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IDENTIFICATION OF THE ANTIGEN RECOGNIZED BY rHIgM22, A REMYELINATION-PROMOTING HUMAN MONOCLONAL ANTIBODY

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SOMMARIO

ABSTRACT

All eukaryotic cells are surrounded by a cellular membrane that functions as a barrier between subcellular compartments and between the cell and its environment. In addition to proteins, they are composed by three different set of lipids: glycerolipids, sphingolipids and sterols. Glycerolipids are the major components of cell membranes and they are produced using phosphatidic acid (PtdOH) as a central precursor. Instead, sphingolipids (SLs), the minor cell components, have sphingosine as basic building block; the additions of oligosaccharides to sphingosine giving rise glycosphingolipids (GSLs). SLs and GSLs are not distribute homogeneously in the outer plasma membrane. They form small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains, called "lipid rafts"; in these semiordered lipid microdomains, SLs are involved in cell adhesion/recognition processes and signal transduction pathway. The organ with the highest enrichment in lipids such as cholesterol e glycosphingolipids, is the brain. Myelin, the fatty white substance that surrounds the axon of nerve cells, is characterized by a high lipid-to-protein ratio, where lipids representing 80% of its dry weight. The myelin membrane contains a high level of two galactosphingolipids, galactosylceramide (GalCer) and 3-*O*-sulfogalactosylceramide (sulfatide), account for about 20 and 5 % of myelin lipids respectively. The specific roles of GalCer and sulfatide seem to be linked to their ability to form and to stabilize specific lateral domains in the membrane of myelin forming-cells and in the myelin sheath, that regulate the correct sorting, trafficking, co-clustering and lateral distribution of the major myelin proteins. Recent reports have suggested that some of the myelin-specific lipids may be key contributors to the pathogenic mechanism of multiple sclerosis (MS), the most common demyelinating disease in the CNS. Individuals with MS disease are reported to have different myelin lipid compositions and elevated levels of anti-sulfatide Ab in biological fluids compared with healthy individuals. On the other hand, anti-myelin antibodies might represent an important immunological tool for the treatment of neurological diseases involving myelin lesions. In particular, it has been shown that human monoclonal antibodies that bind myelin and oligodendrocytes (OLs), as rHIgM22, can initiate dramatic increase in remyelination in animal models of demyelination. Nowadays, the exact mechanism of action of rHIgM22 remains to be elucidated, but some evidence suggest that the mechanism is correlated with the organization of lipid rafts on the surface of myelin and OLs.

The experiments described in this thesis were aimed at the individuation of the molecular target(s) of the antibody and at the characterization of its membrane microenvironment, in order to better understand the characteristics of rHIgM22 and its remyelinating activity.

The binding of rHIgM22 to purified lipids and to lipid extracts from various sources were tested using TLC immunostaining assays and SPR assays. The results obtained show that rHIgM22 binds to sulfatide, and, to a lesser extent, to lysosulfatide in vitro, while it does not bind to other myelin sphingolipids. The binding affinity for both sulfatide and its deacylated derivate is low, even if the binding is specific. On the other hand, 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, phosphatidylinositol (PI) and phosphatidylserine (PS).

To verify whether rHIgM22 can bind sulfatide or other lipids, the binding of rHIgM22 was tested not only to purified lipids, but also to partially purified lipid extracts obtained from wild type, ASM (-/-), CST (+/-) and CST (-/-) mice brains, mouse mixed glial cells (MGC), mouse astrocytes, rat rHIgM22+ OLs, rat microglia, and mouse myelin. In TLC immunostaining experiments for aqueous phases, we observed rHIgM22-immunoreactive bands co-migrating with the pure sulfatide standard and, unexpectedly, a second rHIgM22-immunoreactive band migrating below sulfated, co-migrating with the pure phosphatidylinositol and phosphatidylserine standard, confirming the TLC immunostraining data to purified lipids. The identity of phospholipid species has been confirmed by ESI mass spectrometry experiments; they show that phosphatidylinositol was an 18:0/20:4-PI and phosphatidylserine was 18:0/22:6-PS and 18:0/18:1-PS. In addition, MS analysis for the fractions enriched in sulfatide, shows that rHIgM22 can bind different sulfated species suggesting that this binding is not fatty acid species-specific for the sulfatide.

All these data suggest that not only sulfatide, but also other membrane lipids might play a role in the binding of rHIgM22 to OLs or to other cell types. Moreover, the antigen recognized by rHIgM22 could be associated with plasma membrane lipid rafts in these cells and this target could be including in a multimolecular complex. The identification of the binding target(s) of a rHIgM22, and the characterization of their membrane microenvironment, could greatly contribute to the elucidation of the signaling mechanisms underlying the remyelination promoting activity of this antibody and also of those involved in MS etiology, allowing to define new potential therapeutic strategies.

ABBREVIATIONS

Aq. Ph.	Aqueous phase
CGT	UDP-galactose:ceramide galactosyltransferase
CNS	Central nervous system
CST	3'-phosphoadenosine-5'-phosphosulfate:cerebroside sulfotransferase
EAE	Experimental autoimmune encephalomyelitis
ESI-MS	Electrospray ionisation mass spectrometry
GalCer	Galactosylceramide
GlcCer	Glucosylceramide
GSLs	Glycosphingolipids
LacCer	Lactosylceramide
MS	Mass Spectrometry
MS	Multiple sclerosis
OLs	Mature oligodendrocytes
OPCs	Oligodendrocyte progenitor cells
Or. Ph.	Organic phase
PDGFαR	Platelet-derived growth factor receptor alpha
PtdCho - PC	Phosphatidylcholine
PtdEtn - PE	Phosphatidylethanolamine
PtdIns - PI	Phosphatidylinositol
PtdOH - PA	Phosphatidic acid
PtdSer - PS	Phosphatidylserine
rHIgM22	Recombinant human Immunoglobulin M22
S1P	Sphingosine-1-phosphate
SK1	Sphingosine kinase 1
SK2	Sphingosine kinase 2
SLs	Sphingolipids
SM	Sphingomyelin
SPR	Surface plasmon resonance
Sulfatide	3-O-sulfogalactosylceramide
TLC	Thin layer chromatography
TMEV	Theiler's murine encephalomyelitis virus

INTRODUCTION

All eukaryotic cells are surrounded by a cellular membrane that functions as a barrier between subcellular compartments and between the cell and its environment [1]. The basic structure of all biological membranes is biomolecular leaf arrangement of lipids, in which the amphoteric lipids and cholesterol are oriented so that the hydrophobic portions of the molecules interact, minimizing their contact with water or other polar group, and the polar head groups of the lipids are at the interface with the aqueous environment. In 1972 Singer and Nicholson introduced the "fluid mosaic model" to explain the organization and structure of proteins and lipids that constitute the biological membranes. In this model, proteins are actually immersed in a lipid fluid bilayer while others are attached either to the inner or the outer surface of the membrane [2]. Cellular membranes are formed from a different set of lipids, present in various amounts and proportions. Glycerolipids, sphingolipids and sterols are the major classes of lipids that constituted biological membrane and there is an asymmetric distribution of these lipid components across them [3].

Glycerolipids

Glycerolipids are a classes of lipids that have as basic building block the 1,2-diacylglycerol 3-phosphate (PtdOH, PA or phosphatidic acid), which has a fatty acyl or alkyl group at the sn-1, fatty acyl groups at the sn-2 positions and a characteristic phosphate ester group at the sn-3 position of glycerol [4]. Fatty acids in sn-1 are usually saturated, whereas in sn-2 are unsaturated. The glycerophospholipid classes are defined based on the substituent at the phosphate ester group *at sn*-3 position of PtdOH: choline (PtdCho or PC), ethanolamine (PtdEtn or PE), serine (PtdSer or PS) and phosphatidylinositol (PtdIns or PI). The membranes contain also the plasmalogens (PlsCho and PlsEtn), glycerophospholipids containing vinyl-ether linkages and enriched in polyunsaturated fatty acids at the *sn*-2 position [5].

Glycerophospholipids are amphipathic molecules characterized by a polar (head group) and nonpolar ends (tail). The polar end is charged due to the ionization of the phosphate group and nitrogenous base, instead the nonpolar tail (fatty acid acyl chains) are hydrophobic and tend to aggregate in an aqueous environment (Fig. 1). Physicochemical characteristics of membranes depending by variations in the head group, length of the phospholipid acyl chains and the degree of saturation of them. In fact, there is diversity in the fatty-acyl species because of differences in chain length and degree of unsaturation of the esterified fatty acid and in the distribution of them in the asymmetric bilayer.

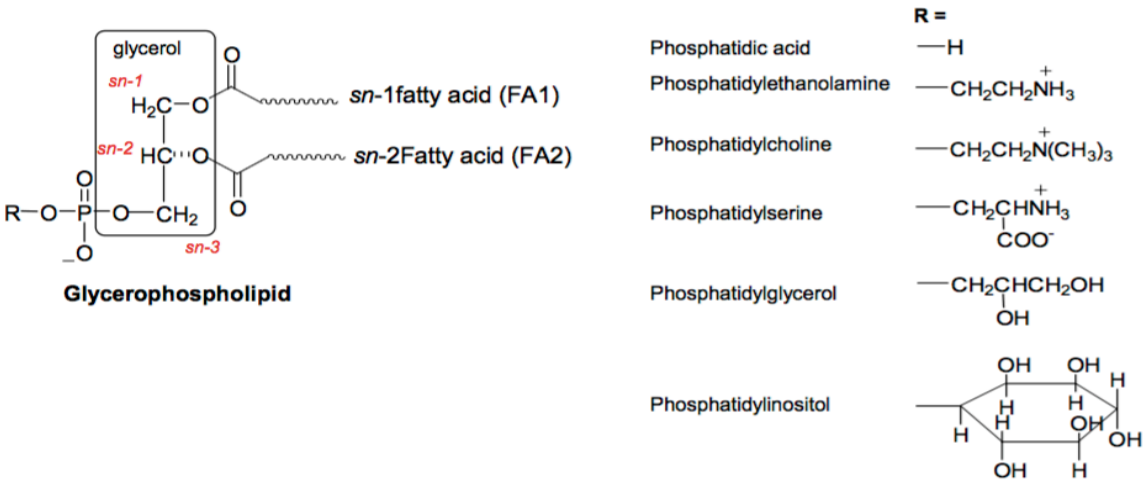


Figure 1. Common glycerophospholipids structure

Glycerolipids metabolism

PtdOH is the precursor of all glycerophospholipids. It consists of glycerol-3-phosphate with long-chain fatty acids esterified at the *sn* -1 and *sn* -2 positions and the glycerophospholipid classes are defined based on the substituent at the *sn*-3 position. Apart from phosphotidylinositols, phospholipids are synthesised on the cytoplasmic leaflet of the endoplasmatic reticulum and to the lesser extent in the Golgi complex and inside mitochondria.

PtdCho and PtdEtn are synthesized mainly via Kennedy cycle [5]. This pathway involves three enzymic steps: (1) choline or ethanolamine kinases, localized in cytosol; (2) choline or ethanolamine phosphate cytidylyltransferases (PCCT and PECT), distributed between cytosol and membrane; (3) CDP-choline or CDP-ethanolamine 1,2-diacylglycerol choline or ethanolamine phosphotransferase, integral membrane proteins predominantly present in endoplasmic reticulurn [6]. The first committed step of PtdCho and PtdEtn synthesis is base phosphorylation by choline or ethanolamine kinase to form phosphocoline and phosphoethanolamine. This is followed by the incorporation of CTP (cytidine triphosphate) to produce CDP-choline or CDP- ethanolamine by PCCT and PECT, respectively. In final step, choline and ethanolamine phosphotransferases catalyze the transfer of phosphocholine or phosphoethanolamine to 1,2-diacyIglycerol from CDP-choline or CDP-effimolamine, with the release of CMP. PtdCho is also synthesized by the repeated methylation of PtdEtn with *S*-adenosylmethionine, reaction catalyzed by PtdEtn *N*-methyltransferase in endoplasmic reticulum and mitochondria [5].

PtdSer is synthesized by base-exchange between PtdEtn or PtdCho with serine, where the phosphodiester bond of PtdEtn or PtdCho is attacked by the hydroxyl group of serine. The reaction is catalyzed by phosphatidylserine synthase I and II localized in endoplasmic reticulum and stimulated by Ca^{2+}. The de novo synthesis of Ptdlns start with the biosynthesis of CDP-diacylglycerol by CDP-diacylglycerol synthase localized in endoplasmic reticulum. This is followed by the reaction between CDP-diacylglycerol and inositol by phosphatidylinositol synthase [7]. PtdIns can be synthesized also by an exchange reaction in which, in the presence of manganese, free myoinositol is incorporated into phosphatidylinositol [8].

PtdIns is the only phospholipid able to undergo reversible and transient changes at its head-group with phosphorylation on position 3,4 and/or 5. This yields seven different phosphorylated forms of PtdIns: PtdIns-4-P, Ptdlns- 4,5-P_2, PtdIns-3-P, PtdIns-3,4-P_2, and PtdIns-3,4,5- P_3.The most abundant of these are PtdIns-4-P and PtdIns-4,5-P2inmammalian cells [9]. In addition, CDP-diacylglycerol is the direct precursor for phosphatidylglycerol (PtdGro) and cardiolipin (di-PtdGro) [10].

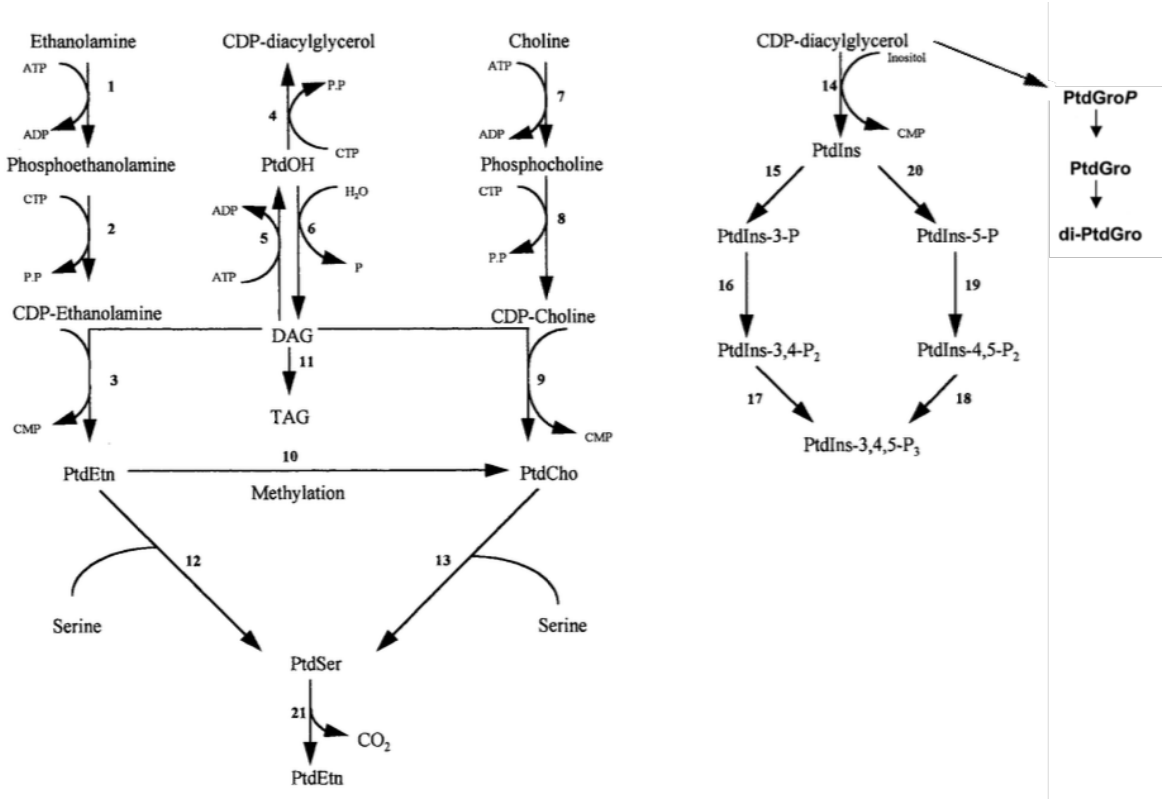


Figure 2. Biosynthesis of glycerophospholipids in brain. *Adapted from Farooqui et al. 2000.*

Biological functions of Glycerolipids

Glycerolipids are a structurally heterogeneous group of lipids that play key structural and functional roles in bacterial, plant, and animal membranes. The role of these lipids in cellular biochemistry is only partly clarified, but it is clear them contribution to the cellular homeostasis. They are involved in the membrane structuring and curvature changing, cellular growth, protein recruitment, signal transduction pathways.

Phospholipids in membranes constituted barriers to protect the cell and for the organelles within those cells. Tougher proteins, phospholipids contribute to the membrane curvature; depending on their biochemical structure, some phospholipids induce negative curvature of a membrane while others induce a positive one [11]. In cellular plasma membrane, the lipid components are asymmetric dispersed and this distribution is not randomly. In general, the external leaflet is dominated by PtdCho, whereas Ptdlns is mostly restricted to the cytoplasmic leaflet (Fig. 3) [12]. Although migration of lipids across the bilayer is a slow mechanism, phospholipids are translocated often by special proteins such as flippases, floppases and scramblases [13].

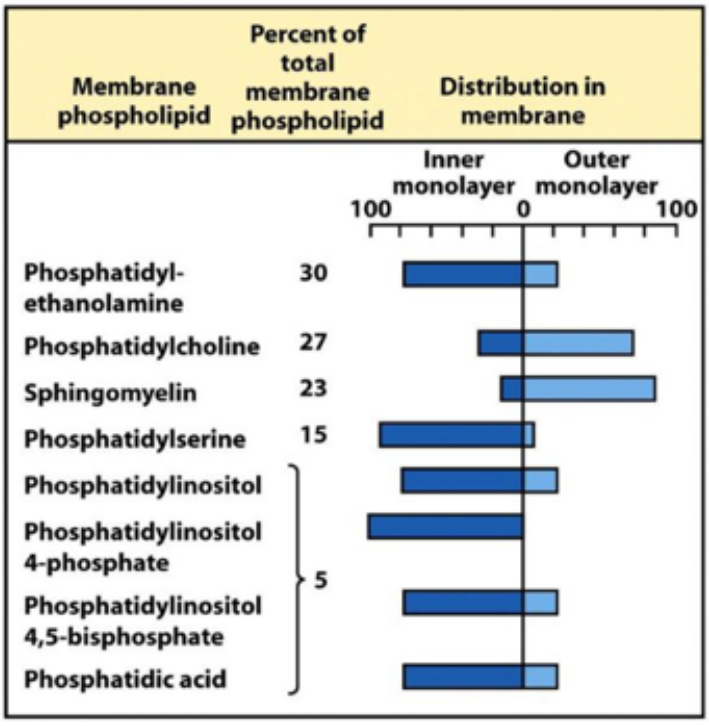


Figure 3. Lipid composition varies across the two leaflets of the same membrane. *Adapted from Lehninger principles of biochemistry.*

Phosphatidic acid (PtdOH) is the most basic phospholipid and serves as a precursor to other glycerophospholipids and due to its conical shape it is involved in curving of membranes [14]. Conversion of PtdOH to diacyl-glycerol, however, is also important for regulatory aspects because diacylglycerol can serve as a lipid second messenger [15] and as a mediator of vesicle formation [16]. Lyso-PtdOH is a naturally occurring phospholipid with hormone and growth factor-like activities. Lyso-PtdOH acts as a signaling molecule that is rapidly produced and released by activated cells. Lyso-PtdOH added extracellularly binds to specific receptors of target cells and stimulates platelet aggregation, smooth muscle contraction, induction of neuronal shape changes and leads to cell proliferation [10]. In addition, PtdOH inhibits mitochondrial division and stimulates mitochondrial outer membrane fusion [17] and it binds to the Rac protein [18].

Phosphatidylcholine (PtdCho) is an ion known as a zwitterion, has a cylinder shape and is the major component of biological membranes, comprising 45-55% of the total lipids [19]. Nonetheless, it was reported to be involved in signal transduction events, serving as a substrate for the phospholipases to generate signal messengers: it is hydrolysed to release DAG, lysophosphatidylcholine, PtdOH (by phospholipase D) and arachidonic acid (by phospholipase A2). In neurons, PC can be additionally cleaved by phospholipase B, yielding one glycerophosphocholine molecule and two free fatty acids [19]. In addition, PtdCho can be converted to sphingomyelins by SM synthase [20]

Phosphatidylethanolamine (PtdEtn) is also a zwitterionic lipid that constituted 15 – 25% of the phospholipids [21]. Due to a small size of the head group respect to PtdCho, PE adopts a moderately conical shape and induces a negative curvature to the membrane [22]. It is enriched in the inner membrane leaflet and it is translocated by across the membrane by the aminophospholipid flippases [23]. In addition to its structural role in membranes PtdEtn has multiple important cellular functions that were until recently unrecognized. PtdEtn have an important role in the phospholipid metabolism, being a substrate for almost all other phospholipids. For example, phosphatidylserine synthase 2 (PSS2) makes PtdSer out of PtdEtn at the ER [24]. PtdEtn is also the donor of the ethanolamine moiety that covalently modifies several proteins [22] and it is the substrate for the hepatic enzyme PE N-methyltransferase [25] that provides approximately one third of the PC in the liver. PtdEtn seems to have an important role in the heart since a decreased PE content of cardiac myocytes causes cell damage after ischemia, and a tered asymmetrical transbilayer distribution of PE in sarcolemmal membranes disrupts these membranes [26]. In the final steps of

autophagy, it is used for attachment to LC3 and for autophagosome formation [27, 28], so it is an important autophagy marker.

Cardiolipin (di-PtdGro) is a cone-shaped phospholipid dimer and is the chief non-bilayer phospholipid found in mitochondria, which are the only organelles to make this lipid [29]. Di-PtdGro have a head group (glycerol) that is esterified to two phosphatidylglyceride backbone fragments rather than one, resulting in a cone-shaped phospholipid dimer.
It is the chief non-bilayer phospholipid found in mitochondria, which are the only organelles to make this lipid and it is almost exclusively associated with the inner mitochondrial membrane.
This exclusivity association between di-PtdGro and mitochondria indicates an important role for PtdGro in mitochondrial bioenergetic processes. Di-PtdGro been shown to interact with a number of proteins, including the respiratory chain complexes, substrate carrier proteins and with a large number of innermitochondrial membrane (IMM) proteins, enzymes and metabolitecarriers [30]. Moreover, cardiolipin is involved in different stages of the mitochondrial apoptotic process and in mitochondrial membrane dynamics [31].

Phosphatidylserine (PtdSer), that makes up 2–10% of the total cellular phospholipid content, have a phosphoserine head-group which make him a anionic amino-phospholipid [32]. PtdSer has important signaling functions both inside and outside of the cell but it is not symmetrically distributed across the two leaflets of the membrane bilayer. It is normally highly enriched in the inner leaflet of the plasma membrane, while PtdCho and sphingomyelin, are enriched in the outer leaflet [33]. 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 PtdSer in the plasma membrane of activated platelets is markedly altered so that PtdSer becomes exposed on the cell surface [34, 35]. During sperm maturation, the asymmetric distribution of PtdSer in the plasma membrane changes and PtdSer becomes exposed on the surface of the sperm [36]. The well-characterized process where there is a transbilayer movement of PtdSer from the inner to the outer leaflet is during the early stages of apoptosis: the exposure of PtdSer 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 [37-40]. In addition, the exposure of PS on the surface of red blood cells serves as a signal for eryptosis [41]. Furthermore, lyso-PtdSer (PS hydrolysis product) is exposed on the surface of activated and dying neutrophils thus initiating the clearance of these cells during acute

inflammation [42]. PtdSer has not only extracellular functions, but it has been demonstrated also its participation in many intracellular processes. For example, PtdSer is the precursor of PtdEtn via the mitochondrial enzyme PtdSer decarboxylase [43]. 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 [44].

PtdIns, also called monophosphoinositide, is an anionic lipid that comprises 10 – 15% of the cellular phospholipids. It is present in various organelles and, like the aminophospholipids, it is enriched in the inner leaflet of the plasmalemma than in the outer leaflet [12, 45]. PtdIns is the unique lipid subjected to reversible and transient changes at its head-group, with phosphorylation on position 3,4 and/or 5. This produces seven distinct phosphoinositides species, involved in the regulation of different cellular metabolic pathways [22]. PtdIns is not only the precursor of the phosphoinositides species, but it is also an important source of the glycosylphosphatidylinositol (GPtdIns), an anchor for the membrane-destined proteins. Itis synthetized on the cytosolic side of ER with subsequent flipping of the product inside the lumen, where the COOH terminus of the proteins can be covalently attached.

