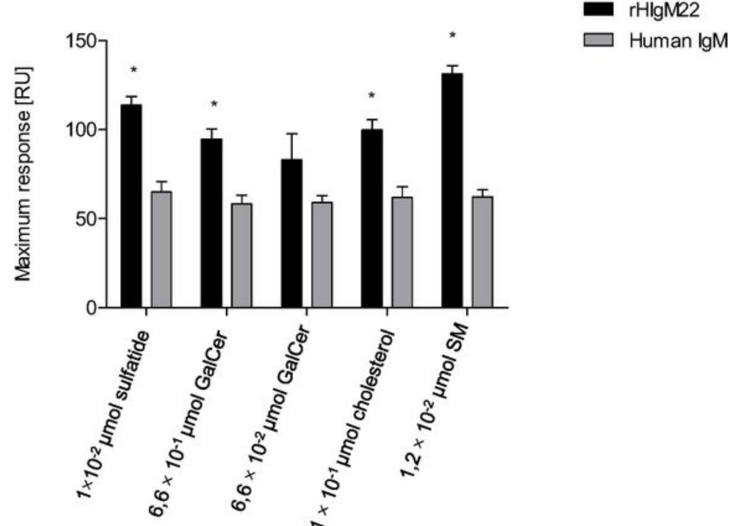


Figure 3

Effect of GalCer, cholesterol and SM on rHIgM22 binding to sulfatide.

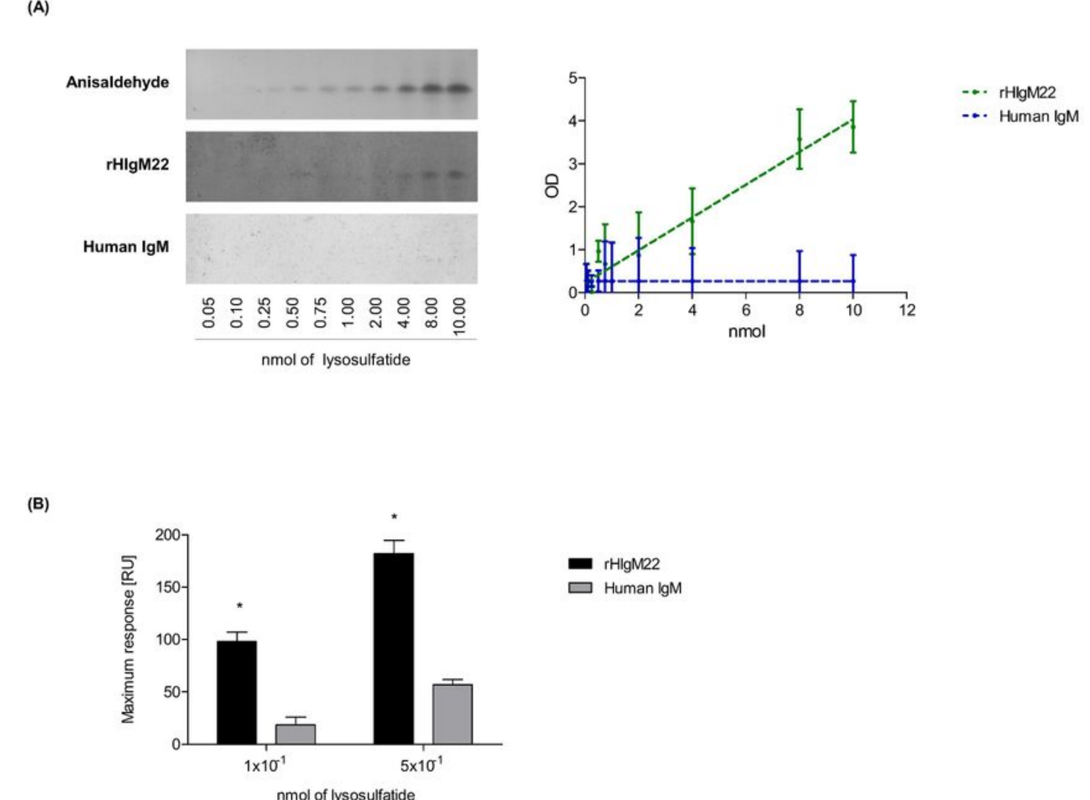
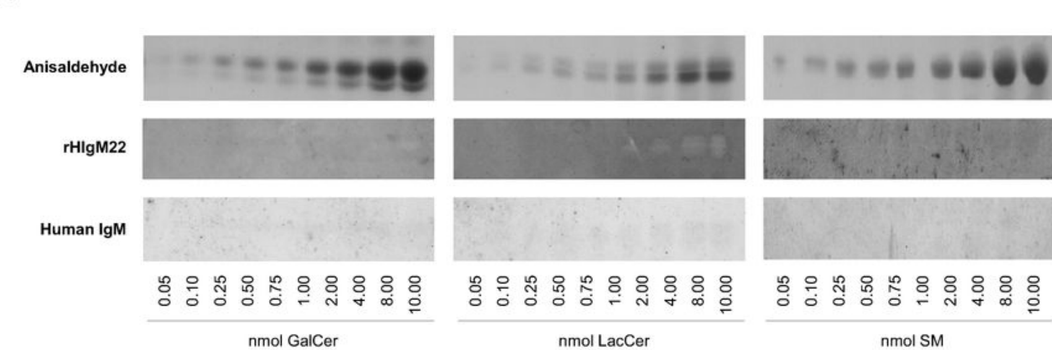


Figure 4

Binding of rHIgM22 to lysosulfatide after chromatographic separation and in DOPC monolayers.