PtdIns(3)P is abundant in the cytoplasmic leaflet of the membrane of early endosomes. It has also been detected on internal vesicles of multivesicular bodies. PtdIns(3)P is made primarily by phosphorylation of PtdIns by class III PtdIns3K (22), secondarily by class II PtdInsP3K. In addition, PtdIns(3)P can be generated by dephosphorylation of PtdIns(3,4)P_2 by Inpp4. PtdIns(3)P is degraded by 3'-phosphatases of the myotubularin family [46] and mutations in members of this family cause myotubular myopathy and Charcot-Marie-Tooth syndrome type 4B [47]. PtdIns(3)P plays very important roles in membrane traffic, directing endosome progression and phagosome maturation and regulating autophagy, and the production of reactive oxygen species (ROS) [48, 49].

PtdIns(4)P is enriched in the Golgi apparatus and, at lower concentrations, it is found on endosomes, plasma membrane, and ER. PtdIns(4)P is generated from PtdIns by two types of PtdIns 4-kinase (PtdIns4K; not to be confused with PtdInsP4K). PtdIns(4)P can be degradated or converted to more complex inositide species. PtdIns(4)P is key to sphingolipid synthesis in the Golgi complex, where it interacts with various proteins that include [50].

PtdIns(5)P is not very abundant so its distribution, metabolism and functions have not been fully characterized. PtdIns(5)P can be made from PtdIns(3,5)P_2 by dephosphorylation operated by myotubularins, which are 3'-phosphatases [51]. It is used as a substrate by type II PtdInsP5K to generate PtdIns(4,5)P_2 (302), but this pathway is likely a minor contributor to the overall biosynthesis of PtdIns(4,5)P_2 (307). PtdIns(5)P may also play a role in traffic between the late endosome and the plasma membrane, where PtdIns(3,5)P_2 and PtdIns(4,5)P_2, respectively, are enriched.

PtdIns(3,4)P_2 can be produced from PtdIns(4)P by phosphorylation at the 3' position of the inositol ring by the class II PtdInsP3K, from PtdIns(3)P by type II PtdInsP4K or from dephosphorylation of PtdIns(3,4,5)P_3 by 5' phosphatases (73). PtdIns(3,4)P_2 can be converted in PtdIns(3)P and PtdIns(4)P by removal of the 4' phosphate or 3' phosphate, respectively, by specific phosphatases. PtdIns(3,4)P_2 is a potent survival signal and it can positively regulate tumor cell invasion [52].

PtdIns(4,5)P_2 constituted 1–2 mol% of the phospholipids [53] at the inner leaflet of the plasma membrane. It is produced from PtdIns(4)P and PtdIns(3)P by specific kinases or from PtdIns(3,4,5)P_3 by dephosphorylation. Furthermore, PtdIns(4,5)P_2 itself can be dephosphorylated by 5'-phosphatases [54]. PtdIns(4,5)P_2 is substrate for lipases and kinases that generate several signaling molecules. For example, it can be phosphorylated by PtdInsP3K to produce PtdIns(3,4,5)P_3, which is a critical mediator of cellular activation or it can be hydrolyzed into IP_3, began a Ca^2 -mobilizing agent[55].

It is produced by phosphorylation of PtdIns(3)P on position 5' by PtdInsK-FYVE [56]. PtdIns(3,5)P_2 is dephosphorylated on the 3' position by myotubularins to generate PtdIns(5)P or, more commonly, it is dephosphorylated on the 5' position by Sac3. Its physiological role is not total clear, but PtdIns(3,5)P_2 seems to be implicated in the osmotic stress response, in autophagy, and in the control of ionic channel activity [57]. PtdIns(3,5)P_2 is found on late endosomal [58].

PtdIns(3,4,5)P_3 makes up 1% of the total lipid (349) and it is one of the major survival signal. PtdIns(3,4,5)P_3 is generate from PtdIns(4,5)P_2 by class I PtdInsP3-kinases. It can be dephosphorylated by both 3'phosphatases and 5'phosphatases to give PtdIns(4,5)P_2 and PtdIns(3,4)P_2, respectively. A number of important signaling proteins, such as PLC-, Akt, Vav, and PDK isoforms, bear PtdIns(3,4,5)P_3-binding domains that recruit and/or activate them at the membrane.

Sphingolipids

Sphingolipids (SLs) are minor cell components and they reside in the external layer of the plasma membrane with the hydrophilic headgroup protruding toward the extracellular environment [59].

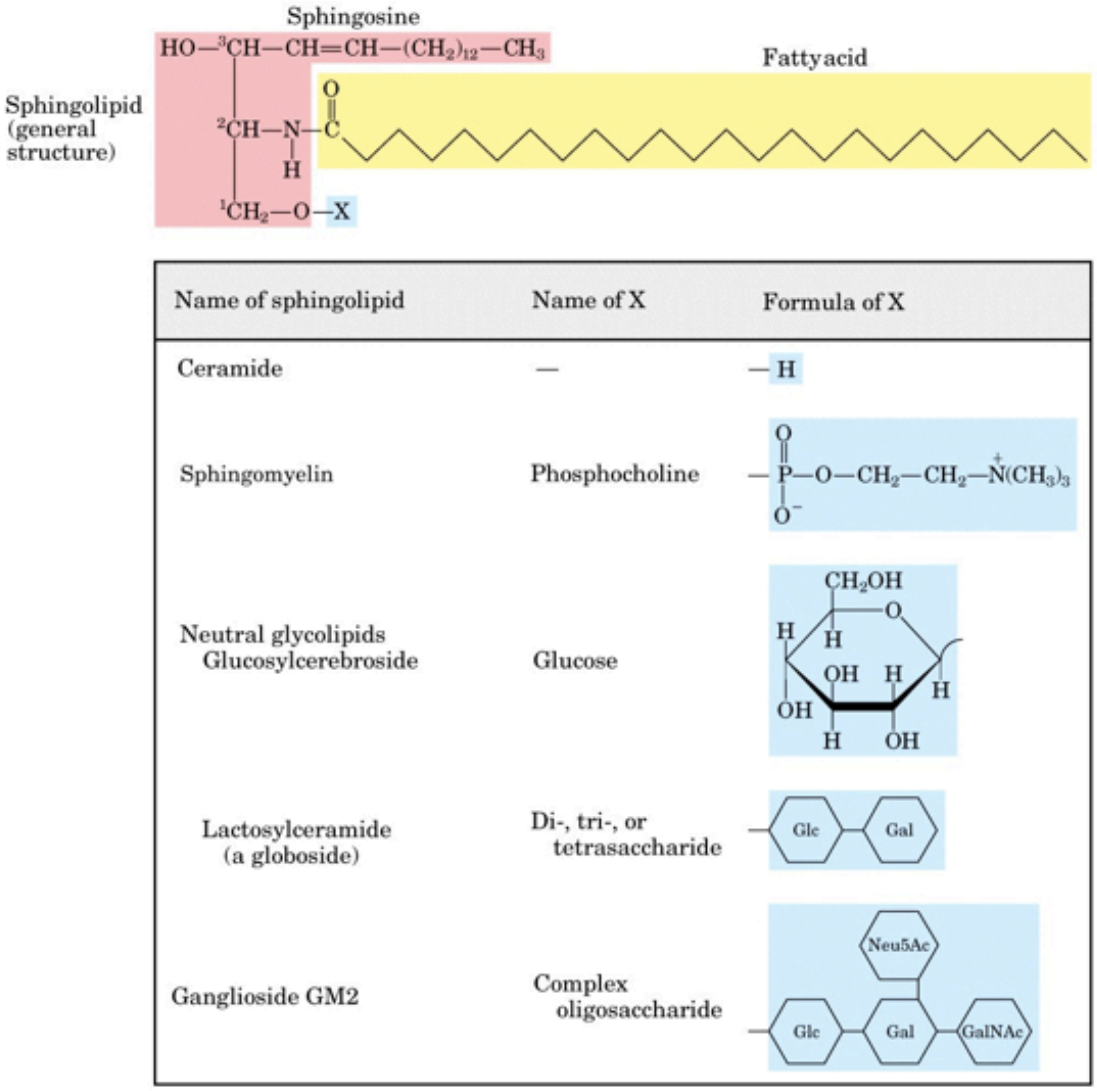


Figure 4. Common sphingolipids structure

The basic building block of sphingolipids is sphingosine, (2S, 3R, 4E)-2-amino-4- octadecene-1,3-diol (Fig. 4); the C1-phosphate ester of sphingosine is commonly named as sphingosine-1-phosphate [60]. Ceramide, the simplest SL, consists of a sphingoid base to which a fatty acid is attached at C-2 via N-acylation; the additions of oligosaccharides to sphingosine giving rise glycosphingolipids (GSLs), as glucosylceramide (GlcCer) and galactosylceramide (GalCer); the

subsequent addition of a sulfate group to a galactosylceramide produces sulfatide, an anionic sulfoglycolipid. By adding a phosphorylcholine moiety, it results sphingomyelin (SM), a very abundant membrane lipid. Glycosphingolipids with a capping N-acetylneuraminic acid are known as gangliosides [61].

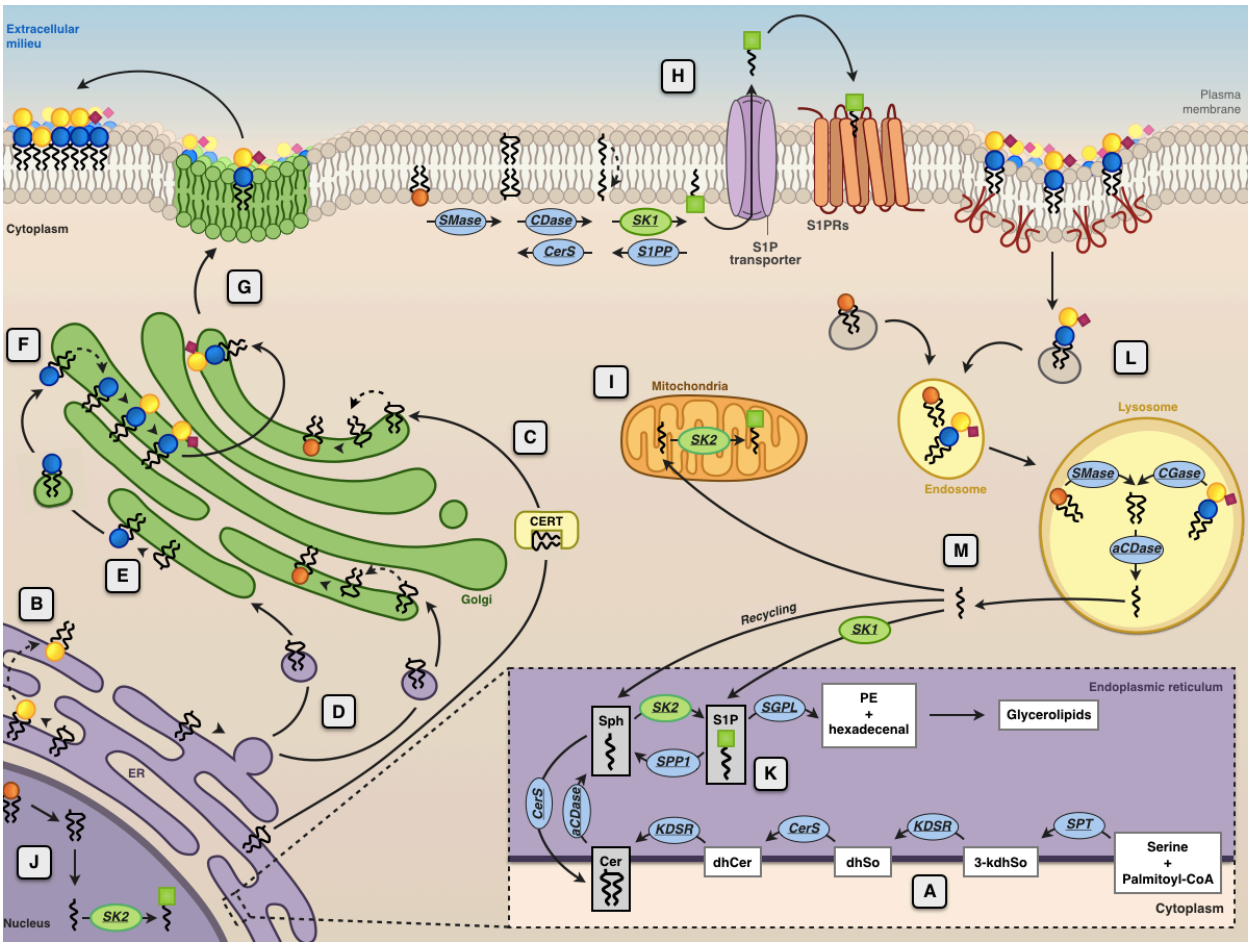


Figure 5. Subcellular compartmentalization of sphingolipid metabolism. *Adapted from Grassi et al, 2019* [62].

Sphingolipid Metabolism

Sphingolipids (SLs) are a class of natural lipids comprised of sphingosine; the predominant mammalian sphingoid bases contain 18 carbon atoms [63]. The *de novo* biosynthesis of SLs starts at the cytosolic leaflet of membranes of the endoplasmic reticulum (ER), with the condensation of serine and palmitoyl CoA by serine palmitoyl transferase [64] to form 3-ketosphinganine, which is subsequently reduced by 3-ketosphinganine reductase to produce sphinganine. Dihydroceramide synthase (CerS) [65] next acylates sphinganine to form dihydroceramide. In humans this is

achieved by six distinct CerS genes (known as LASS genes) that encode for a family of CerS enzymes with differing selectivity for the CoA-activated fatty acid [65]. The majority of dihydroceramide is transformed into ceramide (Cer) by dihydroceramide desaturase/reductase (DES1), which inserts a trans 4 – 5 double bond [66]; ceramide is the central molecule in the sphingolipid biology. Ceramide is also generated during the catabolism of SLs that occurs in lysosomes, and rapidly converted to sphingosine which is largely recycled. Ceramide synthase is able to use as substrate both sphingosine and sphinganine with similar efficiency [67] so ceramide can be also formed by N-acylation of sphingosine produced by the catabolism of complex sphingolipids.

The neo-synthesized ceramide could reach directly the membrane or be transported to the Golgi complex, where it serves as a substrate for production of complex sphingolipids (Fig. 5). Ceramide is the precursor of at least five different products:

(1) Ceramide can be glycosylated to galactosylceramide (GalCer) at the lumenal leaflet of the ER by the transfer of galactose from a UDP-galactose donor [68]. GalCer can be diversified into Sulfatide or GM4 by sulfation or glycosylation at its 3-O-position with Neu5Ac [69].

(2) Ceramide can be phosphorylated by ceramide kinase to generate ceramide-1-phosphate. The subcellular localization of this enzyme is not clear but has been suggested to be the PM [70], Golgi apparatus [71], and the cytoplasm [72] .

(3) Ceramide can be deacylated to sphingosine and free fatty acid by various forms of ceramidases, acting at either neutral (PM [73]), alkaline (ER/Golgi complex [74]), or acidic (lysosomal [75]) pH. Ceramidase activity has also been reported in mitochondria [21].

(4) Ceramide can be converted to SM. The synthesis of SM from ceramide occurs at the lumenal leaflet of the Golgi apparatus by transfer of phosphorylcholine from phosphatidylcholine (PC) to ceramide, with diacylglycerol formed as a by-product [76, 77]. Two SM synthases have been identified: SMS1 is located at the Golgi apparatus and SMS2 at the PM [78, 79]. Since ceramide is synthesized in the ER, there are two mechanisms for transferring ceramide to the Golgi apparatus for its metabolism to SM: vesicular and non-vesicular transport mechanisms. With the non-vesicular transport, ceramide is transported to the cytosolic leaflet of the trans-Golgi membrane by the ceramide transfer protein (CERT), in an ATP-dependent manner [80]. CERT transfers ceramides of relatively short acyl chain length (C16 – 20) from the ER specifically for SM synthesis in the Golgi apparatus [81].

(5) With the vesicular transport, ceramide is transported to the cytosolic leaflet of the cis-Golgi membrane; here, ceramide can be glycosylated to glucosylceramide (GlcCer), by glucosylceramide synthase (GCS). [82].

In alternative pathway, ceramide is phosphorylated in ceramide-1-phosphate at the plasma membrane by ceramide kinase (CERK). Ceramide-1-phosphate can be hydrolyzed by lipid phosphate phosphatase (LPP) to give ceramide [83]. Acid, alkaline and neutral ceramidases are capable of deacylating ceramide to generate pools of sphingosine at specific cellular locations. This sphingosine can be both phosphorylated to sphingosine-1-phosphate (S1P) by sphingosine kinase 1 (SK1), which operates at the plasma membrane and in the extracellular matrix, or by sphingosine kinase 2 (SK2) which is located at the ER, nucleus and mitochondria [62]. S1P levels can be downregulated by S1P phosphatase. The cellular orchestration of these complex interconversions between SLs is called the sphingomyelin cycle, and is an evidence to the role of these SLs in extra- and intracellular signaling pathways. The only currently known exit pathway from this interconnected SL metabolism is the degradation of S1P by S1P-lyase at the ER [61].

In the cytosolic side of the cis-Golgi apparatus membrane, GCS catalyzes the glycosylation of the primary hydroxy group in ceramide, using UDP-glucose as a donor glycoside, to generate GlcCer. GlcCer can either directly reach the plasma membrane, transported in a non-vesicular manner (GLTP transport protein) [84, 85] or be translocated to the luminal side of the Golgi either with FAPP2 or vesicular transport [86, 87]; here GlcCer flips into the Golgi lumen and then it is glycosylated by galactosyltransferase (GalT1) to give lactosylceramide (LacCer). Lactosylceramide, that, once produced, stays at luminal side of Golgi. [88]. Lactosylceramide is extended sequentially at either the 3-O-positon or the 4-O position; most of these GSLs consist of alternating and branched combinations of a- or b- linked glucose, galactose, N-galactosamine and N-acetylglucosamine. Of particular interest are the gangliosides, that is, lactosylceramide-derived sphingolipids containing capping N-acetylneuraminic acid (sialic acid) units. In 1962 Svennerholm, a pioneer in ganglioside research, suggested a nomenclature of brain gangliosides [89]. In this nomenclature G stands for ganglioside, A for asialo-, M for monosialo-, D for disialo-, and T for trisialoganglioside [90, 91]. Specific sialyl transferases convert lactosylceramide stepwise into GM3, GD3, and GT3. Lactosylceramide and each of its sialylated derivatives serve as precursors for complex gangliosides of the 0, a, b, and c series. These different series are characterized by the presence of no (0 series), one (a series), two (b series), or three sialic acid

residues (c series) linked to the 3-position of the inner galactose moiety. Gangliosides from the 0 and c series are only found in trace amounts in adult human tissues.

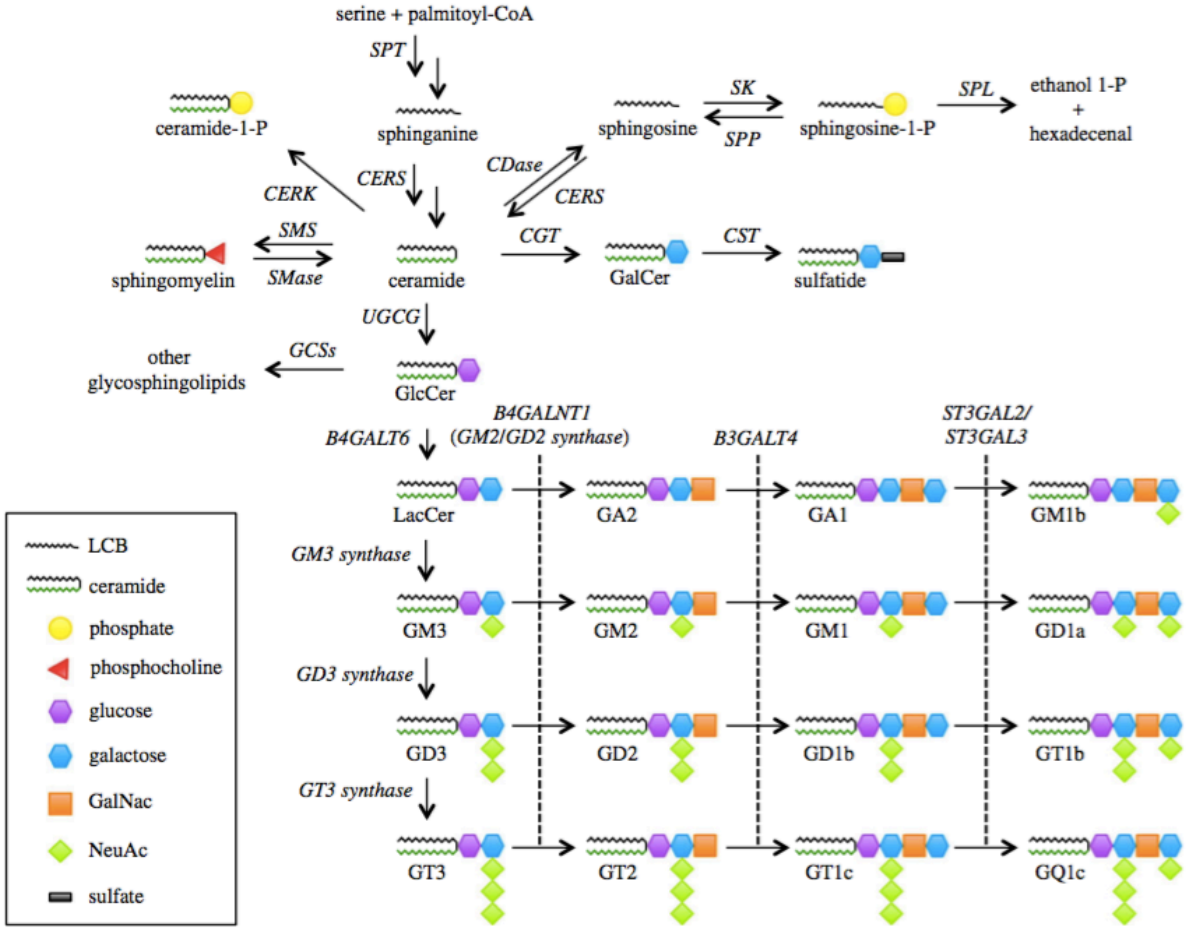


Figure 6. Schematic biosynthetic pathway of glycosphingolipids. *Adapted from Olsen et al, 2017* [92].

Biological functions of sphingolipids

In recent years, there is more and more evidence that SLs are not merely structural components of biological membranes, but are also regulators of cellular events.

The simple SLs, ceramide, ceramide-1-phosphate, sphingosine, and S1P are involved in several cellular events such as proliferation, differentiation, motility, growth, senescence, and apoptosis. Furthermore, complex SLs are involved in cell physiology by acting as antigens, as mediators of cell adhesion, binding agents for microbial toxins and growth factors, and as modulators of signal transduction [66]. Within the simple SLs, Cer and S1P are the best investigated components; they

have an opposite role in apoptosis processes and the levels of these two molecules regulates the balance between cell survival and death.

It has been known for years that ceramide has a role in apoptosis. The ceramide levels are increased by several extracellular signals, inducing apoptosis: heat shock, ionizing radiation, oxidative stress, progesterone, vitamin D3, tumor necrosis factor (TNF)-a, interleukin (IL)-1a, IL-1b, interferon-g, Fas ligand, oxidized low-density lipoprotein (LDL) and nitric oxide [93].

At today, the mechanisms supposed through which ceramide induces apoptosis are two [94]. In the first mechanism, ceramide produced in the plasma membrane acts as a second messenger and it binds to proteins whose activity it regulates; these proteins are the ceramide-activated protein phosphatase, protein kinase Cz , kinase suppressor of Ras, phospholipase A_2, cathepsin D, Jun-N-terminal kinases (JNKs), Ras and Rac, and Src-like tyrosine kinases [66]. In the second mechanism acts a signaling lipid: it can self-associate in the plasma membrane [95] and stimulate the fusion of GSL- and cholesterol-containing rafts into large signaling macrodomains (signaling platforms) [96]. In an additional mechanism, ceramide might induce apoptosis interacting with mitochondria. With a still unknown vehicle, ceramide is transported to mitochondria from ER and here forms membrane channels through which cytochrome c and small proteins are transported, leading to activation of the caspase-dependent apoptotic pathway [97, 98].

Likewise, S1P can act as both a first and second messenger but with opposite roles respect ceramide. As a first messenger, S1P regulates processes such as cytoskeletal rearrangement, cell migration, angiogenesis and immunity and lymphocyte trafficking. As an intracellular second messenger, S1P mediates calcium homeostasis, cell growth, tumorogenesis and suppression of apoptosis [66]. S1P can trigger signal transduction pathways by acting on the same cell from which it is secreted, by acting in an autocrine manner [96].

S1P seems to have opposite effects to ceramide in the pathways relating to cell growth and survival. Ceramide is implicated in growth-inhibitory and pro-apoptotic effects, contrariwise S1P is implicated in cell growth and inhibition of ceramide-mediated apoptosis. Moreover, sphingosine appears to act in a similar fashion to ceramide, whereas ceramide-1- phosphate shares similar functions with S1P [103]. This suggests that the balance between survival and death may depend on a delicate equilibrium between intracellular levels of each of these SLs.

Sphingomyelin is involved in plasma membrane signal transduction, cholesterol efflux and intra-cellular lipid and protein trafficking [99, 100]. As mentioned above, the major cellular site of SM synthesis is the Golgi apparatus [76, 77], but SM have also been observed in nuclear membranes

and in mitochondria. In these membranes, SM are believed to be an important source of ceramides for the nuclear [101] and mitochondrial [102] apoptotic cascades, respectively.

Gangliosides are ubiquitously found in tissues and body fluids and are more abundantly expressed in the nervous system [103]. In cells, gangliosides are primarily, but not exclusively, localized in the outer leaflets of plasma membranes and here are involved in cell-cell recognition and adhesion and signal transduction. In addition to cell plasma membranes, gangliosides have been shown to be present on nuclear membranes where play important roles in modulating intracellular and intranuclear calcium homeostasis and the ensuing cellular functions [104].

(Glyco)sphingolipids in membrane domains

Contrariwise of phosphoglycerolipids, SLs and GSLs are not distribute homogeneously in the outer plasma membrane. They form small (10–200 nm), heterogeneous, highly dynamic (ca. 0.1–1000ms), sterol- and sphingolipid-enriched domains, called "lipid rafts", that compartmentalize cellular processes on the surface of plasma membrane [105, 106]; in these semiordered lipid microdomains, SLs are involved in cell adhesion/recognition processes and signal transduction pathway. These microdomains exist in a gel-like liquid-ordered phase (lo), which has a lower diffusion rate than the surrounding liquid-disordered (ld) phosphoglycerolipid-rich plasma membrane [61]. Initially, proof of the existence of lipid microdomains was their isolation by extraction of membranes at 4°C in the presence of specific detergents: the so-called detergent resistant membrane domains (DRMDs). The presence of lipid rafts *in vivo* is supported by several lines of evidence, including the results of electron microscopy using the freeze-fracture technique [107], a single-particle tracking study [105], a fluorescence resonance energy transfer study [108] and a chemical crosslinking study [109]. Rafts have been shown to exist in both the extracellular, as well as the cytosolic, layer of the plasma membrane [110]. The connection between both layers is not clear, but the crossing of long- and very-long-chain fatty acyl chains of sphingolipids through both layers, interacting with saturated acyl chains on phospholipids, appears likely to contribute to the involvement of both lipid layers [99].

In lipid rafts**,** glycosphingolipids are usually highly asymmetrically enriched in the external leaflet of the plasma membranes, with the oligosaccharide chain protruded toward the extracellular space, where the sugar residues can engage *cis* and *trans* interactions with a wide variety of cell surface and extracellular molecules [111]. In fact, several proteins, including GPI-anchored proteins,

flotillins, caveolins, G-protein- coupled receptors, and certain receptor tyrosine kinases such as the epidermal growth factor receptor and the insulin receptor, appear to associate with these microdomains. Principally, two hypotheses have been developed to explain how lipid rafts accumulate and remain in the membrane as an entity. One hypothesis proposes that the headgroups of sphingolipids interact with each others amide and hydroxy/carboxy group, therefore holding sphingolipids together [112], while cholesterol fills the space between the bulky sphingolipid headgroups and is additionally kept in place by hydrogen bonds and van der Waals interactions between its 3-OH group and the sphingolipid amide groups [113]. The second hypothesis attributes the tight assembly in rafts to the interaction of mainly saturated acyl chains, which also favours cholesterol packing [114]. A major subclass of rafts are caveolae which are invaginations of the cell membrane characterized by the abundance of caveolin, a palmitoylated membrane protein that causes rafts to polymerize [115]. Most of the functions of caveolins are currently unclear, but they have been proposed to act as cholesterol sensors and thereby regulate the number of lipid rafts in the cell membrane [116].

Lipid membrane domains are characterized not only by a peculiar lipid composition, but also by the presence of specific subsets of membrane proteins. For the cell biology of neurons, the most relevant examples of proteins whose functions are modulated by the association with lipid membrane domains are represented by membrane receptors. Some of these receptors are already resident in lipid domains at the resting state, and, upon activation, they can propagate signals to other components intrinsically present in the lipid membrane domain; in other cases, activation of a signaling protein involves its translocation from/to lipid rafts to/from a non-raft membrane region or other intracellular sites [117]. As an example, the lipid raft-dependent lateral interaction involving GM1 ganglioside (and likely other ganglio-series structures, highly expressed in neurons) and the Trk family neurotrophin receptors. Association with ganglioside-enriched membrane domains is important for the cross-talk of Trk receptors with Src family non-receptor tyrosine kinases, which are typically highly enriched in neuronal lipid rafts [118, 119] in a cell- and stage-specific manner, and whose activity is as well regulated by gangliosides. The main roles of lipid membrane domains as signaling platforms involved in events crucial for neuronal survival, synaptic transmission, axon growth, guidance, and regeneration are summarized in Fig. 7.

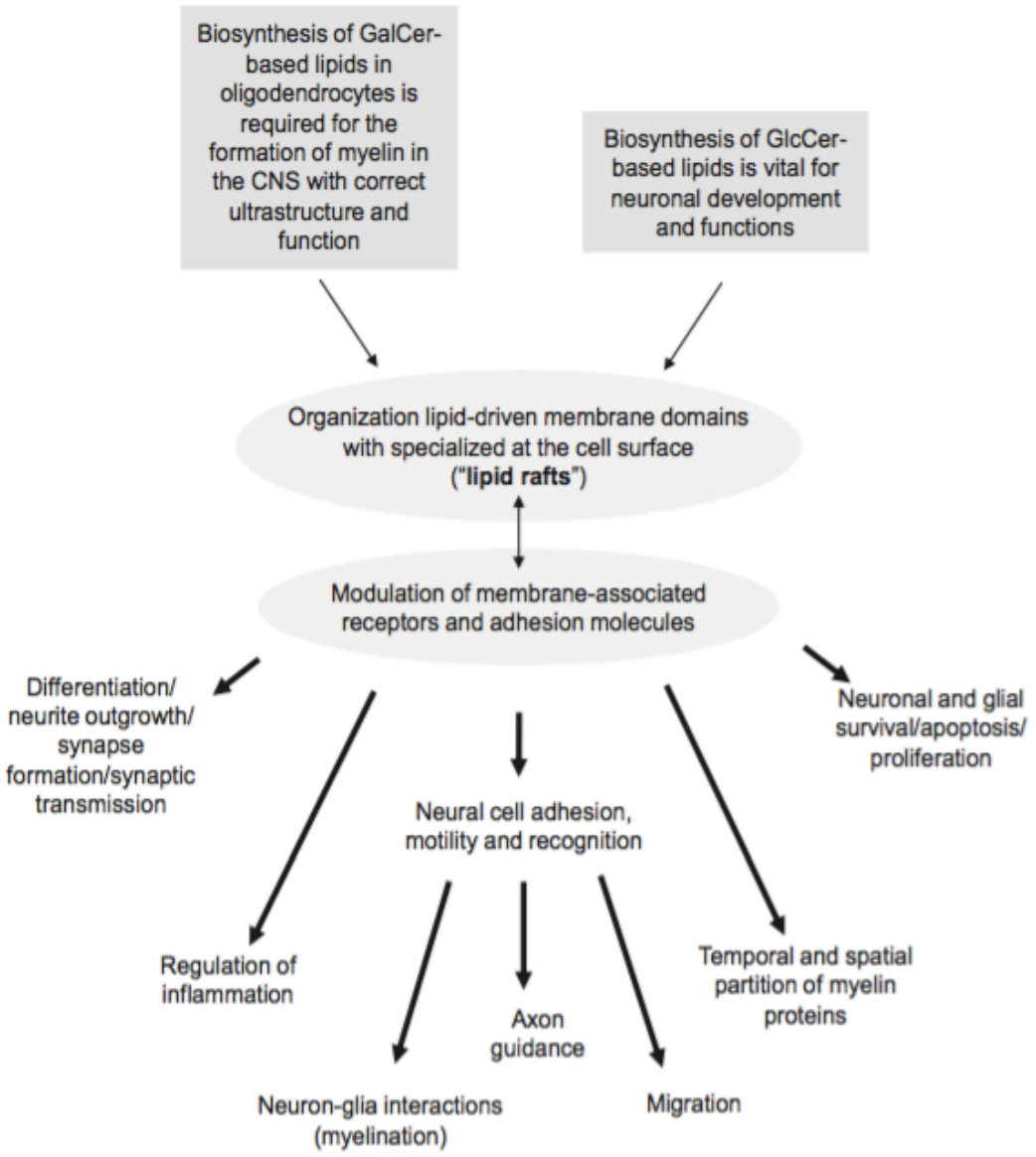


Figure 7. The multifaceted roles of lipid membrane domains in the nervous system. *Adapted from Aureli et al, 2015* [117].

Myelin development, damage and repair

The rapid transduction of electrical impulses is required for the efficient function of the vertebrate nervous system. The conduction velocity of these impulses is increased by the presence of myelin, an insulating layer surrounding the axon [120, 121]. In the CNS, the oligodendrocyte is the resident cell type responsible for the production of myelin that consists of oligodendroglial plasma membrane loops strongly wound concentrically around the axon. Mature myelin consists of up to

160 membrane layers, and internodes extend up to 1.7 mm in the CNS and up to 2 mm in the PNS [122, 123]. In postnatal development, the myelin membranes of an oligodendrocyte may grow by as much as 5,000 μm^2 per day [124].

The CNS myelinating oligodendrocytes are terminally differentiated cells with a limited ability to respond to damage. They depend from the availability of their precursors, the oligodendrocyte progenitor cells (OPCs) that can proliferate, migrate and differentiate into defined progeny [125], either oligodendrocytes or astrocytes depending on the context [126, 127]. Generation of OPCs in the spinal cord occurs in two steps. The first is ventral, with OPCs observed initially within the ventricular zone of the motor neuron progenitor (pMN) domain of neuroepithelium, in early embryonic life [128, 129]. Subsequently, the second step occurs in the dorsal spinal cord and hindbrain/telencephalon of the brain in late embryonic development and early post-natal life [130-132]. These proliferating cells migrate into the developing white matter, exit the cell cycle, undergo differentiation into mature oligodendrocytes, and begin to express a subset of myelin-associated proteins and other specific markers, several of which are glycosphingolipids [133] (Fig. 8).

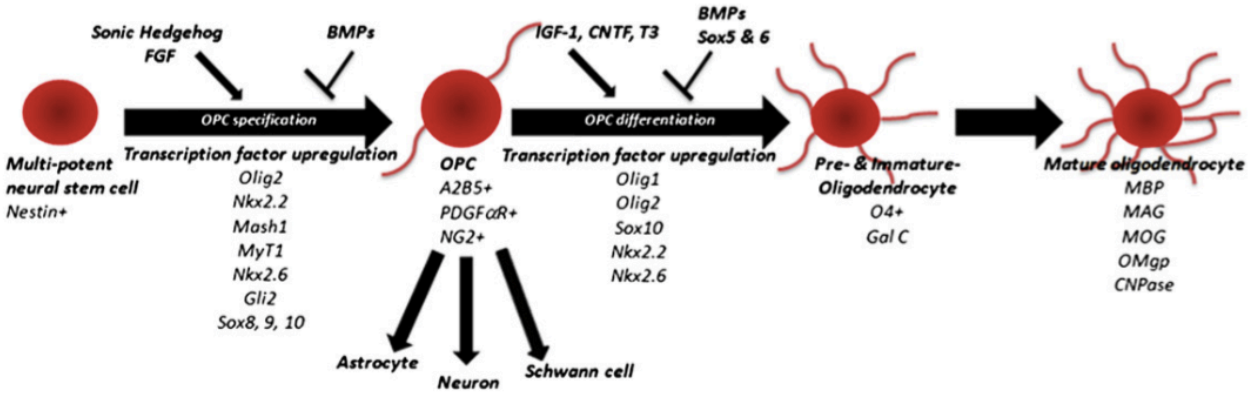


Figure 8. Schematic representation of the morphological and antigenic characteristics of cells of the oligodendroglial lineage differentiating from mitogenic progenitor cells to mature myelinating OLGs. Multi-potent neural stem cells (nestin+) are specified into oligodendrocyte progenitor cells (OPCs; A2B5+, PDGF R+, and NG2+) by exposure to sonic hedgehog (SHH) and fibroblast growth factor (FGF), whereas bone morphogenic proteins (BMPs) inhibit this specification. This specification involves the transcription factors Olig2, Nkx2.2, Mash1, MyT1, Nkx2.6, Gli2, and Sox 8/9/10. OPCs can differentiate into multiple cell types including astrocytes, neurons, and Schwann cells. Differentiation along the oligodendroglial lineage to pre-oligodendrocytes/immature oligodendrocytes (O4+/ GalC+) is potentiated by the growth factor IGF-1, the cytokine CNTF, and the thyroid hormone T3, and requires the transcription factors Olig1/2, Sox10, and Nkx2.2/2.6. Conversely, BMPs and transcription factors Sox5/6 prevent this differentiation. Differentiation into mature oligodendrocytes is associated with acquisition of myelin-related proteins, MBP, MAG, MOG, oligodendrocyte–myelin glycoprotein (OMgp), and 2 ,3 -cyclic nucleotide 3 - phosphodiesterase (CNPase). *Adapted from Miron et al, 2011 [134].*

Upon first contact with an axonal membrane, the extremity of the OPC membrane is either retracted or stabilized (Fig. 9); when it is stabilized, a specialized membrane domain for continued axoglial communication is created. This initial contact stimulate a number of molecular rearrangements in the future myelinating cell, including the association of the tyrosine-kinase fyn with lipid-rich membrane microdomains [135], the suppression of RhoA activity [136], the local enrichment of phosphoinositides in the glial membrane, as PIP_2 and PIP_3 [137-139], the localization and activity of polarity proteins change [140, 141] and reorganization of cytoskeletal.

Myelin lipids have a high lateral mobility and the cholesterol content is likely to limit the membrane's fluidity, thereby affecting its intracellular trafficking and myelin compartmentalization. The compaction of multiple membrane layers progresses from the abaxonal layers toward the inner tongue with a delay of two to three wraps behind the leading edge [139], allowing the growing sheath to expand underneath the previous layer. This compaction requires specialized adhesive proteins and the removal of molecules that prevent compaction. Initially, the intracellular surface of the glial membrane is inhibitory to tight appositions owing to highly charged phospholipids, such as PIP_2. However, negatively charged membrane surfaces attract basic proteins MBP [142]. MBP is abundant (8% by mass) and shows a high affinity to PIP_2 [143]. By neutralizing membrane phospholipids, MBP pulls together two bilayers similar to a zipper [144], forming a the major dense line that allows myelin to grow [145]. Oligodendrocytes that lack MBP fail to form compact myelin, while peripheral myelination can occur without the incorporation of MBP because seems to be compensated for by the basic intracellular domain of P0 [146] and by protein 2 (P2/*Pmp2*) [147].

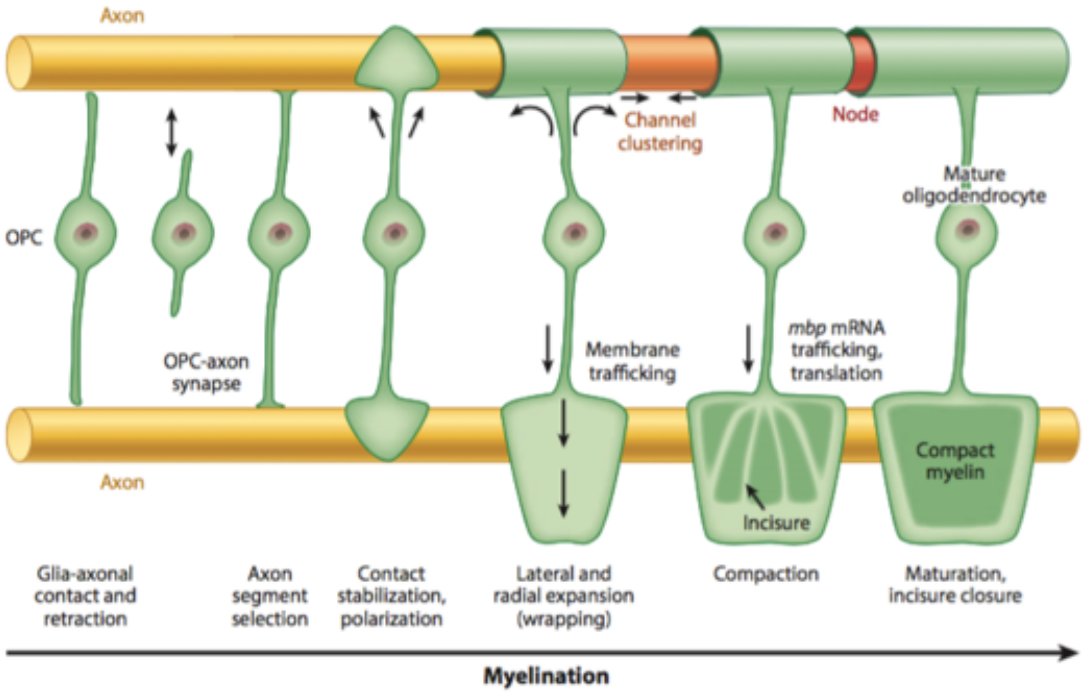


Figure 9. Schematic model of the differentiation of a committed oligodendrocyte progenitor cell (OPC) into a mature myelinating oligodendrocyte. *Adapted from Nave et al, 2014* [148].

Lipids in myelin

Myelin is a concentrically laminated membrane structure surrounding an axon around [149]. The myelin lamella is formed by fusion of the apposed inner leaflets of the plasma membrane in glial cells, with no intervening cytoplasm. Myelin provides the structural basis for saltatory action potential propagation, which accelerates nerve conduction 20–100-fold compared with nonmyelinated axons of the same diameter [148].

The myelin membrane originates from and is a part of the oligodendroglial cells in the central nervous system (CNS: brain and spinal cord) and the Schwann cells in the peripheral nervous system (PNS: nerves). In the CNS one oligodendrocyte makes myelinate multiple segments on many axons, while in the PNS one Schwann cell forms a single myelin sheath. Although some molecular or morphological differences, the basic sheath arrangement, characteristics and function between peripheral and central myelin is essentially the same.

A myelinated axon is interrupted by non-myelinated regions, called nodes of Ranvier, and can be divided in four distinct domains, node (N), paranode (PN), juxtaparanode (JXP) and internode (INT) (Fig. 10). At the nodes of Ranvier, Neurofascin 186 (Nf-186) supports the clustering of Na^+

channels, that regulate electrical impulse conduction. The nodal Na^+ channels are separated from the juxtaparanodal K^+ channels via the paranode, where Neurofascin 155 (Nf-155) binds tightly to the axonal complex of Contactin and Contactin-associated protein (Caspr). At the juxtaparanode, clustered K^+channels are associated to Caspr-2 and Contactin-2.

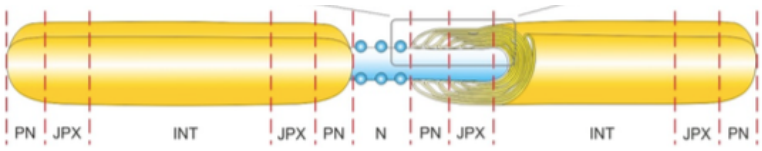


Figure 10. Schematic of node of Ranvier organization. *Adapted from Podbielska et al, 2013 [150].*

One of the biochemical characteristics distinguishing myelin from other biological membranes is its high lipid-to-protein ratio, where lipids representing 80% of its dry weight [151]. The lipids are assembled with myelin-specific membrane proteins: myelin proteolipid protein PLP), an intrinsic (integral) membrane protein, myelin basic protein (MBP) an extrinsic (peripheral) membrane protein, myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG), and 2′3′-cyclic-nucleotide 3′-phosphodiesterase (CNP).

The three main classes of lipids comprising CNS myelin are cholesterol, sphingolipids and phospholipids with a constant molar proportion of 2:1:2 [152].

Substance	Myelin		White matter	Grey matter	Whole brain
	Human	Rat	Human	Human	Rat
Protein	30.0	29.5	39.0	55.3	56.9
Lipid	70.0	70.5	54.9	32.7	37.0
Cholesterol	27.7	27.3	27.5	22.0	23.0
Cerebroside	22.7	23.7	19.8	5.4	14.6
Sulfatide	3.8	7.1	5.4	1.7	4.8
Total galactolipid	27.5	31.5	26.4	7.3	21.3
Ethanolamine phosphatides	15.6	16.7	14.9	22.7	19.8
Phosphatidylcholine	11.2	11.3	12.8	26.7	22.0
Sphingomyelin	7.9	3.2	7.7	6.9	3.8
Phosphatidylserine	4.8	7.0	7.9	8.7	7.2
Phosphatidylinositol	0.6	1.2	0.9	2.7	2.4
Plasmalogens	12.3	14.1	11.2	8.8	11.6
Total phospholipid	43.1	44.0	45.9	69.5	57.6

Table 1. Comparison of the human and rat brain composition. Protein and lipid values are given in percentage of the dry weight; everything else in total lipid weight percentage. *Adapted from Quales et al, 2002 [153].*

The myelin membrane contains a high level of two galactosphingolipids, galactosylceramide (GalCer) and 3-O-sulfogalactosylceramide (sulfatide), account for about 20 and 5 % of myelin lipids respectively [154]. How described in the above sections, the synthesis of these galactosphingolipids involves two sequential steps (Fig. 11), the addition of galactose from UDP-galactose (UDP-Gal) to ceramide, catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT) and the subsequent addition of the sulfate group by the enzyme 3'-phosphoadenosine-5'-phosphosulfate:cerebroside sulfotransferase (CST) [155]. Galactosylceramides and sulfatides with long chain fatty acid moieties, in particular 24:0 and 24:1 fatty acids, are the most typical myelin lipids [156].

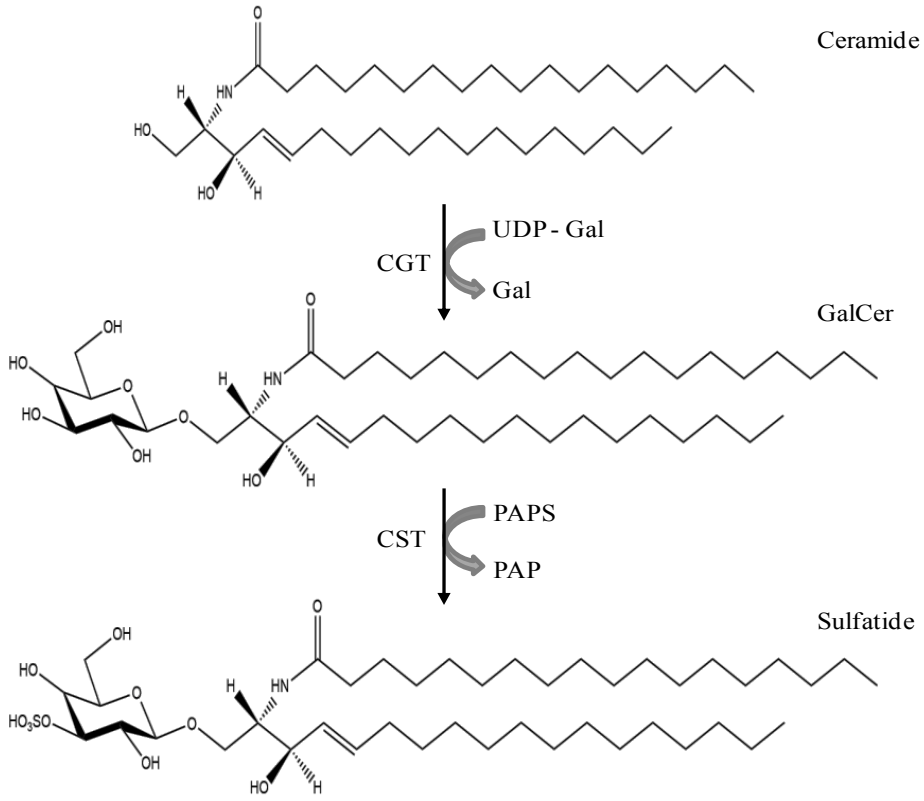


Figure 11. Structure and biosynthetic pathway of sulfatide, the major sulfoglycolipid in the nervous system. 3-O-sulfogalactosylceramide is highly heterogeneous in its fatty acid composition. The main fatty acids found in mature CNS myelin are long chain fatty acids (24:0 and 24:1), including a significant amount of 2-hydroxylated fatty acids. Sulfatide synthesis requires the addition of galactose from UDP-galactose (UDP-Gal) to ceramide, catalyzed by the UDP-galactose:ceramide galactosyltransferase (CGT, EC 2.4.1.45, encoded by the ugt8 gene), and the subsequent addition of the sulfate group by the enzyme 3'-phosphoadenosine-5'-phosphosulfate:cerebroside sulfotransferase (CST, EC 2.8.2.11, encoded by the gal3st1 gene).

The essential role of sphingolipids, in particular GalCer and sulfatide, in myelin structure has been studied using animal models lacking the enzymes responsible for their synthesis.