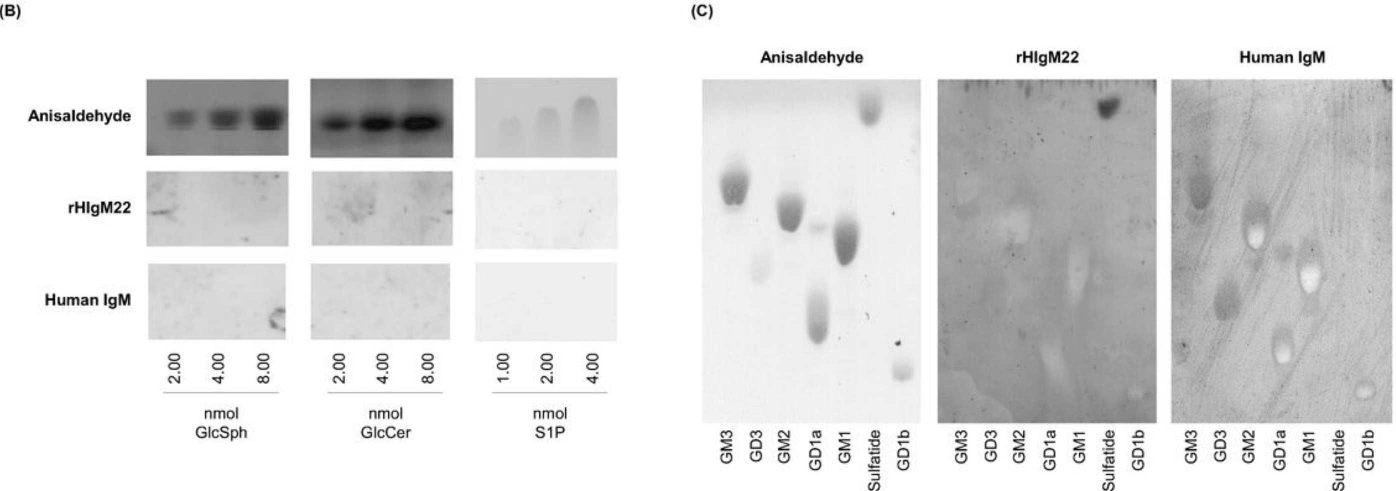


Figure 5

Binding of rHIgM22 to neutral glycolipids, S1P and gangliosides.

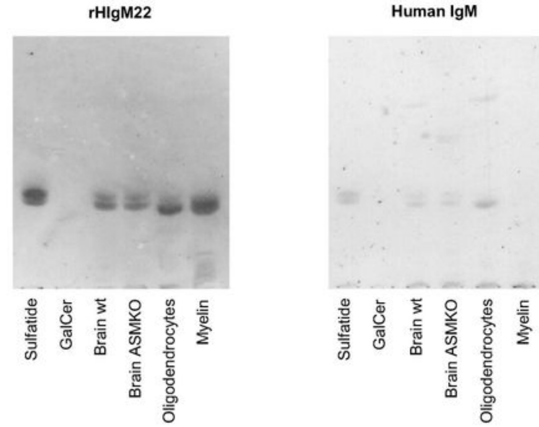


Figure 6

Biding of rHIgM22 to methanolyzed organic phases after chromatographic separation.

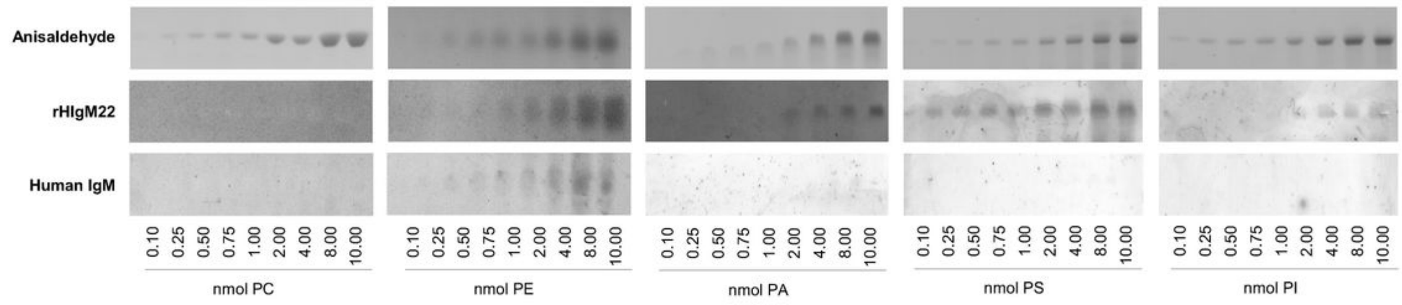


Figure 7

Binding of rHIgM22 to purified phospholipids.