CerS2 null mice have greatly reduced levels of sphingolipids with very long chain fatty acyl chains (C22:0-C24:0) and a massive decrease in galactosylceramides and sulfatides in myelin [157]. Nevertheless, CerS2 null mice form relatively normal myelin with mild structural defects, such as focal detachments of individual or groups of myelin lamellae.

CGT knock-out mice, that completely lack GalCer or sulfatide, able to form myelin with an apparently normal structure with normal major dense and intraperiod line periodicity [158]; this could be due to a partial compensation of the loss of these galactolipids by synthesizing 2-hydroxylated GlcCer, usually not present in myelin. Despite the presence of compact myelin, these animals exhibit tremor, splaying of the hind limbs and ataxic locomotion that worsens progressively, resulting in death of most animals by the third month of age [159]. In fact, they present disruption of nerve conduction with an action potential decrease respect a wild type mice [159] and, a more detailed analysis of the structure of their myelin revealed ultrastructural abnormalities; the CNS myelin sheaths are thinner, nodal length is increased, lateral loops are widely spaced, the tight junctions are destroyed, suggesting that the formation of these junctions may be dependent on the presence of this lipid. In addition, CGT (-/-) mice exhibit a significant increase in cellularity in the spinal cord [160, 161] and a decrease of proliferation and survival rate of oligodendrocyte precursors. In the other hand, the PNS myelin in the knock-out mice appears normal suggesting that galactolipids might be less critical in the formation and maintenance of PNS myelin sheath structure [158].

CST (-/-) mice cannot synthesize sulfatide, while the levels of GalCer, other glycolipids, and phospholipids are not significantly altered [162]. These mice produce compact myelin, even if it is thinner than that of normal mice and shows alterations in the paranodal structure similar to those of CGT knock-out mice. The phenotype of these mice is similar but milder than that of CGT (-/-) mice in terms of age of onset, life span and severity of symptoms and they survive for more than 1 year [163]. CST-deficient mice are born healthy but start displaying hind limb weakness around 6 weeks of age, followed by pronounced tremor and progressive ataxia. Electron microscopy analysis of myelinated nerve fibers of these mice, revealed disorganized termination of the lateral loops at the node of Ranvier and deteriorated clusterization of Na^+ and K^+ channels [164]. These ion channels alterations are present both in CNS and in PNS in

mutant mice and are accompanied by an altered distribution of proteins involved in the structure of nodes, such as Caspr, contactin and neurofascin155 (NF155) [164]. In addition, in these animals is observed an increased number of oligodendrocytes and oligodendrocytes mature earlier and in greater number [165, 166]. This increase is determined by an increased proliferation and by a reduced rate of apoptotic death in the oligodendrocytic lineage cells [166].

1-month-old CGT mutant mice form abundant myelin but it is characterized by structural features associated with sheath immaturity such as uncompacted and redundant myelin profiles [158, 159, 167]. In contrast, the same age CST mutant mice showed no difference in the frequency of redundant myelin and revealed only a modest increase in the frequency of uncompacted myelin, suggesting that myelin development is relatively normal. The difference between the CGT and CST mutants indicates that GalCer is critical for myelin development while sulfatide is less essential at early stages of maturation [168]. Sulfatide, therefore, seems to play a role in maintaining the integrity of the major dense line, either directly or indirectly by helping to organize the plasma membrane components responsible for intra-myelin stability and adhesion [169].

The structure-specific roles seem to be linked to the ability of GalCer and sulfatide to form and to stabilize specific lateral domains in the membrane of myelin forming-cells and in the myelin sheath [154]. In particular, GalCer- and sulfatide-rich domains in the oligodendrocyte membranes regulate the correct sorting, trafficking, co-clustering and lateral distribution of the major myelin proteins [170]. While in the early stages of myelin formation galactolipid levels are absent or very low and only a few of the typical myelin proteins are associated with lipid rafts, during the mid-myelination stage, GalCer and sulfatide are synthesized at detectable levels and the PLP and MOG proteins tend to localize in lipid rafts; later, in the final stages of myelination, myelin- MAG and MBP are also translocated into lipid rafts [171-173]. Recent studies have demonstrated a differential involvement of GalCer-rich and sulfatide-rich membrane domains in modulating the function of this specific myelin proteins, in particular has been analyzed the effect of myelin galactolipids on the lateral organization and membrane dynamics of two myelin proteins, PLP and MBP [171].

The differential involvement of GalCer-rich and sulfatide-rich membrane domains in modulating the function of this specific myelin proteins has been studied by separately overexpressing CGT or CST on a galactolipid-null background, using the rat immature

oligodendrocyte cell line OLN-93, that does not produce galactolipids [154]. This study revealed that membrane association and dynamics (as lateral motility mobility in intact cells) of MBP is ordered by GalCer, while those of PLP are regulated by sulfatide (Fig. 12). This clearly indicates the importance of the formation of distinct lipid membrane domains in the myelin membrane for the lateral organization of specific myelin proteins as MBP and PLP.

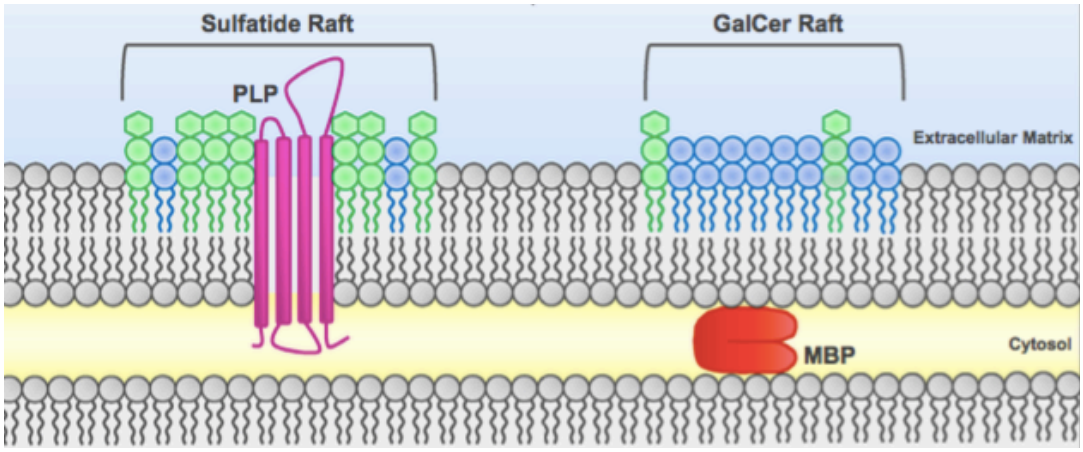


Figure 12. Association with specific sulfatide-rich domains regulates the function of PLP. The membrane association and dynamics of PLP are sulfatide-dependent while those of MBP are GalCer-dependent. *Adapted from Grassi et al, 2015 [154].*

Additionally, GalCer and sulfatide present in the apposed extracellular surfaces of the myelin sheath can do trans-interactions and form specialized "glycosynapse", which stabilized the myelin wrapping [174, 175]. Moreover, for long-term axon-myelin stability is required another oligosaccharide mediated trans interactions between the gangliosides GD1a and GT1b, in specialized domains of the axonal surface, and MAG [176], whose correct localization in the myelin membrane, is in turn regulated by the association with galactolipid rich domains [173] (Fig. 13).

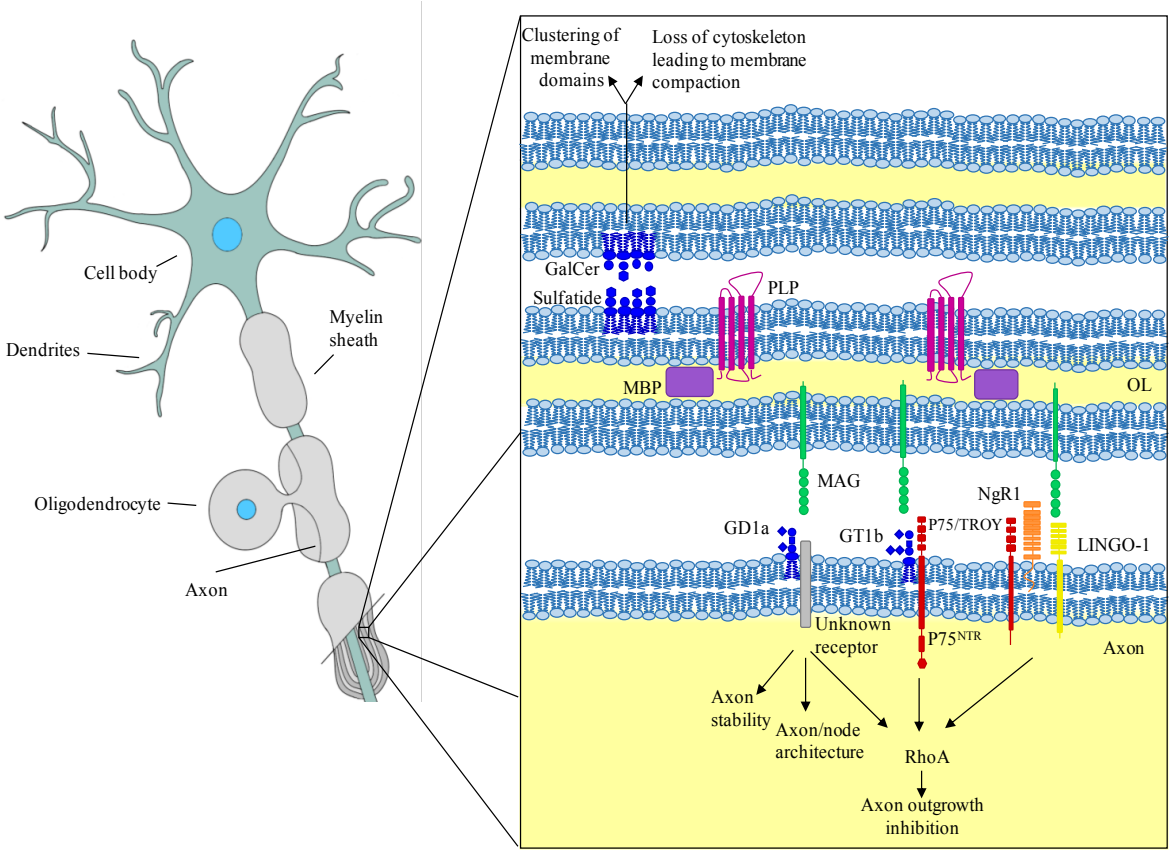


Figure 13. Glycolipid-enriched membrane domains in myelin. Glycolipid–glycolipid and glycolipid–protein interactions play multiple roles in myelin formation, maintenance and functioning but also in axon-myelin stability and communication. GalCer and sulfatide on opposing surfaces of the myelin wrap interact with each other through *trans* carbohydrate–carbohydrate interactions forming a "glycosynapse" causing transmembrane signaling which results in clustering of membrane domains and loss of cytoskeleton integrity, leading to compaction and formation of mature myelin. On the other hand, GD1a and GT1b gangliosides, enriched in axonal lipid rafts, interact with MAG resulting in transmembrane signaling. MAG can also interact with Nogo-R1 (NgR1) which in turn interacts with signaling molecules p75/TROY and LINGO-1, leading to RhoA activation and axon outgrowth inhibition. Lateral interaction of GD1a and GT1b with p75 is important for the organization of NgR1 complex. *Adapted from Aureli et al, 2015 [117].*

The available information indicate that GalCer is primarily involved in myelin formation and maturation, while sulfatide contributes to the long term stability of myelin structure, in particularly affecting the integrity of the nodal and paranodal regions [145].

In addition to cerebroside, the other major myelin lipids are cholesterol, phosphatidylcholine, phosphatidylethanolamine, much of the latter are plasmalogens and there is less sphingomyelin. Minor glycosphingolipid components (0.1% to 0.3%) include gangliosides, especially GM_1

and GM4 [177]. The role of plasmalogens in membranes has not been elucidated, but they seem to play a role in membrane fusion processes and membrane dynamics. In fact, in knockout mouse for Dhapat, a peroxisomal enzyme essential for plasmalogen synthesis, reduced CNS myelination is observed together with abnormal paranodal structure and reduced corpus callosum conduction velocity [151].

Demyelination Process in Multiple Sclerosis

The term "demyelination" indicates a loss of myelin with a consequent axonal damage, determining impairment of the conduction of signals. According to pathogenesis, demyelinating diseases of the CNS can be classified into several categories: demyelination due to inflammatory processes, viral demyelination, demyelination caused by acquired metabolic derangements, hypoxic–ischaemic forms of demyelination and demyelination caused by focal compression [178]. MS is the most common human demyelinating disease it is thought to be caused by the interaction of multiple genetic and environmental factors. The exact cause of multiple sclerosis still remains elusive, but it established that Multiple sclerosis is an immune-mediated disease in which the body's immune system attacks the CNS. In the most widely accepted hypothesis concerning MS pathogenesis, CD4+ Th1 cells release cytokines and mediators of inflammation that may cause tissue damage, CD4+ Th2 cells may be involved in modulation of these effects and, as recent evidence suggests, Th17 cells, CD8+ effector T cells and CD4+, CD25+ regulatory T cells play a prominent role in MS immunopathology [179].
Data collected from experimental autoimmune encephalomyelitis (EAE) studies suggest the he following immunopathological events: (1) initial T cell priming, (2) activation phase in the periphery, (3) migration of the pro-inflammatory T cells and monocytes across the blood-brain barrier (BBB), (4) amplification of local inflammation and activation of resident antigen presenting cells (APCs), such as microglia, (5) invasion of CNS parenchyma, resulting in damaging of oligodendrocytes, myelin sheath and axons [150, 179, 180]. The T cell priming occurs within systemic immune sites and it started by myelin antigens sensitization, among which myelin lipids. There are two different mechanisms for lipid antigen uptake, depending on antigen origin and its structure: lipids containing longer alkyl chains are transported to late endosomes, where CD1b molecules, specialized for binding long chain lipids, are located [181-183]; lipids constituted by with alkyl chains whit multiple unsaturation sites or shorter saturated

tails are directed to early or recycling endosomes, where CD1c and CD1a molecules are located [184, 185]. Activated APCs, within secondary lymphoid organs, induce the activation and expansion of myelin-specific T cells, that consequently circulate searching for their specific antigens to become re-activated [186]. Upon acrossing the BBB, via interactions between adhesion molecules found on the surface of lymphocytes and endothelial cells [70], the autoreactive $CD4^+$ T cells initiate the local pro-inflammatory cascade. In addition, a variety of effector mechanisms are induced: antibody-mediated cytotoxicity, oxygen and nitrogen radicals, pro-inflammatory cytokines and apoptosis-mediating molecules that damage oligodendrocytes, myelin sheaths and axons. The results of the local inflammation are the characteristic demyelinated plaques and astrocytic activation (gliosis), the are the major pathological hallmarks of the disease [187].

In MS, the demyelination is associated with disorganization of paranodal and juxtaparanodal domains: the paranodal and juxtaparanodal proteins become diffusely distributed along denuded axons and the aggregates disappear [188, 189]. Early alterations include the overlapping of Neurofascin (Nf)-155-positive paranodal structures with juxtanodal Kv1.2 channels adjacent to actively demyelinating white matter lesions associated with injured axons [190]. Consequently, nodal sodium channels are then directly adjacent to juxtaparanodal potassium channels, leading to impaired saltatory conduction of action potentials (Fig 14). These data suggest that paranodal junctions might be the initial site of demyelination [150].

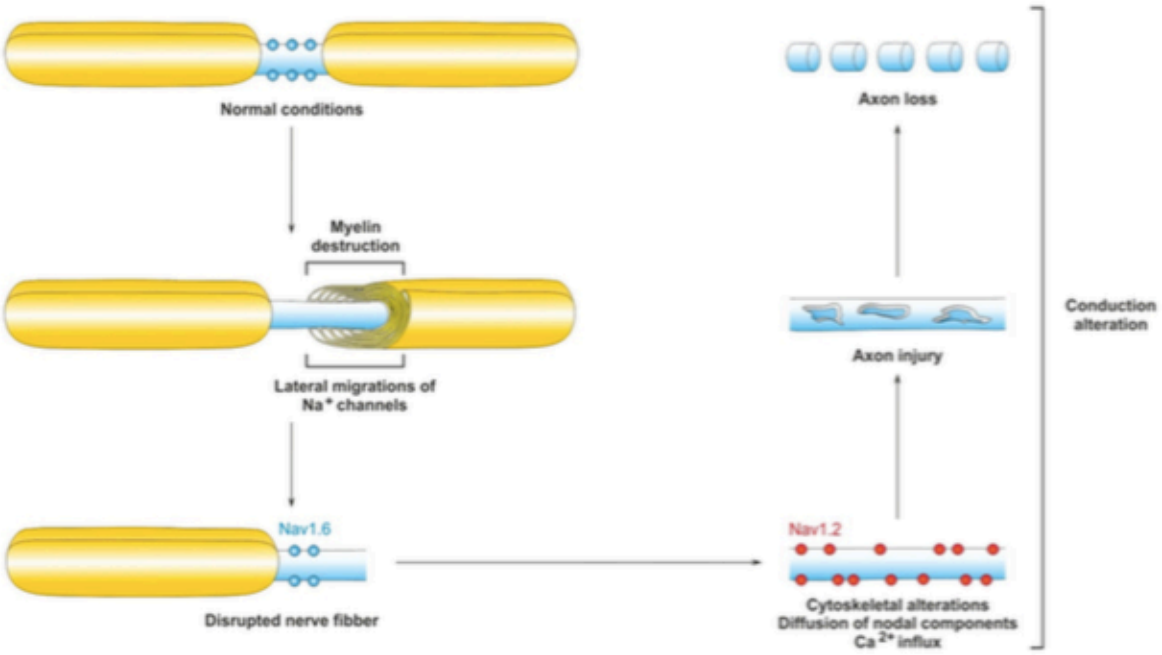


Figure 14. Characteristics of the demyelination process. Demyelination destroys the paranode and sodium channels migrate laterally. Following this is sodium channels (Nav) redistribution and re-expression of the immature isoform of sodium channels, Nav1.2. Persisting currents may cause conduction block and calcium overload, leading to axon injury and eventually loss. *Adapted from Podbielska et al, 2013* [150].

Recent reports have suggested that some of the myelin-specific lipids may be key contributors to the pathogenic mechanism of MS. Individuals with MS disease are reported to have different myelin lipid compositions and elevated levels of anti-sulfatide Ab in biological fluids compared with healthy individuals [191]. Additionally, recent reports suggest that sulfatide might be a target autoantigen and abnormally released sulfatide from disrupted myelin could act as a glial activator [189]. Despite this insight into pathophysiology, the cause of MS remains unclear and definitive treatment of this frequent and chronic disease still obscure.

Therapeutic approaches and remyelination promotion

Remyelination, the phenomenon by which new myelin sheaths are generated around axons, is a common repair mechanism following an insult to the nervous system. The development of therapies aimed to promote remyelination in conditions in which axons are demyelinated, such as in multiple sclerosis, is an important therapeutic goal. Theoretically, remyelination is

beneficial in people with MS for its potential to restore myelin structure, recover neuronal conduction deficits and it could help to protect denuded axons from degeneration.

Myelination begins with OPCs differentiating into oligodendrocytes, followed by their maturation into myelinating oligodendrocytes [192]. In developing zebrafish, myelination is rapid and oligodendrocytes produce myelin only during a short hour window, after this time, no new myelin sheaths are made by these oligodendrocytes. Likewise, in toxin-mediated demyelination rodent models, only newly differentiated oligodendrocytes formed from OPCs are able to remyelination and pre-existing mature oligodendrocytes do not remyelinate [193, 194].

In higher vertebrates, it is not still clear at what point after differentiation oligodendrocytes can no longer product new myelin sheaths. If also in these organisms, the oligodendrocytes have myelinogenic potential for only a few hours, there is a very brief window of opportunity for remyelination; consequently, remyelination mechanism probably requires ongoing oligodendrocyte differentiation. In this scenario, events that slow remyelination or oligodendrocyte differentiation, such as inflammation, could block the myelin repair, because oligodendrocytes become unable to myelinate axons.

While in murine models, remyelination is rapid, extensive and associated with functional recovery [195], in MS patients this mechanism is highly variable: extensive in some cases, absent in others [196-198]. In MS subjects, the presence of extrinsic inhibitors in lesions, insufficient pro-regenerative factors and an impaired intrinsic capacity in oligodendrocyte lineage cells could be some of the reasons leading a not efficient remyelination mechanism. Remyelination failure can be divide in two distinct phases: (1) impaired recruitment of OPCs into lesions and (2) an inability to differentiate or mature into remyelinating oligodendrocytes [199, 200]. For example, CSPGs (chondroitin sulfate proteoglycans) inhibit the recruitment of OPCs by attenuating their migration and harming the morphological differentiation of oligodendrocytes [201, 202]. However, oligodendrocyte differentiation and myelination in culture are facilitated by achieving a sufficient OPC density [203], so the incapacity of OPCs to differentiate in MS plaques lesions could be related to an insufficient density within those lesions. Differentiation failure occurs also because chronically demyelinated MS lesions are associated with a low density of mature oligodendrocytes [204, 205].

Strategies that help to promote the proliferation and/or recruitment of OPCs within the demyelinated lesion in the CNS is an important therapeutic goal [206]. Several studies have

identified compounds that promote the recruitment, survival and differentiation of oligodendrocytes that could lead to an improved remyelination (Fig. 15). Benztropine, clemastine, quetiapine and GSK239512S target either muscarinic acetylcholine receptors (such as M1) and/or histamine receptors (such as H1 and H3). Domperidone elevates prolactin levels, which improves remyelination through prolactin receptor (PRLR). Other medications act through specific nuclear receptors: clobetasol activates glucocorticoid receptor (GR), IRX4204 is a retinoid X like receptor (RXR) agonist, liothyronine stimulates thyroid hormone receptor (THR) and vitamin D activates vitamin D receptor (VDR). XAV939 and indomethacin both attenuate inhibitory WNT–β-catenin signalling. AXIN, axis inhibition protein; FZD, Frizzled.

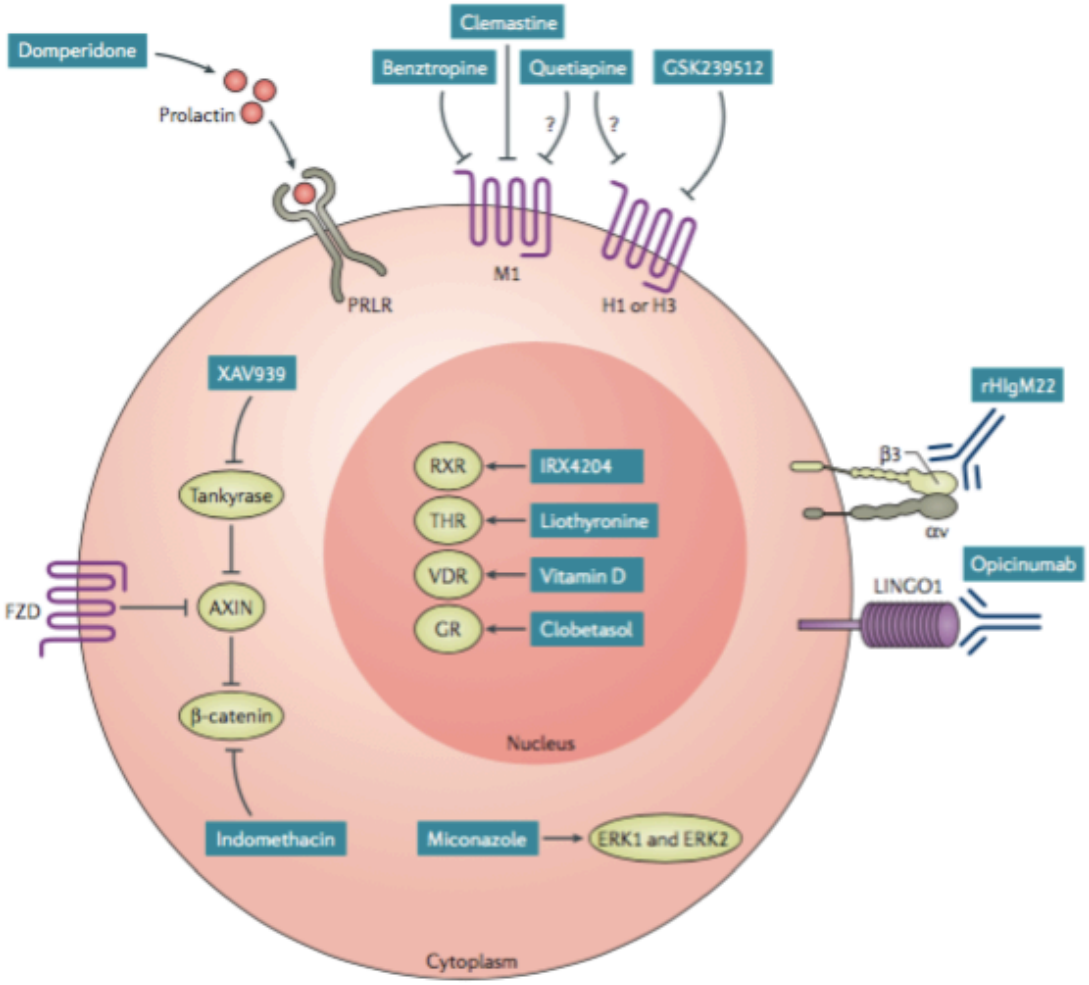


Figure 15. Drugs that affect myelin reformation and are prospective medications for repair in multiple sclerosis. *Adapted from Plemel et al, 2017* [206].

One additional factor that severely affects remyelination is the presence of an immune response following demyelination. The robust clearance of myelin debris is crucial following the degenerative phase [136, 207]; it was observed that when the myelin debris clearance is prevented owing to an impaired phagocyte response, then remyelination is slowed [208-210]. T cells, macrophages or microglia can damage OPCs and oligodendrocytes through mechanisms that include the release of free radicals, granzyme B and inflammatory cytokines [211]. In the other hand, immune cells also can produce a variety of growth factors that promote OPC recruitment and differentiation [212].

As mentioned above, in MS activated myelin-reactive T cells are recruited leading to the activation of microglia and to the recruitment of circulating macrophages. Fingolimod, a structural analog of sphingosine, is converted *in vivo* to fingolimod-P, a structural analog of S1P, which acts as a nonselective agonist for $S1P_1$, $S1P_3$, $S1P_4$, and $S1P_5$ receptors, causing the irreversible internalization and degradation of bound S1P receptors; this preventing their recycling back to the cell surface and the consequently recruitment of T cells.

An alternative therapeutic approach is the use of CNS reactive antibodies to promote remyelination [213, 214]. LINGO1, a membrane-bound protein, is one of the numerous molecules present in the lesion environment that have been identified to be impediments to OPC differentiation and thus potential remyelination inhibitors. Overexpression of LINGO1 in oligodendrocytes, axons [215] or astrocytes inhibits oligodendrocyte maturation via homotypic *trans*-interaction [216], meaning that LINGO1 in the lesion microenvironment can interact with oligodendrocyte LINGO1 to stall remyelination. Neutralization of LINGO-1 using anti-LINGO IgG antibodies has been found to promote remyelination in several animal models [217].

Another group of identified remyelination promoting antibodies, including rHIgM22, have natural autoreactive antibodies (NAbs) features and they are generally of the IgM isotype. These antibodies react to self antigens and, bind them with rather low affinity but high avidity, compared to conventional antibodies [218]. In addition, all remyelination promoting antibodies with identified antigens are polyreactive, as a result of their flexible antigen-binding site. Several of these monoclonal antibodies (mAbs) recognize not only protein antigens but also at least one or multiple sphingolipids. Since only the hydrophilic carbohydrate moiety of the sphingolipids is exposed to the cell surface, it is detectable by antibodies, therefore, it is truthful

not consider the lipid backbone as essential part of the antigen. Currently the most widely accepted hypothesis concerning the remyelination-promoting antibodies mAbs is the lipid-raft hypothesis: mAbs targeting SLs stabilize existing rafts or stimulate the formation of new lipid rafts at the plasma membrane, thereby enhancing the effects of extracellular stimuli via existing cellular signaling pathways [219]. The discovery of these molecules highlights a number of promising approaches to improve remyelination for people with MS.

Proposed mechanism of action of remyelination-promoting IgM

All identified remyelination-promoting antibodies have germline origin or near germline with few somatic mutations, thus all cardinal features of NAbs and represent a subclass of NAbs.
The discovery of natural antibodies for CNS reparative was serendipity. The first successful attempt to stimulate remyelination using NAbs was performed in the Theiler's murine encephalomyelitis virus (TMEV)-induced model of demyelination by Rodriguez et al. in 1987 [220]. To test the hypothesis of virus-induced autoimmunity TMEV-infected SJL mice were immunized with spinal cord homogenates (SCH) of normal mice in order to stimulate a polyclonal antibody response directed against a variety of CNS antigens, including myelin components. Instead of an expected exacerbation of the disease course, mice immunized with SCH showed four-times' higher levels of remyelination than non-immunized mice. These results demonstrated for the first-time a beneficial effect of antibodies in stimulating CNS remyelination. In particular, two monoclonal mouse antibodies of the IgM isotype (SCH79.08 and SCH94.03) proved effective in promoting remyelination [221]. and both antibodies were able to target mature oligodendrocytes (OLs) in vitro. In order to identify monoclonal remyelination-promoting antibodies of human origin from sera of patients with monoclonal gammopathies, cerebellar slice cultures were used in addition to cultured OLs for preliminary screening [214]. This resulted in the identification of two serum-derived human remyelination-promoting antibodies, sHIgM22 and sHIgM46, then engineered to make a recombinant antibody (rHIgM22 and rHIgM46) [48,49]. rHIgM22 has recently been approved by the FDA for Phase I, multi-center, double-blind, randomized, placebo-controlled, dose-escalation study designed to evaluate safety, tolerability, pharmacokinetics, and immunogenicity of single IV administrations of rHIgM22 in patients with all clinical presentations of MS.

Although to date the mechanism of remyelination is not entirely known, what is established is that rHIgM22 is capable to access to the brain. It has been generally accepted that IgMs with a molecular weight of close to 1 million are too large to cross the blood brain barrier (BBB) from the circulation to enter the CNS ([222]. Direct evidence of IgM access to the brain comes from a MRI study using antibodies labeled with ultra-small superparamagnetic iron oxide particles (USPIO) [223]. While MRI imaging showed a co-localization of rHIgM22 with demyelinating lesions in mice spinal cords in Theiler's virus- infected mice, no IgM accumulation in the CNS was apparent in non-infected animals or animals without demyelination. In addition, dysfunction of the BBB is a typical feature of MS [224, 225] therefore, it is plausible to accept IgM access to demyelinated brain lesions as well in the human situation.

Despite mouse and human remyelination-promoting IgMs stimulate repair in TMEV- and lysolecithin-demyelinated mice [214, 221, 226-230], the precise mechanism of action is still unknown. Several pieces of evidence suggest that the rHIgM22 inhibits apoptotic signaling in OPCs and the differentiation of them to mature (MBP+, MOG+) OLs in vitro [231], promoting OPC survival and proliferation [232, 233]. The signaling complex responsible for the anti-apoptotic effect consists of platelet-derived growth factor receptor alpha (PDGFαR), integrin avb3 and the Src family kinase (SFK) Lyn [231] (Fig. 16). PDGFaR is a phenotypic marker of OPCs [234] and retinal astrocytes [235] and its ligand, PDGF, is produced by neurons [236] and astrocytes as PDGF-AA [237] and -AB dimers [238]. Since OPC-signaling complex responsible for rHIgM22-mediated actions including PDGF receptor [231], this suggests an involvement of PDGF. Literature data shows that PDGF stimulates OPC proliferation and survival in vivo [239-241] and in vitro [241-244], but does not mediate survival in cells of the OL-lineage that are more mature than OPCs. Most likely PDGF is required for rHIgM22-mediated inhibition of apoptotic signaling and differentiation in isolated OPC cultures. In fact, rHIgM22-mediated OPC proliferation is detectable only in mixed glial cultures (cultures containing substantial amounts of astrocytes, microglia and OPCs) but not in highly enriched OPC populations [233]; secreted astrocytic or microglial factors, or direct cellular contact between OPCs and other glia seems to be essential for the proliferative response. Considering that PDGF is secreted by astrocytes and possibly microglia [237, 238] but not by oligodendrocyte-lineage cells, it demonstrates an involvement of cells other than OLs in IgM-mediated OPC proliferation and, possibly, remyelination. It appears that three cell types (OPCs, microglia and astrocytes) are required for IgM stimulated proliferation of OPCs *in vitro*. The

growth factor PDGF and potentially other secreted microglial and astrocytic factors are important mediators for this effect.

Although it is clear that the rHIgM22 promotes OPC survival and proliferation, the actual target and cellular type involved in rHIgM22-mediated action are still under investigation. Several pieces of evidence suggest that the antigen recognized by this antibody might be a plasma membrane lipid and that the binding target of rHIgM22 could be associated with detergent-resistant membranes (DRM)/lipid rafts organization [245, 246]. This hypothesis is based on the observation that the well known anti-sulfatide antibody O4 and rHIgM22 have a similar binding pattern to CNS tissues [245], and that binding of rHIgM22 is abolished in CNS tissue sections from CST (-/-) mice [246], suggesting that likely sulfatide might represent a critical player in rHIgM22 biological activity.

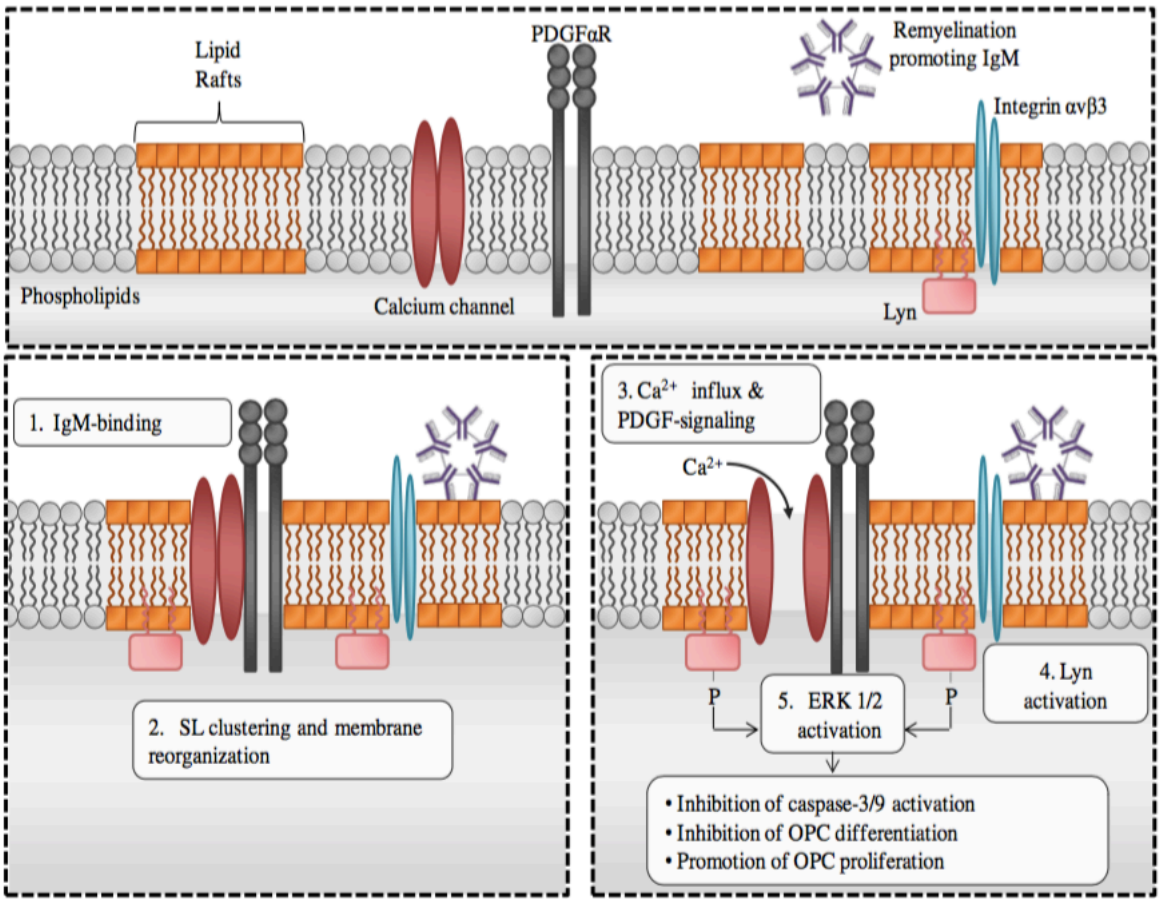


Figure 16. Proposed mechanism of action of rHIgM22. Binding of rHIgM22 to the surface of oligodendrocyte determines a reorganization of the membrane, favoring the interaction of Lyn, integrin αvβ3 and PDGFαR. IgM-stimulated activation of Lyn, with consequent activation of ERK 1/2 determines the inhibition of the apoptotic pathway and of OPC differentiation. Other factors (e.g. PDGF) might be required to promote the proliferation of these cells. *Adapted from Watzlawik et al, 2013* [219].

AIM OF THE STUDY

Treatments for MS have come a long way over the last two decades. All of these drugs act by reducing the immune attack, which causes inflammation and damage to the myelin coating around nerve cells in the brain and spinal cord. Although these drugs can reduce damage to myelin, they can't arrest it completely or repair the injury. Although the body, via remyelination, can replace damaged myelin, this process is impaired in MS. Remyelination, the physiological response after most demyelinating conditions, is a regenerative process whereby demyelinated axons are ensheathed with new myelin sheaths; it occurs through the activity of OPCs that migrate into damage sites [199]. This repair process is characteristic of the early disease phase of MS [247]; however, in late phase of MS, remyelination seems to be limited by oligodendrocyte density, which could be the product of a deficient oligodendrocyte recruitment [200]. When the remyelination fails, the axon, devoid of its myelin sheath, undergoes several physiological changes, resulting in axonal dysfunction, degeneration, and loss of sensory and motor function [248]. Treatments which promote remyelination would offer the potential to delay, prevent or reverse disability and a great deal of research is exploring this.

One of the therapeutic approaches that is currently being developed to improve the remyelination involves the use of CNS reactive antibodies to promote remyelination [227]. One of these antibodies is rHIgM22, a recombinant form of a human IgM, which is able to bind to myelin and to the surface of mature, O4-positive oligodendrocytes in vitro [214], to enhance remyelination in three different mouse models of demyelination [214, 226, 227, 249, 250] and to promote OPC proliferation. Recently, this antibody has completed a Phase 1a/b clinical trial in adults with both stable and active MS [251]. Despite the encouraging clinical data, the exact mechanism of action of rHIgM22 remains to be elucidated.

The aim of this study is to identify the molecular binding target(s) of rHIgM22 and to identify the target cell(s) within CNS, in order to better understand the *in vivo* remyelination capability of rHIgM22. The binding target of rHIgM22 in oligodendrocytes and myelin could be represented by a complex that includes glycolipids, likely sulfatide, and could be associated with plasma membrane lipid rafts. In fact, the anti-sulfatide antibody O4 and rHIgM22 have a similar binding pattern to CNS tissues [245], and in both cases binding is abolished in CNS tissue sections from CST knock-out mice [246]; this suggests that the antigen(s) recognized by rHIgM22 could be a CST-sulfated antigen present in myelin and on the surface of differentiated OLs.

In addition, rHIgM22 has been shown to bind directly to differentiated OLs but binding to immature OPCs has not been detected, therefore no rHIgM22 effects have been observed in these cultures [227, 252]. Since OPCs apparently do not respond to rHIgM22, and differentiated oligodendrocytes are unlikely to be sufficient for substantial remyelination, it is likely that other cells are involved in remyelinating processes. Indeed, rHIgM22 seems to induced OPC proliferation by activating PDGFαR in mixed glial cultures, but not in isolated OPCs, suggesting that the stimulation of OPC proliferation by rHIgM22 requires factors produced by astrocytes [253] and/or microglia [233].

Defining the identity of the binding partners, the molecular organization of their membrane microenvironment and to identify the cell types with which rHIgM22 interacts should greatly contribute to the elucidation of the signaling mechanisms underlying the biological activity of rHIgM22. In addition, the characterization of the mechanism and the factors involved in the proliferation, recruitment, and differentiation of oligodendroglial progenitor cells is key in designing strategies to improve remyelination in demyelinating disorders. This, in turn, would allow to gain a better knowledge regarding the molecular mechanisms involved in MS etiology thus allowing to define new potential therapeutic targets.

MATERIALS AND METHODS

Materials— Ca^{2+} and Mg^{2+}-free HBSS, D-Glucose, BSA fraction V, trypsin, HEPES, phosphate-buffered saline (PBS), poly-D-lysine, poly-L-lysine, Na_3VO_4, NaOH, Na_2HPO_4,$CHCl_3$, CH_3OH, polyisobuthylmethacrylate, *O*-phenylenediamine (OPD), H_2O_2, citric acid, HCl, sucrose, Tris-HCl, 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, StemPro Accutase were purchased from Invitrogen*;* DNaseI from *Roche Spa*; 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 and Silica gel 60 (0.040-0.063 mm) were purchased from *Merck*; CH_3COOH was purchased from *Fluka*; HPA sensor chips and HBS-N Buffer were purchased from *GE Healthcare Srl*. Chrompure Human IgM was purchased from *Jackson Immuno Research, Inc*. and HRP-conjugated anti-Human IgM μ-chain antibody was 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 purified in our laboratories.

Animal specimens— For the experiments reported in this thesis, the sources of animal specimens were:

- wild type (WT) C57BL/6N mice and acid sphingomyelinase knock-out (ASM(-/-)) mutant C57BL/6N mice [254], used for brain lipid extraction, myelin purification and preparation of mixed glial cultures;
- primary cultured rat oligodendrocytes positive for rHIgM22 staining (kindly prepared by Dr. Yana Zorina, Acorda Therapeutics, Ardsley, NY);
- primary cultured rat microglia [255], kindly provided by Dr. Michela Matteoli, Department of Medical Biotechnology and Translational Medicine, University of Milano;
- hemibrains from wild type and cerebroside sulfotransferase (CST) (+/-) and (-/-) mice [162], kindly provided by Dr. Xianlin Han, Sanford-Burnham Medical Research Institute, FL, USA.

Mixed glial cell (MGC) culture— A primary mixed glial culture, composed of astrocytes, oligodendrocytes, and microglia, is obtained when newborn disaggregated cerebral brain cells from rat or mouse are plated at high cell density in serum-supplemented medium[233, 256]. In this culture model, neurons fail to survive and, after one week, mixed glial cell cultures are free of neurons, meningeal cells, and fibroblasts. MGC cultures were prepared according to Watzlawik et al [233]. Briefly, the hemispheres from P2 mice brains were minced with a surgical blade and then incubated for 30' at 37°C in 0.05% trypsin in modified HBSS (Ca^{2+} and Mg^{2+} free HBSS containing 5 g/L D-glucose, 3 g/L BSA fraction V, 20 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin). Following the addition of $MgSO_4$ and DNase I, the sample was centrifuged at 200 g at 8°C for 5 minutes and resuspended in modified HBSS. The tissue was then further dissociated by trituration through a sterile flame narrowed glass pipette, centrifuged at 200 g at 8°C for 10 minutes, resuspended in culture medium and plated on Petri dishes or T75 flasks coated with poly-D-lysine (25 µg/mL). The cells were cultured in DMEM high glucose containing 10% heat inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 2 mM glutamine and the culture medium is changed every 3/4 days. With this protocol it is possible to obtain cultures with about 60-70% of astrocytes, 30- 40% of OPCs, and less than 3% of microglial cells.

Oligodendrocytes isolation—Oligodendrocytes were harvested, through shaking procedure, from 8-10 days old mixed glial cell cultures, prepared as described in the previous paragraph, when the cells 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 surnatant was discarded and the pellet resuspended in 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. 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. Mixed glial cell culture that underwent a shaking procedure

to isolate oligodendrocytes precursors were used maximum three times. After the third time, the adherent layer was represented mostly by astrocytes, thus allowing to collect these cells. 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 [257]. 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. 50 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 resuspended 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, resuspended 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 resuspended 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, resuspended 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 resuspended 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, resuspended 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 were thawed at RT, resuspended 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, the pellet was resuspended 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.) [258]. In brief, 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 resuspended in 1M NaOH and incubated overnight at RT before being diluted 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 resupended 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 [258].

Chromatography

Chromatography is an important biophysical technique that allows the separation, identification, and purification of the components of a mixture for quantitative and qualitative analysis [151]. The word "chromatography", used by Tswett in 1906, derived from the Greek words *chroma* (color) and *graphien* (to write) and it is defined as: "A physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction."

There are different types of chromatography but they all work on the same principle; all chromatographic systems have a mobile and a stationary phase: sample is dissolved in the mobile phase, which travels through the stationary phase. Separation is possible because different compounds have different affinities for the mobile and stationary phases, thereby affecting their distribution between the two phases.

Chromatographic methods are classified according to mobile phase, stationary phases, separation mechanism or type of support. The mobile phase may be either a gas (GC, Gas Chromatographic) or liquid (LC, Liquid Chromatographic); the stationary phase may be a liquid or, more usually, a solid. *Table 2* summarizes some of the chromatographic methods that have been developed on the basis of different mobile–stationary phase combinations.

By state of phases and mechanism			
Mobile phase	**Stationary phase**	**Acronym**	**Mechanism**
Gas	Solid	GSC	Adsorption Chromatography
	Liquid	GC	Partition Chromatography
Liquid	Solid	LSC	Adsorption Chromatography
	Liquid	LC	Partition Chromatography
Liquid		CE	Electromigration

By the polarity of phases			
	Stationary phase	**Mobile phase**	
Normal phase Chromatograpy	More polar	Less polar	LC
Normal phase Chromatograpy	Less polar	More polar	LC

By geometry of the separation region	
Planar Chromatograpy	2-dimensional
Column Chromatography	1-dimensional

Table 2. Chromatographic methods.

The chromatographic procedures used to identify the antigen recognize by rHIgM22 are discussed in the next section.

Thin-layer chromatography

Thin layer chromatography (TLC) is the easiest way to analyze the total lipid mixtures extracted from tissues and cultured cells.

In TLC the stationary phase is a thin layer of a solid such silica supported on an inert base such as glass o aluminum foil. The mixture is applied at the bottom of the TLC plate and allowed to dry, then the plate is placed in a closed tank containing solvent (the mobile phase) so that the liquid level is below the spot (Fig. 17).

This method allows to have an economical first information about the possible structure of the separated molecules on the basis of their chromatographic mobility in comparison with standards and of their reactivity to the staining procedures [258, 259].

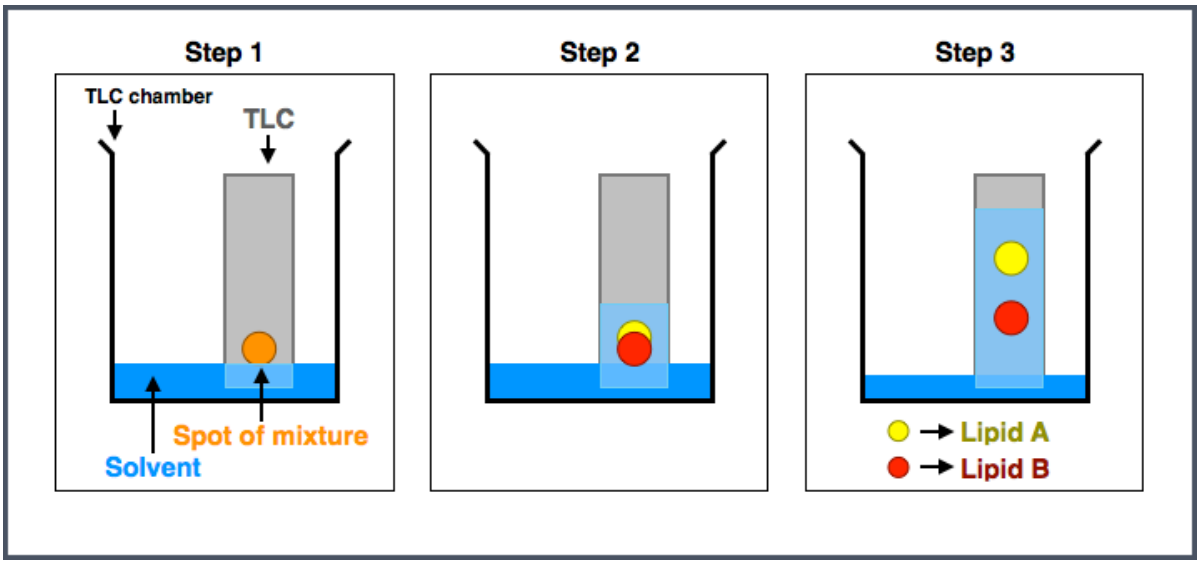


Figure 17. Development of a TLC plate. An orange spot separates into a red and yellow spot.

TLC Procedure. Lipids and samples were separated using HPTLC plate (silica gel 60 HPTLC, Merck): the samples were spotted on a 3 mm imaginary lane at 1.5 cm from the plate bottom edge and were applied maintaining a 3–5 mm distance. The plate was immersed into the chromatographic solvent system (1 cm deep) and put in a closed tank allowing the solvent to reach the TLC top edge (TLC size, 10×10 cm or 10x20 cm). Chromatography was carried out at room temperature in the range of 20–25°C. Depending on the samples to be analyzed, the following solvent systems have been used:

- $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) for the analysis of pure sulfatide, lysosulfatide, neutral glycolipids, pure phospholipids, total lipid extracts, organic phases and methanolized organic phases;
- $CHCl_3$:CH_3OH:0.2% $CaCl_2$ in H_2O 50:42:1 (v/v/v) for the analysis of pure gangliosides and aqueous phases;
- $CHCl_3$:CH_3OH:$CH_3CH(OH)CH_3$:H_2O 70:20:5:3 (v/v/v/v) for the analysis of column chromatography fractions;

After separation, lipids were detected by spraying the TLC plates with Anisaldehyde reagent for the general detection of lipids.

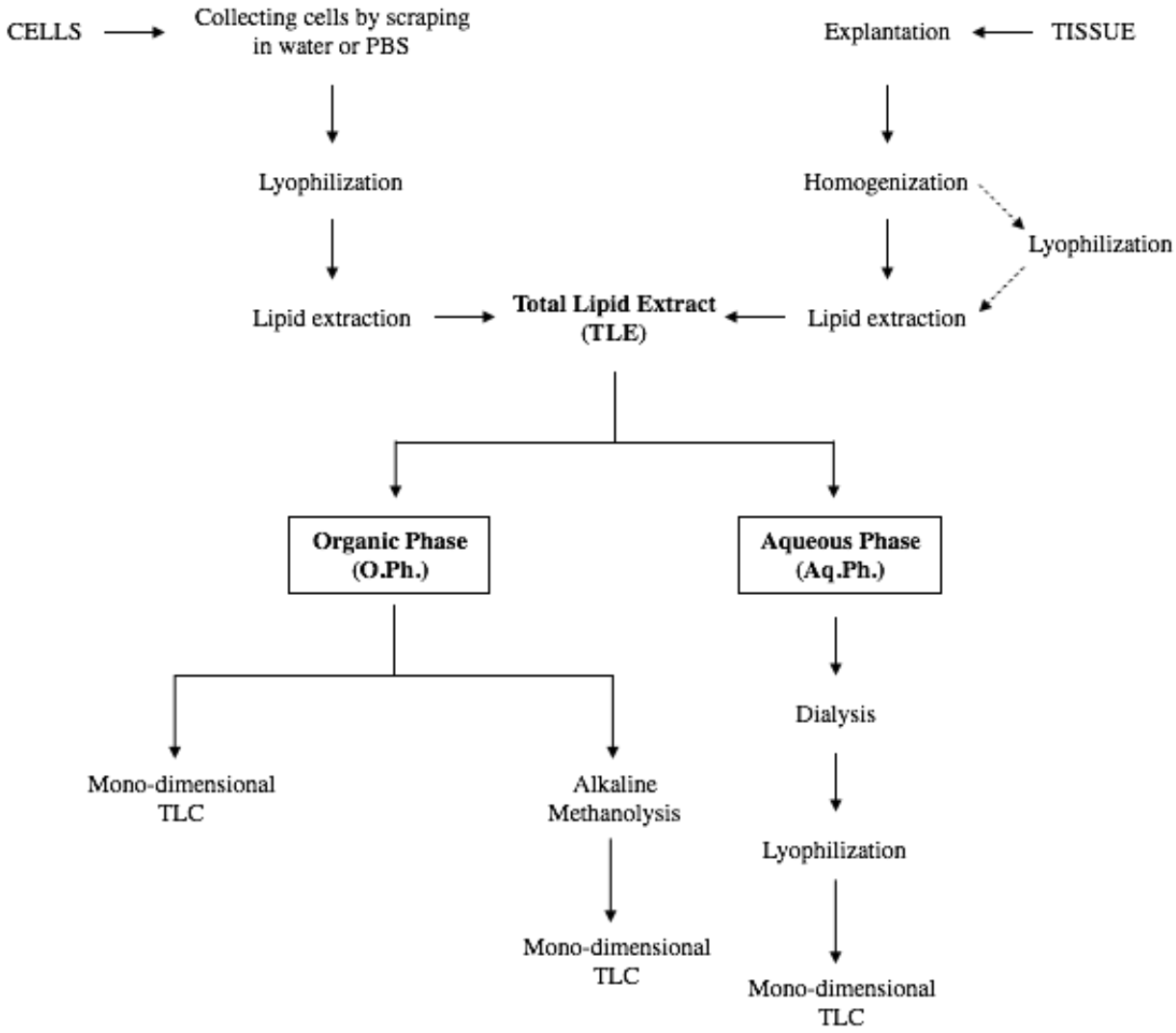


Figure 18. Schematic representation of the analytical pathways for complex lipids TLC. *Adapted from Scandroglio et al, 2009* [258].

TLC immunostaining

TLC immunostaining is a simple, rapid and sensitive method for the detection of antigenic targets of antilipid antibodies; as described by Magnani et al. [259], the procedure consists in two essential steps: separation of lipids on a TLC plate and detection of lipids *in situ* with specific antibodies (Fig. 19).

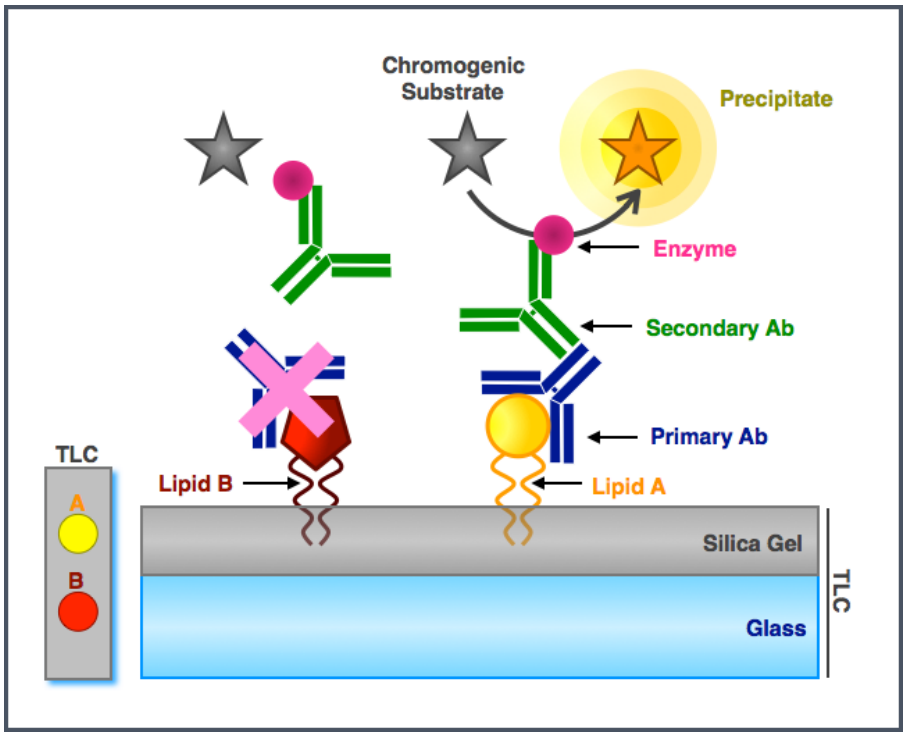


Figure 19. TLC overlay assay schemes of antibody-mediated GSL detection. *Adapted from Meisen et al, 2011* [260].

TLC immunostaining Procedure. After chromatographic separation (as described above), the TLC plates were coated three times with a polyisobuthylmethacrylate solution [258], and air dried for 1 hour before being immersed in blocking solution (3% BSA in PBS) for 1 hour. After washing thrice (1 min each) with washing buffer (PBS), the plates were then incubated with rHIgM22 or isotype human IgM (Chrompure Human IgM; Jackson Immunoreasearch; negative control) at 0.5, 1.0, 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 and 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.

Column Chromatography

Column chromatography allows to separate a mixture into the pure individual components, to remove impurities and to identify unknown compounds. In column liquid chromatography, the mobile phase is liquid (an organic solvent or a mixture of solvents) and the stationary phase (silica or alumina) can be either solid or liquid supported by an inert solid. The stationary phase is packed

into a column (glass or metal); its length and diameter vary by the amount of sample to be analyzed, the separation mode to be used, and the degree of resolution required. There are two procedure to prepare the column by packing with silica: dry or wet method. In dry method the column is filled with dry powdered silica and then the mobile phase is flushed through it until all the silica is evenly wet (equilibrated). The sample is dissolved in a small volume of mobile phase and it is applied at the top of the column. Classical or low- pressure chromatography utilizes gravity flow or a peristaltic pump to maintain a flow of eluent (mobile phase) through the column. Flash chromatography is an alternative to gravity-fed chromatography and it is a rapid with high resolution technique. It utilizes smaller silica gel particles (250-400 mesh) and for this reason it needs at pressurized gas (i.e. nitrogen gas) to restricted flow of solvent caused by the small gel particles.

Column Chromatography Procedure. The aqueous phase from WT mouse brain (~ 30 mg) was purified by flash chromatography with silica gel 60 column (1 cm, internal diameter, with 20 cm of Silica gel 60, 0.040-0.063 mm) equilibrated and subsequently eluted with $CHCl_3$:CH3OH:$CH_3CH(OH)CH_3$:H_2O 70:20:5:3 (v:v:v:v). The eluting solvents and the lipids obtained in each eluate were collected as fractions, each with a volume of 3 mL. TLC is then used to determine which fractions contain the compound. The structural analysis of the purified lipids was performed by ESI-MS.

Electrospray Ionisation Mass Spectrometry (ESI-MS)

Mass spectrometry is an analytical technique used to identify unknown compounds within a sample. The process consists in the conversion of the sample into gaseous ions which are then characterized by their mass to charge ratios (m/z). The mass spectrometry experiment consists in (1) the ionization of molecules in the gaseous phase, in (2) the separation of the different ions according to their specific mass to charge ratio (m/z) and in (3) their detection. For this reason, the mass spectrometry instrument consists of three major components: (1) ion source, for producing gaseous ions from the sample under investigation; (2) analyzer, for resolving the ions into their characteristics mass components according to their mass-to-charge ratio; (3) detector system, for detecting the ions and recording the relative abundance of each of the resolved ionic species (Fig. 20).

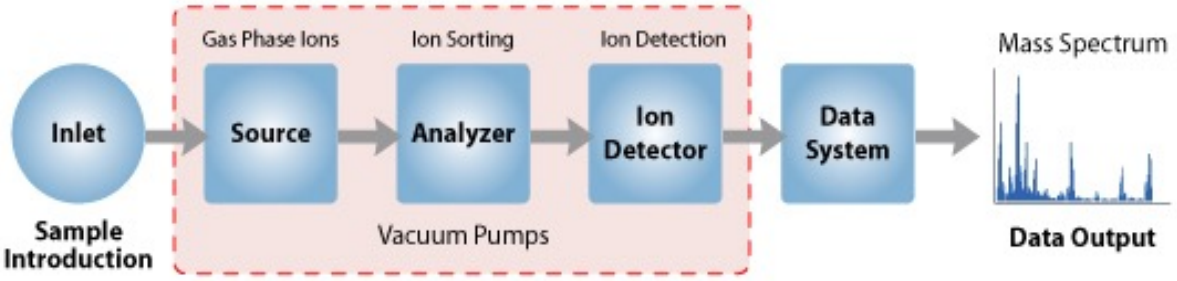


Figure 20. Basic components of the ESI-mass spectrometer.

In ESI-mass spectrometer, electrical energy is used to assist the transfer of ions from solution into the gaseous phase by protonation or cationisation. The transfer of ionic species from solution into the gas phase by ESI involves three steps: (1) dispersal of a fine spray of charge droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplets [261] (Fig. 21).

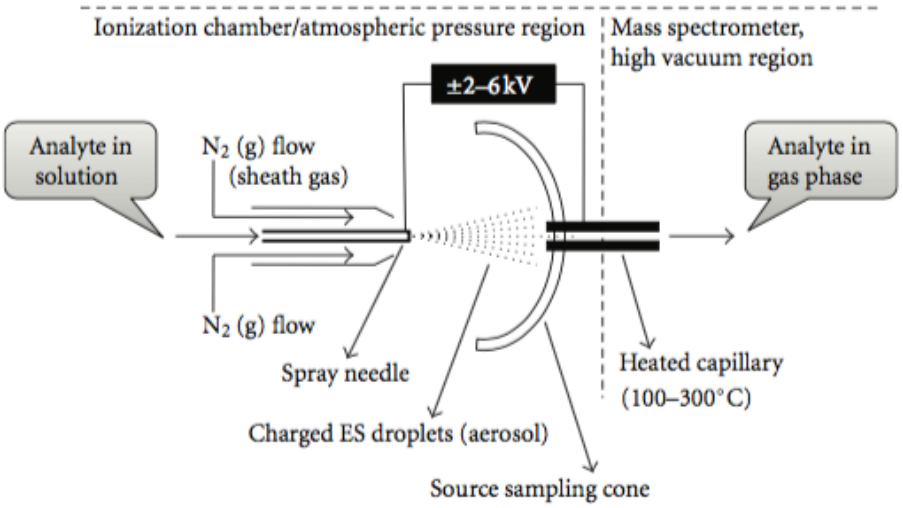


Figure 21. A schematic representation of the ESI-ion source. *Adapted from Banerjee et al, 2012* [262].

The molecular ions produced in the ionization chamber are then transferred in the mass analyzer region via several ion optics (electromagnetic elements like skimmer, focusing lens, multipole, etc.). The mass analyzer separates the ions according to their *m*/*z* value. The separated ions are then passed to the detector systems to measure their concentration, and the results are displayed on a chart called a mass spectrum. The ions in the gas phase are very reactive and often short lived; for

this reason, the ion optics, analyzer and the detectors are kept at very high vacuum (typically from 10^{-3} torr to 10^{-6} torr pressure).

Tandem mass spectrometry, also known as MS/MS, involves multiple steps of mass spectrometry selection. It is a technique to break down selected ions (precursor ions) into fragments (product ions). The fragments then reveal aspects of the chemical structure of the precursor ion. Once samples are ionized by ESI to generate a mixture of ions, a precursor ion of a specific *m/z* is selected (MS1) and then it is fragmented (MS2) applying a voltage 30-50 times higher than the previous one to generate a product ion for detection.

ESI-MS Procedure. The analyses of the compound were carried out using a ThermoQuest Finnigan LCQ Deca ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization (ESI) ion source, an Xcalibur data system, and a TSP P4000 quaternary pump. The conditions for MS analysis, in negative mode, were the following: sheath gas flow, 50 arbitrary units; spray voltage, 4 kV; capillary voltage, -47 V; capillary temperature, 260°C; and fragmentation voltage (used for collision- induced dissociation), 50%.

Surface plasmon resonance

Surface plasmon resonance (SPR) is an optical technique utilized for detecting molecular interactions. Binding of a mobile molecule (analyte) to a molecule immobilized on a thin metal film (ligand) changes the refractive index of the film [263].

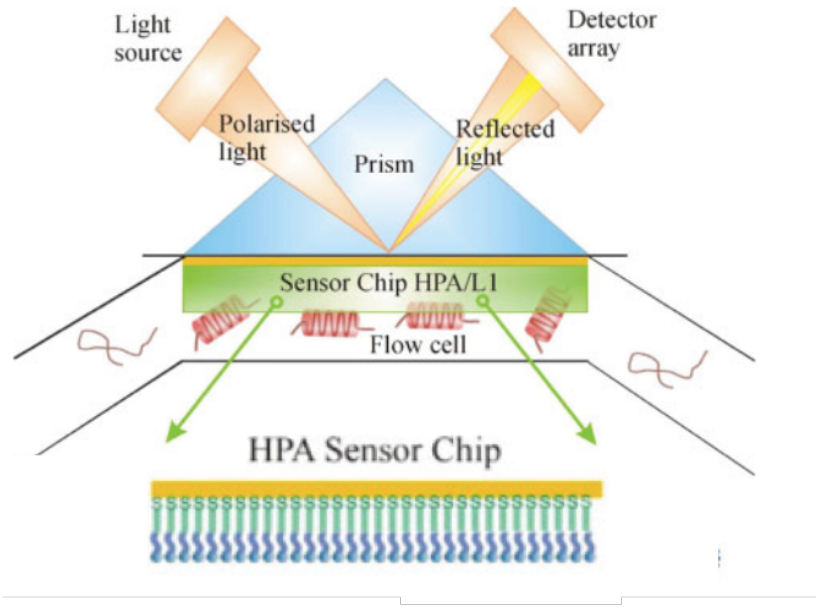


Figure 22. Binding of peptides onto the immobilized lipids (HPA) surfaces in the BIACORE system. *Adapted from Mozsolits at al, 2003* [264].

SPR represents a much more physiological experimental setting respect to the TLC immunostaining (where the lipids are adsorbed on a silica gel surface, not being organized as a lipid monolayer), and should allow to obtain information on the effect of the presence of other lipids on the ability of antibody to bind the target.

The affinity of IgM22 for different purified lipids in supported lipid monolayers has been assessed using the sensor chip HPA (Fig. 22), which bears a flat hydrophobic surface consisting of long-chain alkane thiol molecules attached directly to a gold film. Preformed liposomes adsorb spontaneously to the surface of the chip to form a supported lipid monolayer, with polar head groups directed out towards the solution containing the antibody.

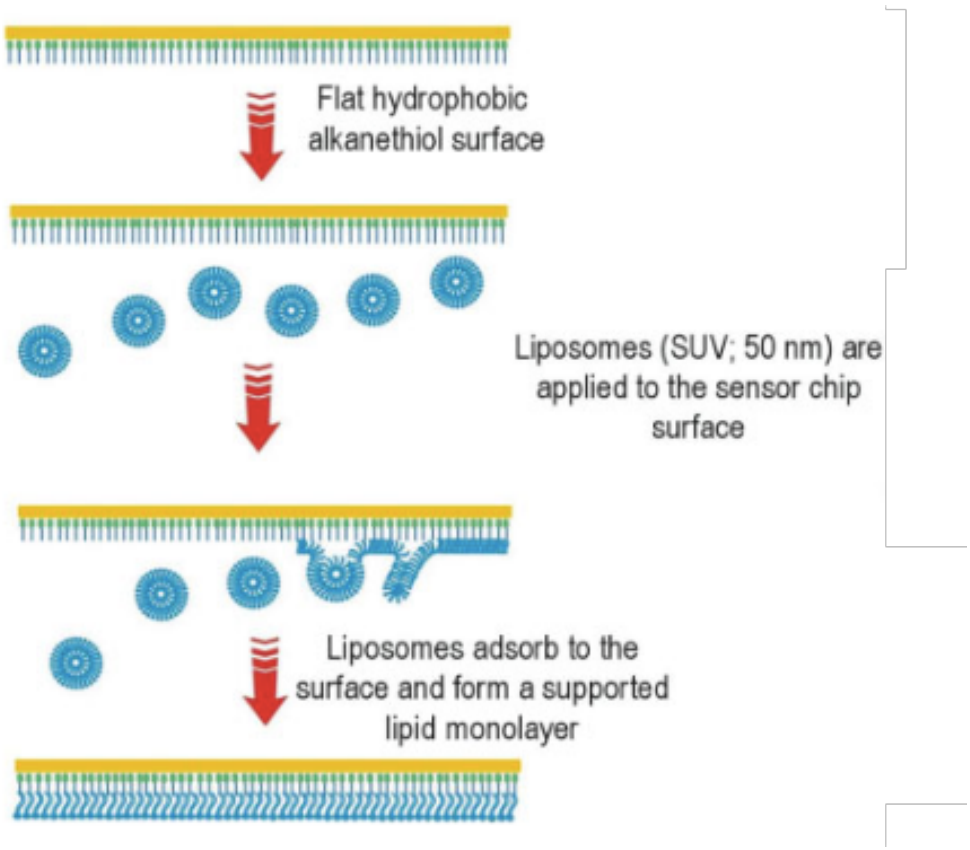


Figure 23. The preparation of a monolayer and a bilayer model membrane system on an HPA sensor chip. *Adapted from Mozsolits at al, 2003* [264].

SPR Procedure. The affinity of rHIgM22 for different purified lipids in supported lipid monolayers was assessed by SPR using a BIAcore 3000 analytical system (GE Healthcare, Uppsala, Sweden) with HPA sensor chips. Chips were incubated in the presence of DOPC
liposomes containing different amounts of the target lipids. Liposomes containing the target lipids were prepared following standard procedures. Briefly, different amounts of sulfatide (1 – 0.1 – 0.01 – 0.001 - 0.0005 μmol) in chloroform/methanol 2:1 were mixed with 1 μmol of DOPC, either alone or in presence of different amounts of a second lipid (cholesterol, GalCer, SM, lysosulfatide), 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. The sensor chip were 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 without or with

1 mM $CaCl_2$ and 1 mM $MgSO_4$ 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 [265].

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

In vitro binding of rHIgM22 to purified lipids

In order to identify the antigen(s) recognized by rHIgM22, we have tested the binding of rHIgM22 to purified lipids *in vitro* using TLC immunostaining and surface plasmon resonance on lipid monolayers with different composition.

Binding to sulfatide

Data published by Rodriguez et al. demonstrated that rHIgM22 is able to bind to myelin and to the surface of oligodendrocytes *in vitro* [214, 246] and that it binds to CNS tissue sections with a pattern similar to that of O4, an anti-sulfatide antibody [245], suggesting that sulfatide could be one of the antigens recognized by this remyelination-promoting antibody.

Starting from this observation, the first purified lipid we analyzed was sulfatide. **Fig. 24** shows rHIgM22 binding to different amounts of pure sulfatide, from 0.05 to 10 nmol; the binding was proportional to the amount of sulfatide up to 10 nmoles. Increased binding was observed by increasing the concentration of antibody from 2.5 μg/mL to 5.0 μg/mL, however below 2.5 g/mL the binding was not quantitatively proportional to the antibody concentration. Binding was very weak at antibody concentrations of 1.0 μg/mL or lower. Significant binding was observed also for isotype IgM control; however, this binding was significantly lower than that of rHIgM22 in all the experimental points, except for the IgM concentration of 0.5 μg/mL.

To confirm the specificity of rHIgM22 binding to sulfatide, we set up SPR experiments using lipid monolayers, an experimental setting that allows an antigen presentation closer to the one occurring in a biological membrane.

As shown in **Fig. 25**, rHIgM22 at 5 g/mL binding to sulfatide in DOPC monolayers on HPA chip, at all the amounts of the target lipid (0.0005-1 mol), was significantly higher than the binding of control IgM. Moreover, the shape of the sensorgrams was consistent with a specific binding for rHIgM22. With a DOPC:sulfatide 1:0.01 molar ratio, the maximum response for rHIgM22 was two-fold higher than the maximum response for control IgM.

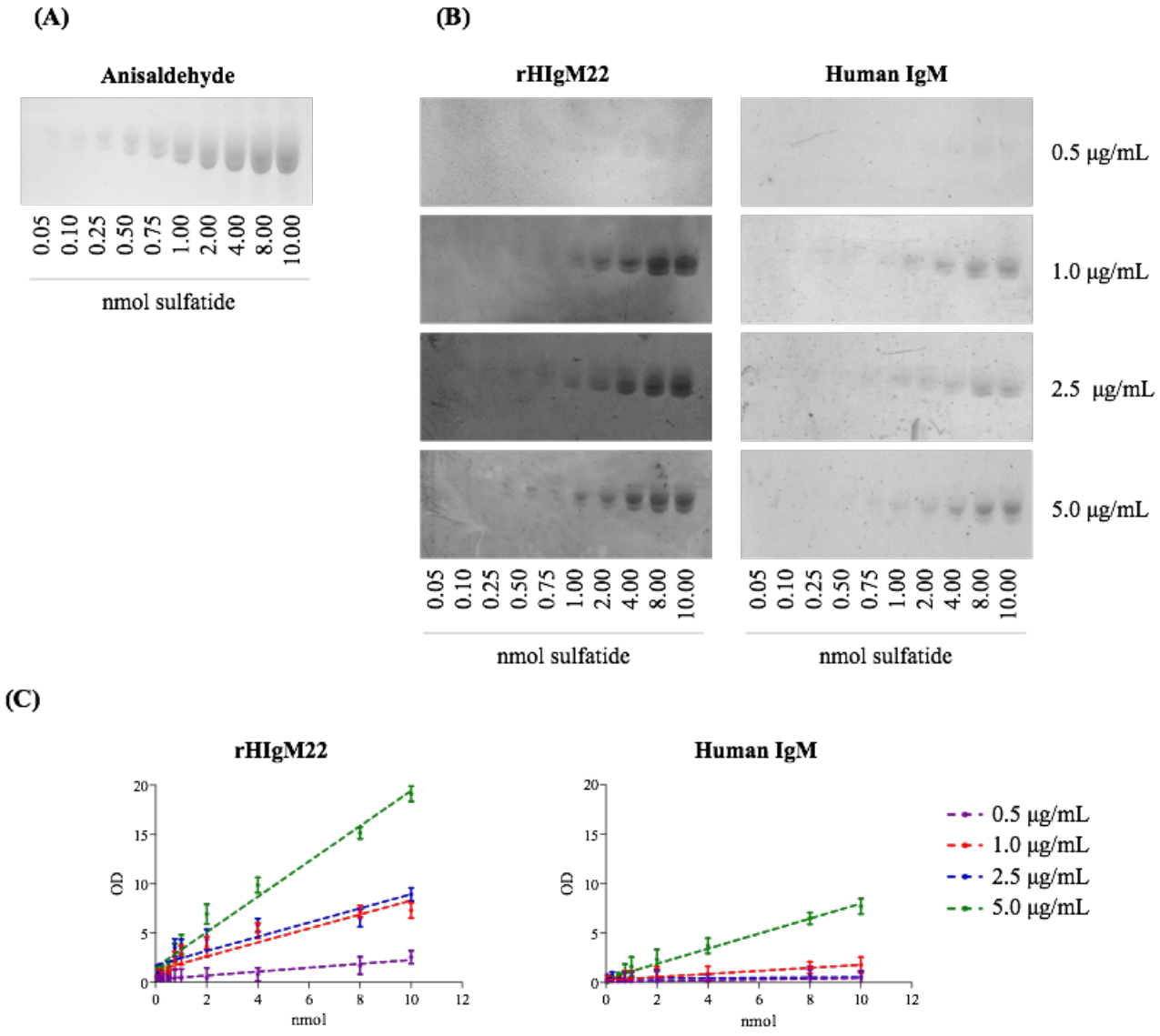


Figure 24. Thin layer chromatography (TLC) and TLC-immunostaining of purified sulfatide with rHIgM22.

(A) Different amounts of purified sulfatide (from 0.05 to 10.00 nmol) were separated by TLC using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent. (B) After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with different concentrations (0.5, 1.0, 2.5 and 5.0 µg/mL) of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods". (C) Optical density (OD) of each band was calculated by densitometry; the data are expressed as mean ± SD of three independent experiments.

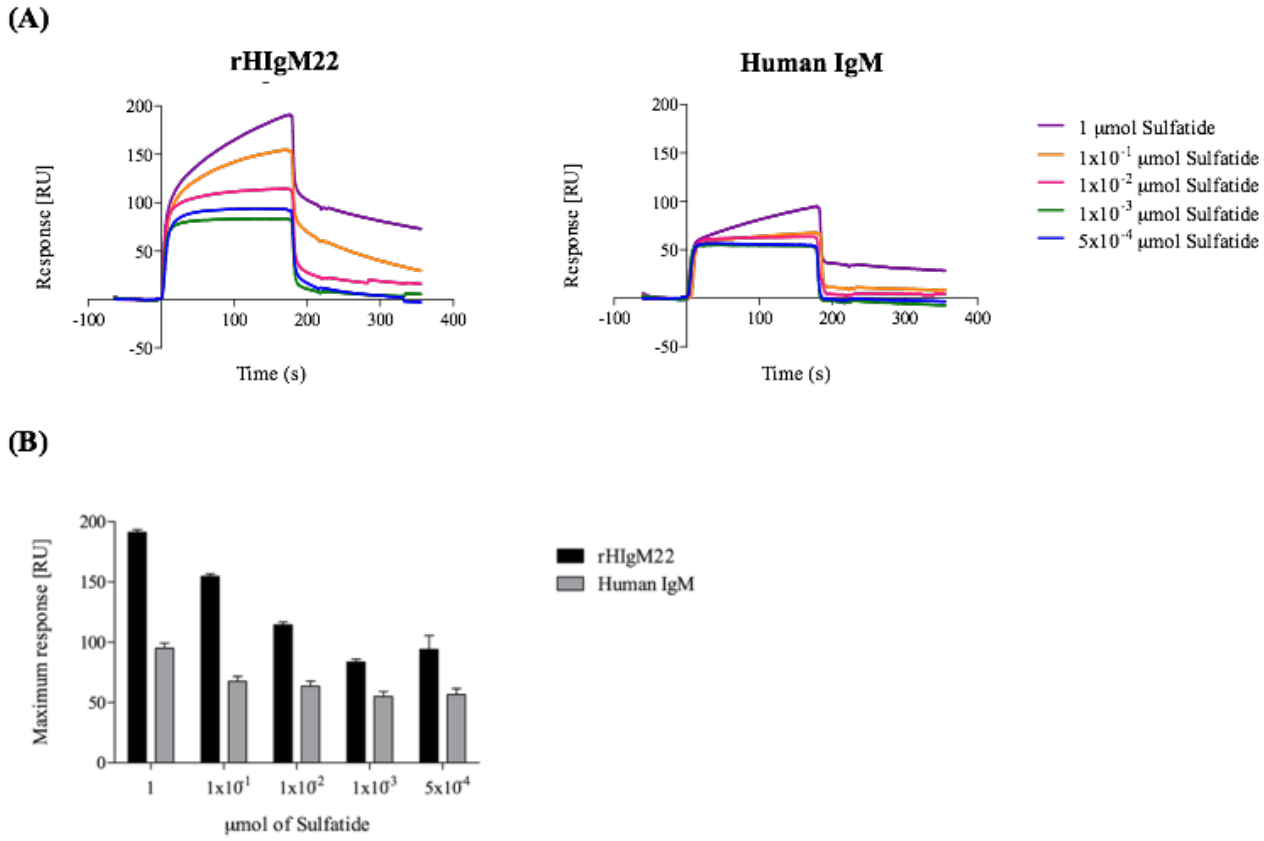


Figure 25. Binding of rHIgM22 to sulfatide in DOPC monolayer.

(A) Lipid monolayers were formed in HBS-N buffer as described in "Methods" from small unilamellar vesicles composed of DOPC (1 µmol) and different amounts of sulfatide (from 5×10-4 µmol to 1 µmol); the liposome solutions were immobilized on an HPA sensor chip. The ability to anchor sulfatide was then assayed by injections of rHIgM22 or Human IgM (5.0 µg/mL) at a flow rate of 10 µL/min across the different monolayers. (B) The binding of the two antibodies is represented also as maximum response; the data are expressed as mean ± SD of three independent experiments, * $p<0.05$

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 [266-268]. 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, rHIgM22 was able to recognize lysosulfatide and the surface plasmon resonance experiments confirmed that these binding was specific (**Fig. 26**).

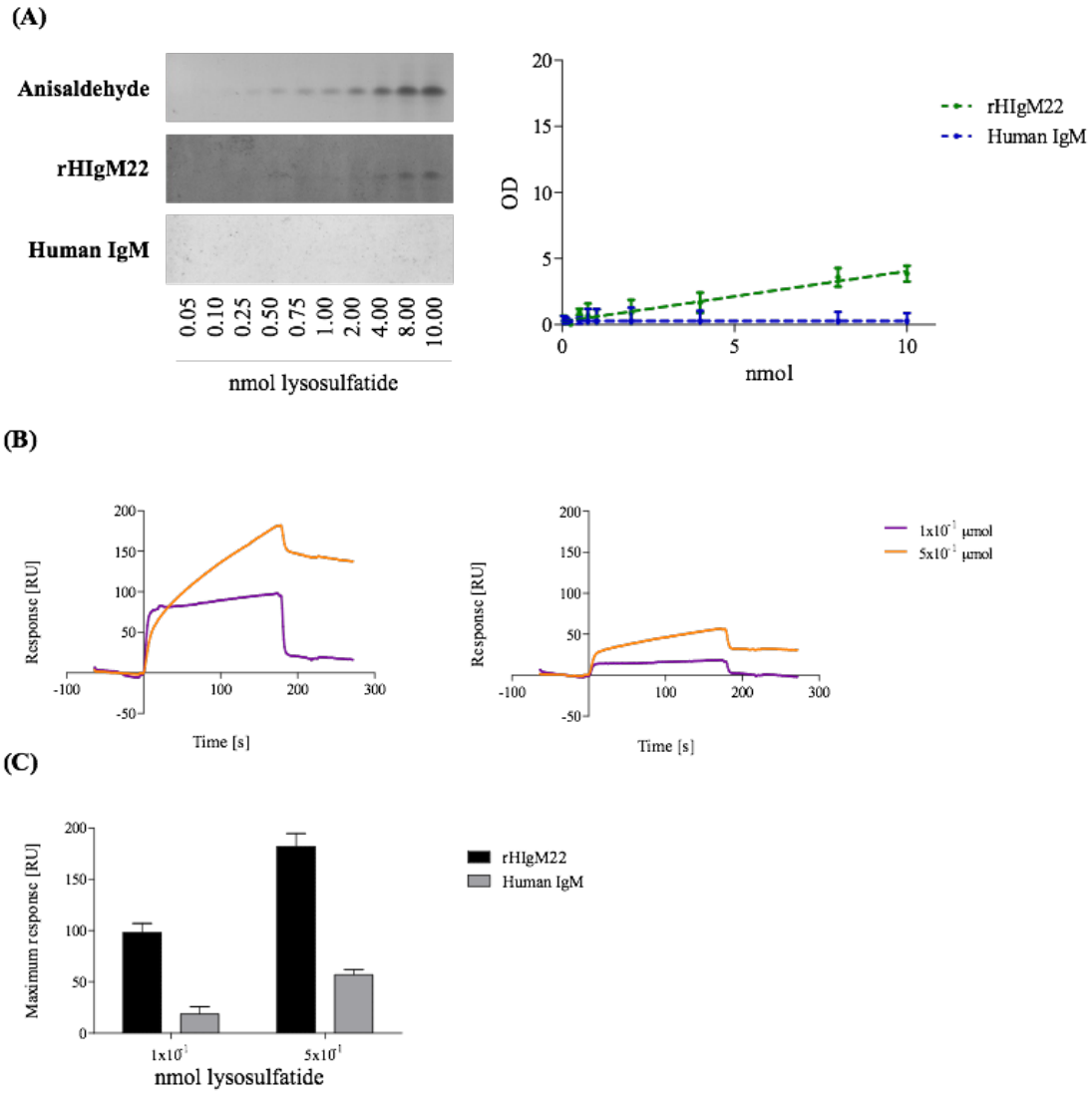


Figure 26. Thin layer chromatography (TLC) and TLC-immunostaining of purified lyosulfatide with rHIgM22.

(A) Different amounts of purified lyosulfatide (from 0.05 to 10.00 nmol) were separated by TLC using CHCl3:CH3OH:H2O 110:40:6 (v/v/v) as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent. After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 µg/mL of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods". The optical density (OD) of each band was calculated by densitometry; the data are expressed as mean ± SD of three independent experiments. (B) Lipid monolayers were formed in HBS-N buffer as described in "Methods" from small unilamellar vesicles composed of DOPC (1 µmol) and different amounts of lysosulfatide (0.1 µmol or 0.5 µmol); the liposome solutions were immobilized on an HPA sensor chip. The ability to anchor lysosulfatide was then assayed by injections of rHIgM22 or Human IgM (5.0 µg/mL) at a flow rate of 10 µL/min across the different monolayers. (B) The binding of the two antibodies is represented also as maximum response; the data are expressed as mean ± SD of three independent experiments, * $p<0.05$

Binding to glycolipids

The myelin membrane is enriched in glycolipids (31% vs. 7% for liver cell plasma membranes) [151], in particular galactosylceramide (~20% of total lipids) [154]. On the other hand, gangliosides, the most abundant glycosphingolipids in the nervous system, are a minor component of the myelin membrane (<1% of total lipids) [269]. So, using the previous experimental setting, other potential glycolipid targets have been screened.

rHIgM22 at 5.0 g/mL showed no significant binding to sphingolipids such as GalCer, SM, LacCer (**Fig. 27A**), GlcSph and GlcCer (**Fig. 27B**) and mono- (GM3, GM2, GM1) and poly-sialogangliosides (GD3, GD1a and GD1b) (**Fig. 28**). While control IgM at 5.0 g/mL showed no significant binding to GalCer, LacCer, SM, in the case of gangliosides the binding was slightly higher than binding of rHIgM22.

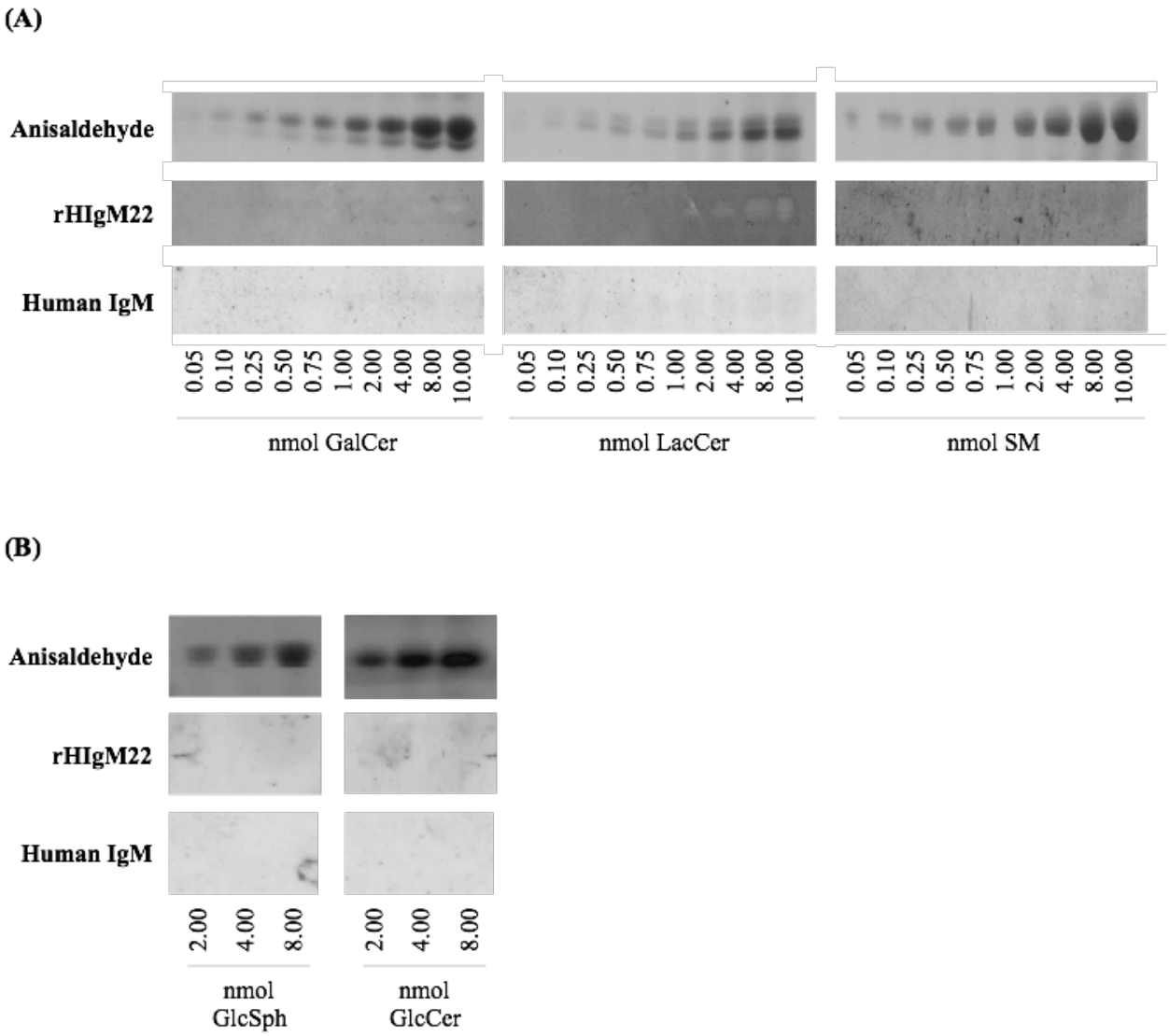


Figure 27. Thin layer chromatography (TLC) and TLC-immunostaining of neutral glycolipids with rHIgM22.

(A) Different amounts of purified GalCer, LacCer and SM (from 0.05 to 10.00 nmol) or (B) GlcSph and GlcCer (from 2.00 to 8.00 nmol) were separated by TLC using CHCl3:CH3OH:H2O 110:40:6 (v/v/v) as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent. After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 μg/mL of the two primary antibody of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods".

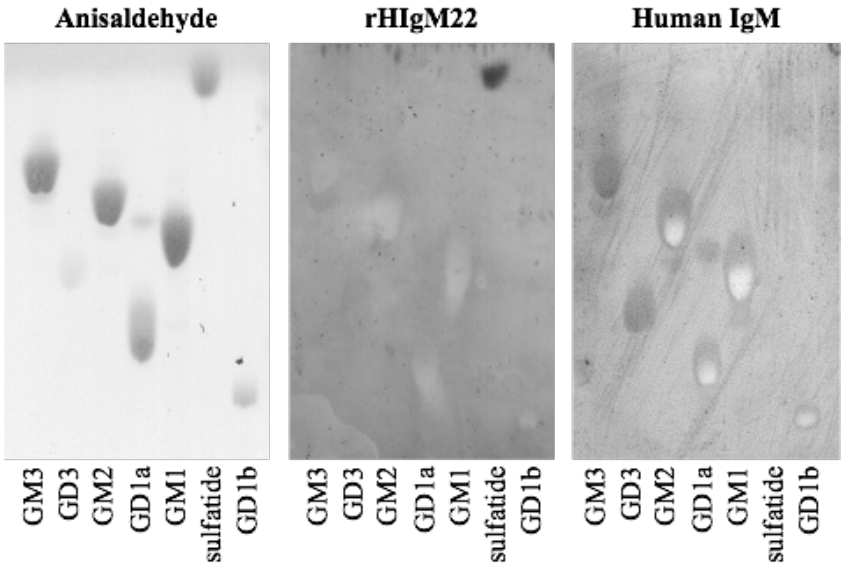


Figure 28. Thin layer chromatography (TLC) and TLC-immunostaining of purified gangliosides with rHIgM22.

4.00 nmol of each purified gangliosides were separated by TLC using CHCl3:CH3OH:CaCl2 0,2% 50:42:11 (v/v/v) as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent. (B) After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 μg/mL of the two primary antibody of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods".

Binding to glycerophospholipids

Since glycerophospholipids are the major component of biological membranes, the binding of rHIgM22 to glycerophospholipids was assessed through TLC immunostaining.

Under experimental conditions similar to those used to assess binding to sulfatide, rHIgM22 showed no significant binding to PC and a non-specific binding to PE (**Fig. 29**). rHIgM22, on the other hand, showed a significant binding to phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidyinositol (PI); in this case, the control IgM gave no significant binding, suggesting that binding of rHIgM22 to these lipids might be specific.

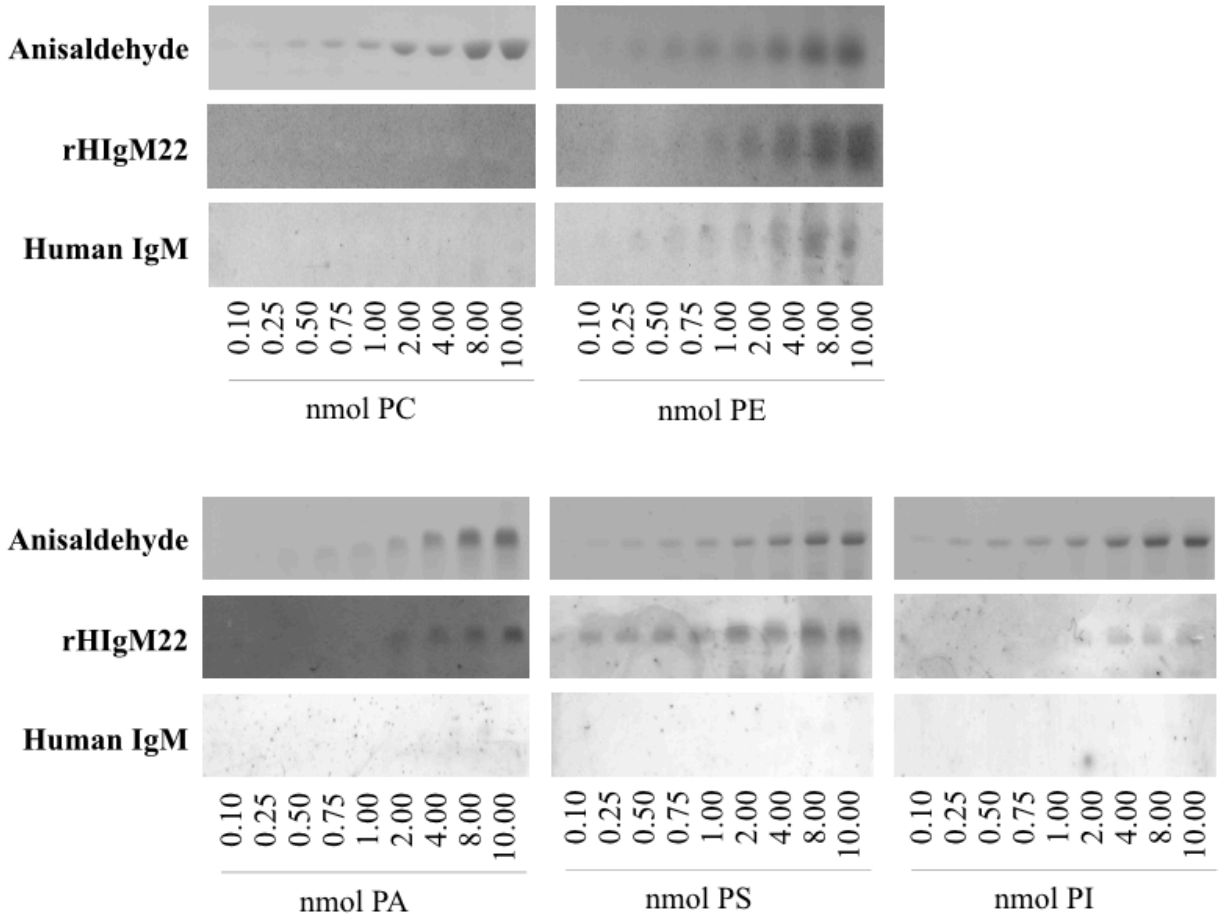


Figure 29. Thin layer chromatography (TLC) and TLC-immunostaining of purified phospholipids with rHIgM22.

Different amounts of purified PC, PE, PA, PS and PI (from 0.10 to 10.00 nmol) were separated by TLC using CHCl3:CH3OH:H2O 110:40:6 as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent. After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 μg/mL of the two primary antibody of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods".

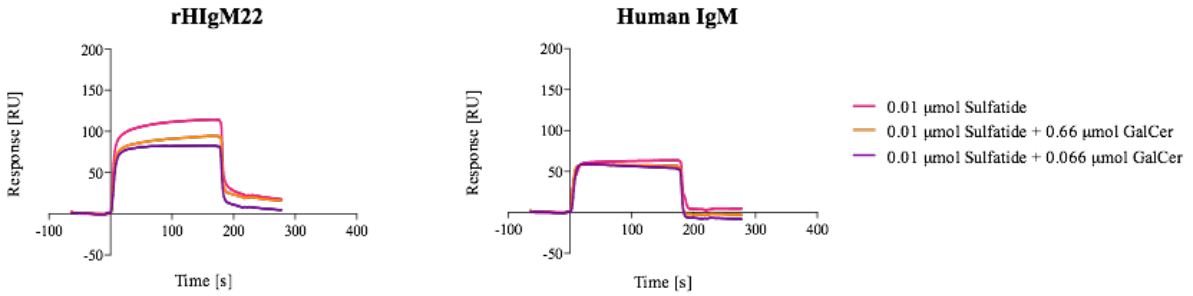
Effect of different lipids on the binding of rHIgM22 to sulfated containing monolayers

Model membrane studies have shown that sulfatides are protected against antibody recognition in a sphingomyelin/cholesterol environment compared to a phosphatidylcholine/cholesterol environment, suggesting that also the binding of rHIgM22 could be affected by lipid microenvironment [270, 271].

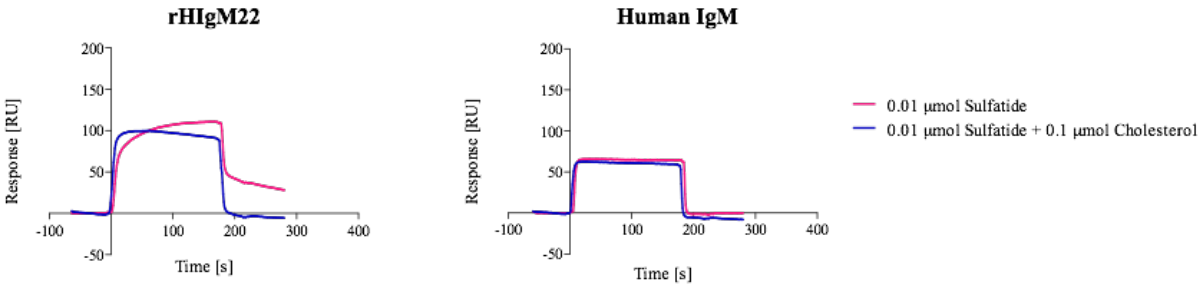
Therefore, SPR experiments have been used also to analyzed the effect of the presence of a second lipid on rHIgM22 binding to sulfatide. In these experiments, monolayers were prepared by mixing a fixed amount of DOPC and a fixed amount of sulfatide (0.01 μmol) with a third lipid using molar ratios corresponding either to those found in the myelin membrane or to a 10 fold higher amount of the third lipid (Figure 20-21), reflecting the molar ratio expected for a lipid raft-like microenvironment.

Binding analysis revealed that the binding response of rHIgM22 to sulfatide containing monolayers is reduced in the presence of either GalCer (0.66 or 0.066 μmol, the molar ratio calculated for GalCer:sulfatide in myelin and a 10-fold higher ratio, possibly resembling the situation in glycolipid-enriched membrane rafts) or cholesterol (0.1 μmol, at a molar ratio cholesterol:sulfatide 0.1:0.01), while the addition of SM (0.12 μmol, at a molar ratio SM:sulfatide 0.012:0.01) to the monolayers determined a increase of the binding (**Fig. 30**). These finding suggest that the binding of rHIgM22 to sulfatide is affected by the membrane lipid microenvironment and that the presence of different lipids, at a certain density, might be required to allow an optimal recognition of the antigen by rHIgM22.

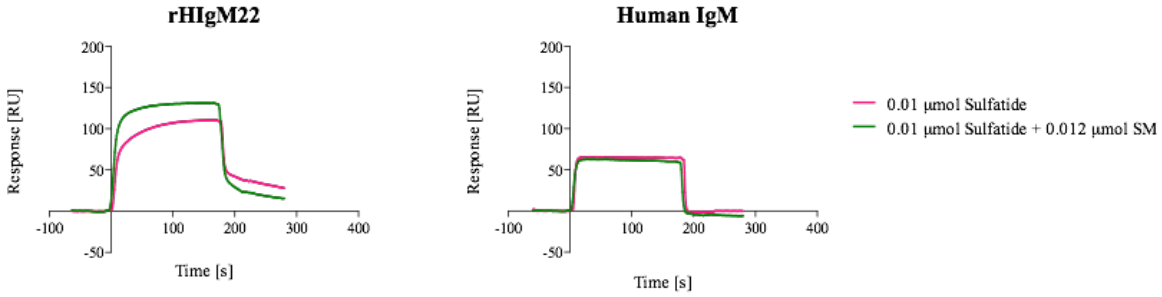
(A)


rHIgM22
Human IgM
Response [RU]
Time [s]
0.01 µmol Sulfatide
0.01 µmol Sulfatide + 0.66 µmol GalCer
0.01 µmol Sulfatide + 0.066 µmol GalCer

(B)


rHIgM22
Human IgM
Response [RU]
Time [s]
0.01 µmol Sulfatide
0.01 µmol Sulfatide + 0.1 µmol Cholesterol

(C)


rHIgM22
Human IgM
Response [RU]
Time [s]
0.01 µmol Sulfatide
0.01 µmol Sulfatide + 0.012 µmol SM

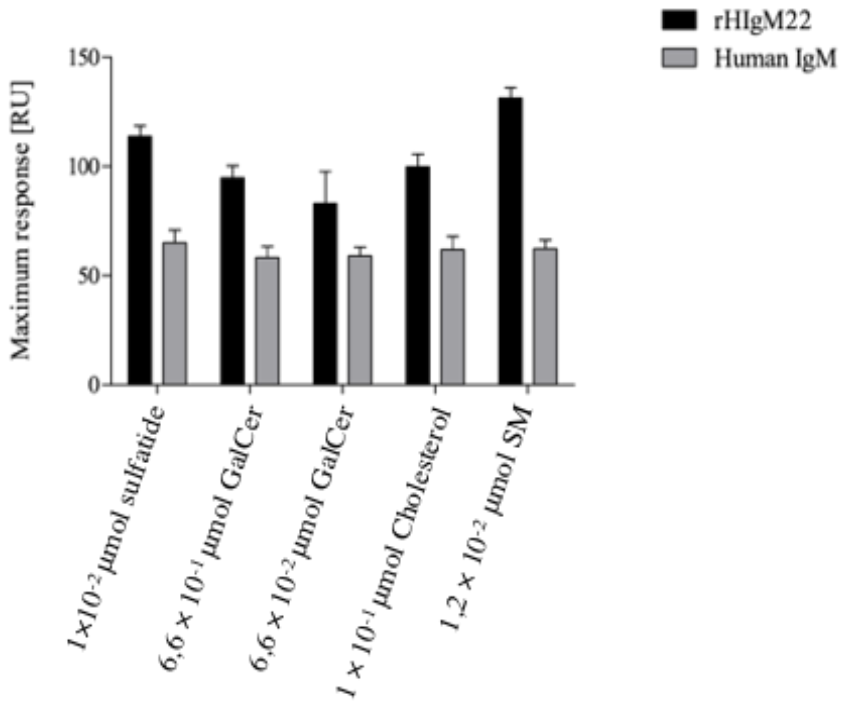


Figure 30. Effect of GalCer, Cholesterol and SM on rHIgM22 binding to sulfatide.
(A) Lipid monolayers were formed in HBS-N buffer as described in "Methods" from small unilamellar vesicles composed of DOPC (1 µmol), a fixed amount of sulfatide (1×10-2 µmol) and different amounts of (A) GalCer (0.66 µmol or 0.066 µmol), (B) Cholesterol (0.1 µmol), (C) SM (0.012 µmol); the liposome solutions were immobilized on an HPA sensor chip. The ability to anchor lipid was then assayed by injections of rHIgM22 or Human IgM (5.0 µg/mL) at a flow rate of 10 µL/min across the different monolayers.

In vitro binding of rHIgM22 to lipid extracts

Previous experiments were aimed at the individuation of the molecular target of the antibody, to better understand mechanisms underlying its activity. The data collected on purified lipids, show that rHIgM22 binds to sulfatide *in vitro* but it does not bind to other myelin sphingolipids, such as galactosylceramide, lactosylceramide and sphingomyelin. To verify whether rHIgM22 can bind sulfatide or other lipids in myelin extracts and other CNS cells, the binding of rHIgM22 was tested not only to purified lipids, but also to partially purified lipid extracts obtained from mice and rat brain, cerebellum, brain myelin, mixed glial cultures, microglial cells and cultured oligodendrocytes (listed in the Materials and Methods section).

Total lipid extracts prepared under the experimental conditions described in Materials and Methods do contain all cellular lipids, including hydrophobic (cholesterol and triglycerides) and amphipathic lipids: glycerophospholipids and sphingolipids, and, among those, sphingomyelin, neutral glycosphingolipids (GlcCer, LacCer, and, in myelin and myelin-producing cells, GalCer), and acidic sphingolipids (gangliosides, enriched in neurons, and sulfatide, enriched in myelin).

As shown in **Fig. 31**, when applied to total lipid extracts, the TLC immunostaining procedure was strongly affected by the binding of both rHIgM22 and control IgM to the glycerophospholipids, giving multiple signals whose intensity was not proportional to the amount of lipid samples. It was thus impossible, in these samples, to identify a signal surely ascribable to a specific binding to sulfatide (even if the amount of sulfatide theoretically present in these samples was comparable to the amount detectable using pure sulfatide using similar experimental conditions).

Since in total lipid extracts from biological samples, glycerophospholipids are present in amounts much higher than sulfatide and sphingolipids, representing a significant source of interference in the TLC immunostaining procedure, lipid extracts been subjected to a two-phase Folch's partitioning (with minor modifications), allowing to obtain aqueous phases enriched in gangliosides and a minor portion of sulfatide, and organic phases enriched in the more apolar lipids, including galactolipids and glycerophospholipids.

To obtain lipid mixtures devoid of potentially interfering glycerophospholipids and enriched in sulfatide and other sphingolipids, the organic phases have been further treated with alkali in methanol; this treatment allows to remove almost completely the interfering glycerophospholipids

from the sample, obtaining organic methanolized phases, which is enriched in sulfatide and galactolipids.

As shown in **Fig. 32**, in TLC immunostaining experiments for phospholipid-void methanolized organic phases it was clearly possible to detect a double band co-migrating with the sulfatide standard in samples from wt and ASMKO mouse brain, myelin and rHIgM22-positive cultured oligodendrocytes. All these samples showed a weak reactivity with the control IgM, however the ratio of the rHIgM22 signal and the control IgM signal was comparable to that observed for pure sulfatide.

In TLC immunostaining experiments for aqueous phases, we observed rHIgM22-immunoreactive bands co-migrating with the pure sulfatide standard (**Fig. 33**) and a second rHIgM22-immunoreactive band migrating below sulfated, co-migrating with the pure PI and PS standard, confirming the observation that the antibody is able to bind sulfatide, PI and PS.

It is common to have a small contamination of these two lipids in the aqueous phase, but since PI and PS are glycerophospholipids, they are usually recovered in the organic phase; for this reason, the experiments showed in the next section were aimed to isolate phosphatidylinositol and phosphatidylserine species from aqueous phase in order to better understand the characteristics of these two glycerophospholipids.

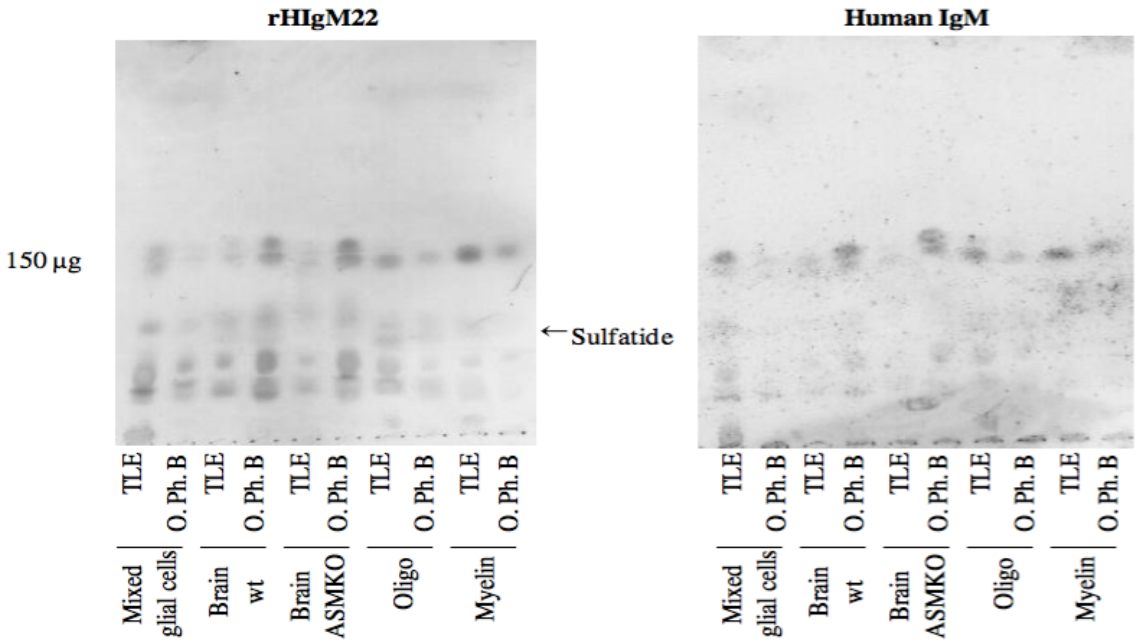


Figure 31. rHIgM22 binding to total lipid extracts and organic phases. Using TLC immunostaining we assessed rHIgM22 binding to different total lipid extracts (TLE) and organic phases (O.Ph.) obtained from different cells and tissues such as mixed glial cells, wild type mouse brain, ASMKO mouse brain, rHIgM22 positive OPC, and mouse myelin. For each extract and organic phase, either 150 µg protein were loaded on the TLC plates. The organic phases used for the analysis were obtained through partition. After chromatographic separation (solvent system: $CHCl_3$:CH_3OH:H_2O 110:40:6), After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 µg/mL of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods".

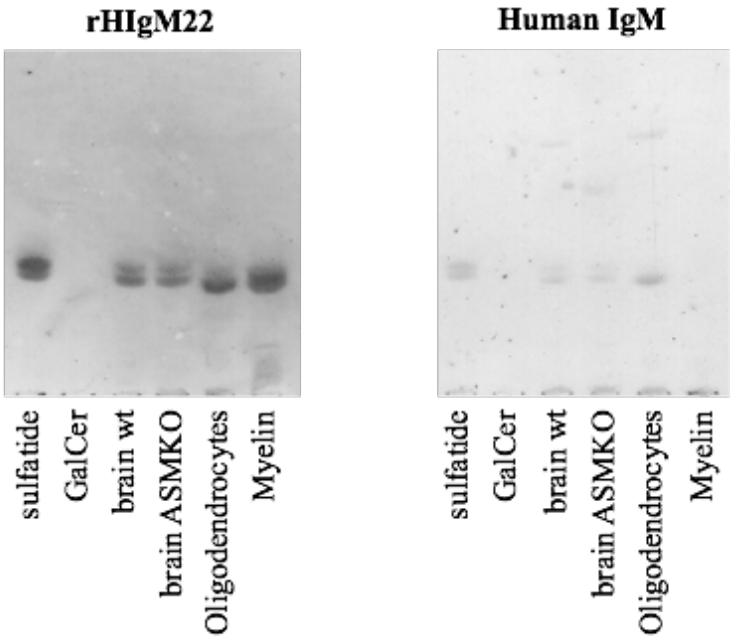


Figure 32. TLC-immunostaining of methanolyzed organic phases with rHIgM22.
O.Ph.Met (amounts equivalent to 150 μg protein) obtained from different tissues, such as wild type mouse brain, ASMKO mouse brain, rHIgM22 positive OPC and mouse myelin were separated by TLC using $CHCl_3$:CH_3OH:H_2O 110:40:6 (v/v/v) as solvent system. After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 μg/mL of the two primary antibody rHIgM22 and Human IgM (negative control) as described in "Methods".

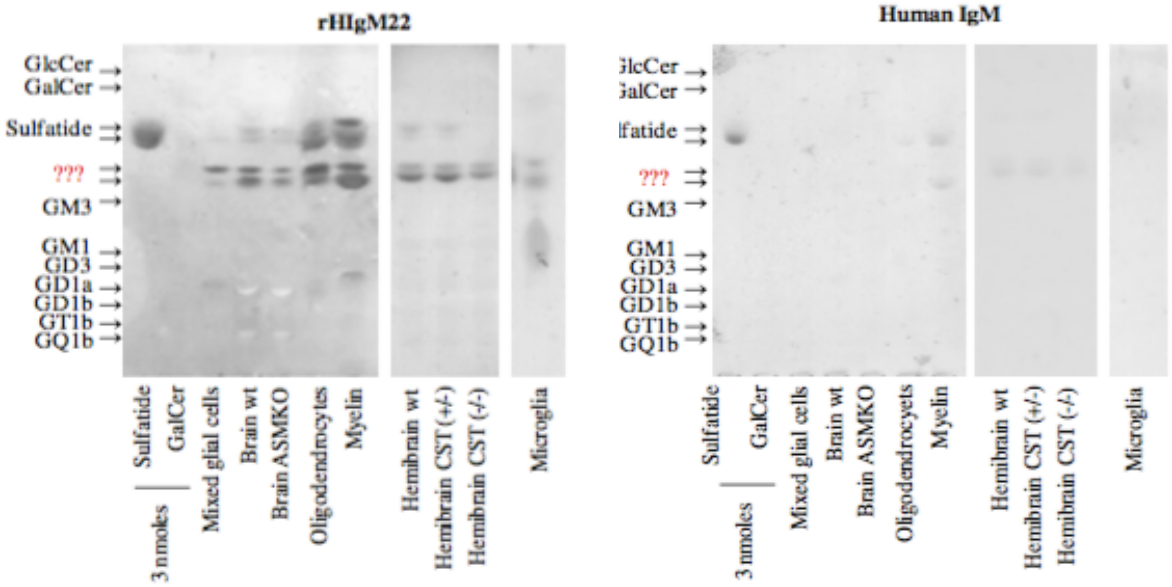


Figure 33. rHIgM22 binding to aqueous phases in TLC immunostaining.

Using TLC immunostaining we assessed rHIgM22 binding to aqueous phases (A.Ph. obtained by partitioning the TLE with water) obtained from different cells and tissues such as mixed glial cells, wild type mouse brain and hemibrain, ASMKO mouse brain, rHIgM22 positive OPC, mouse myelin, CST (+/-) and (-/-) hemibrain, and rat microglia. For each A.Ph. amounts equivalent to 150 μg protein were loaded on the plate, while for each pure lipid 3 nmol were loaded. After chromatographic separation (solvent system: $CHCl_3$:CH_3OH:$CaCl_2$ 50:42:11), After chromatographic separation, TLC plates were fixed with a polyisobuthylmethacrylate solution and immunostained with 5.0 μg/mL of the two primary antibody rHIgM22 and Human IgM (negative control) as described in “Methods”.

Mass spectrometry analysis

To confirm the identification of phosphatidylinositol and phosphatidylserine species aqueous phase from mouse brain was purified using column chromatography (**Fig. 34**), as described in Materials and Methods. Following the column chromatographic separation, the fractions enriched in phosphatidylinositol and phosphatidylserine species were analyzed by ESI mass spectrometry in negative mode (the specific conditions are reported in the section Materials and Methods).

The MS spectrum of the fraction enriched in phosphatidylinositol revealed the presence of one ion with an m/z of 885 and its MS/MS spectra, obtained by the fragmentation of the 885 ion, shows ions with an m/z of 581 and 419. These spectrum features are characteristic of Phosphatidylinositol with C18:0 and C20:4 (**Fig. 35**). The MS spectrum of the fraction enriched in phosphatidylserine revealed the presence of two ions with an m/z of 788 and 834. The MS/MS spectra obtained by the fragmentation of the 788 ion, shows ions with an m/z of 701 and 419 and MS/MS spectra obtained by the fragmentation of the 834 ion, shows ions with an m/z of 747 and 419. These spectrum features are characteristic of phosphatidylserine with C18:0 and C20:4 for 788 ion and phosphatidylserine with C18:0 and C22:6 for 834 ion (**Fig. 36**). The presence of five hydroxyl groups in inositol, the polar head of phosphatidylinositol, and the presence of α-amino group and one hydroxyl group in serine, the polar head of phosphatidylserine, could justify the small contamination of these two lipids in the aqueous phase.

In order to better understand the characteristics of rHIgM22 binding, also fractions enriched in sulfatide species were analyzed by ESI mass spectrometry in negative mode (**Fig. 37**). The MS spectrum of these fraction revealed the presence of different ions:

- 806 ion; this spectrum feature is characteristic of Sulfatide C18:0 FA
- 850 ion; this spectrum feature is characteristic of Sulfatide C20:0 OH FA
- 866 ion; this spectrum feature is characteristic of Sulfatide C23:0 OH FA or C22:1 2OH
- 878 ion; this spectrum feature is characteristic of Sulfatide C22:0 OH FA
- 904 ion; this spectrum feature is characteristic of Sulfatide C24:1 OH FA

The data collected from fractions enriched in sulfatide, show that rHIgM22 can bind different sulfated species, with a wide variety of acyl chains of different lengths and degree of hydroxylation.

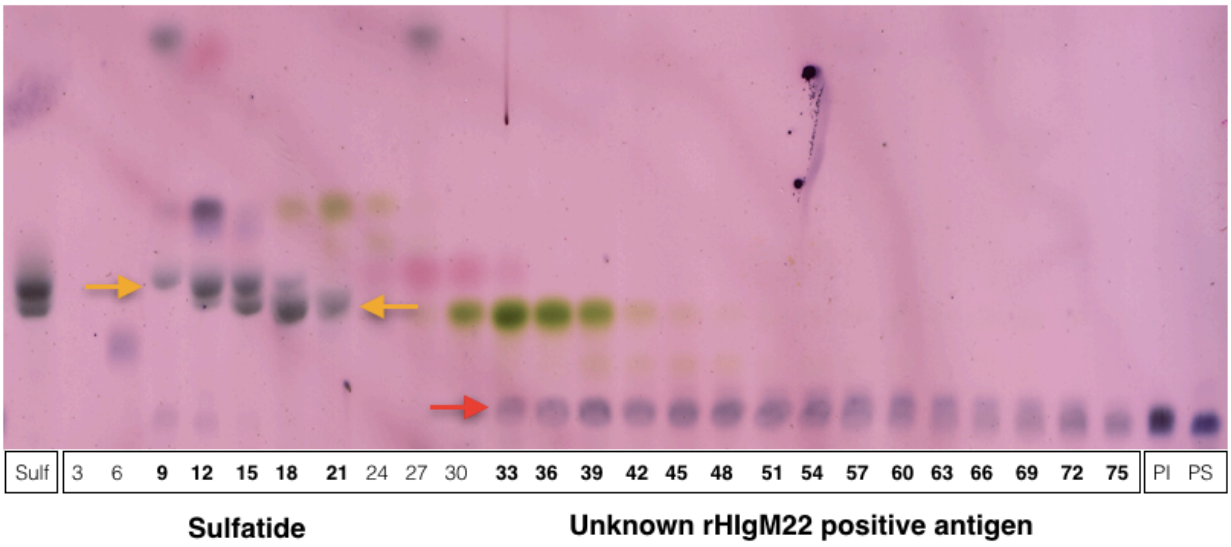


Figure 34 Thin layer chromatography (TLC) of column fractions. After column chromatography separation of aqueous phases from mouse brain, the fraction were separated by TLC using $CHCl_3$:CH_3OH:$CH_3CH(OH)CH_3$:H_2O 70:20:5:3 (v/v/v/v) as solvent system and the chemical detection of lipid was carried out with Anisaldehyde reagent.

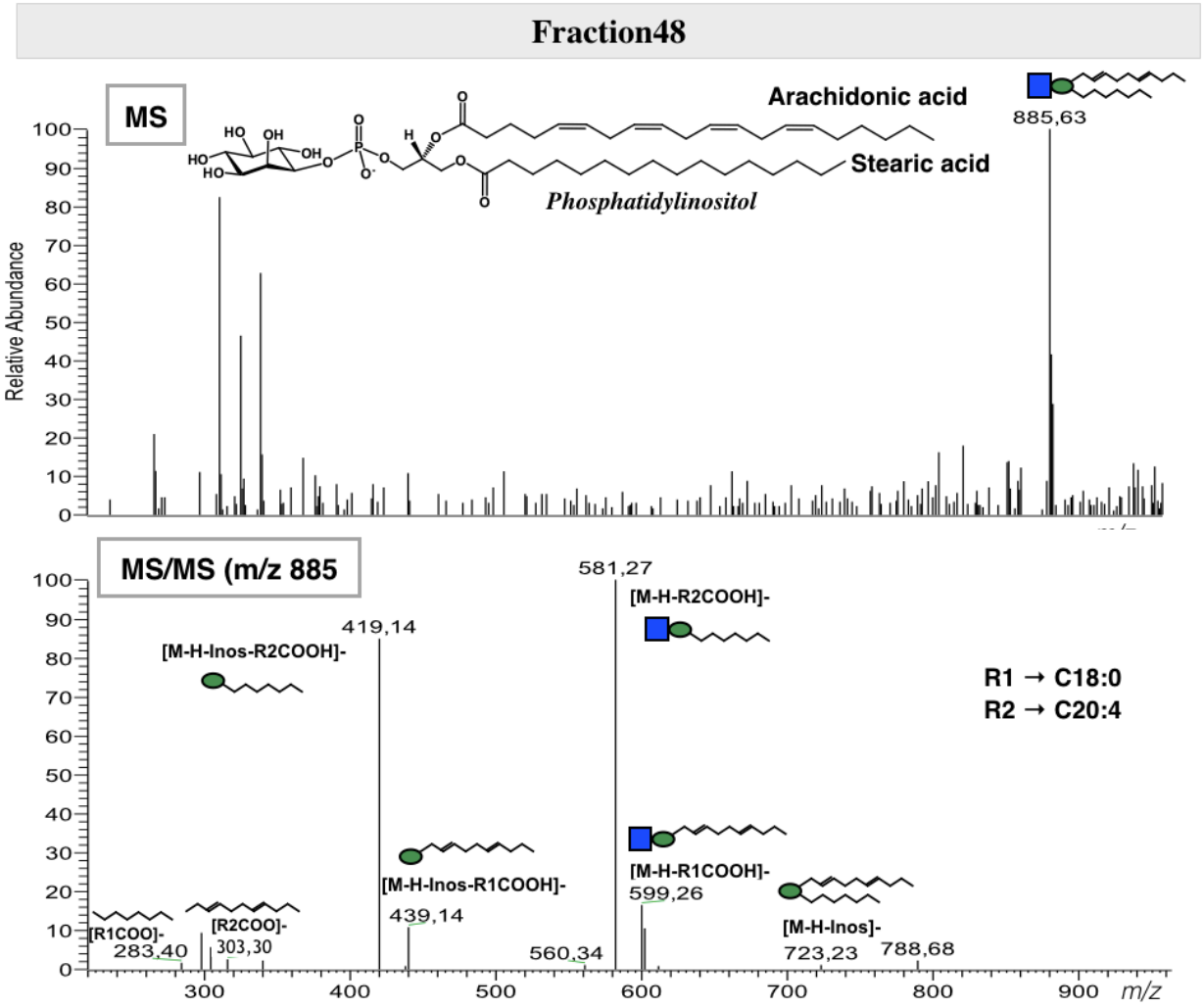


Figure 35. ESI-MS analysis of phosphatidylinositol. After column chromatography separation of aqueous phases from mouse brain, the fractions enriched in phosphatidylinositol were subjected to ESI MS analysis and subsequently to ESI MS/MS.

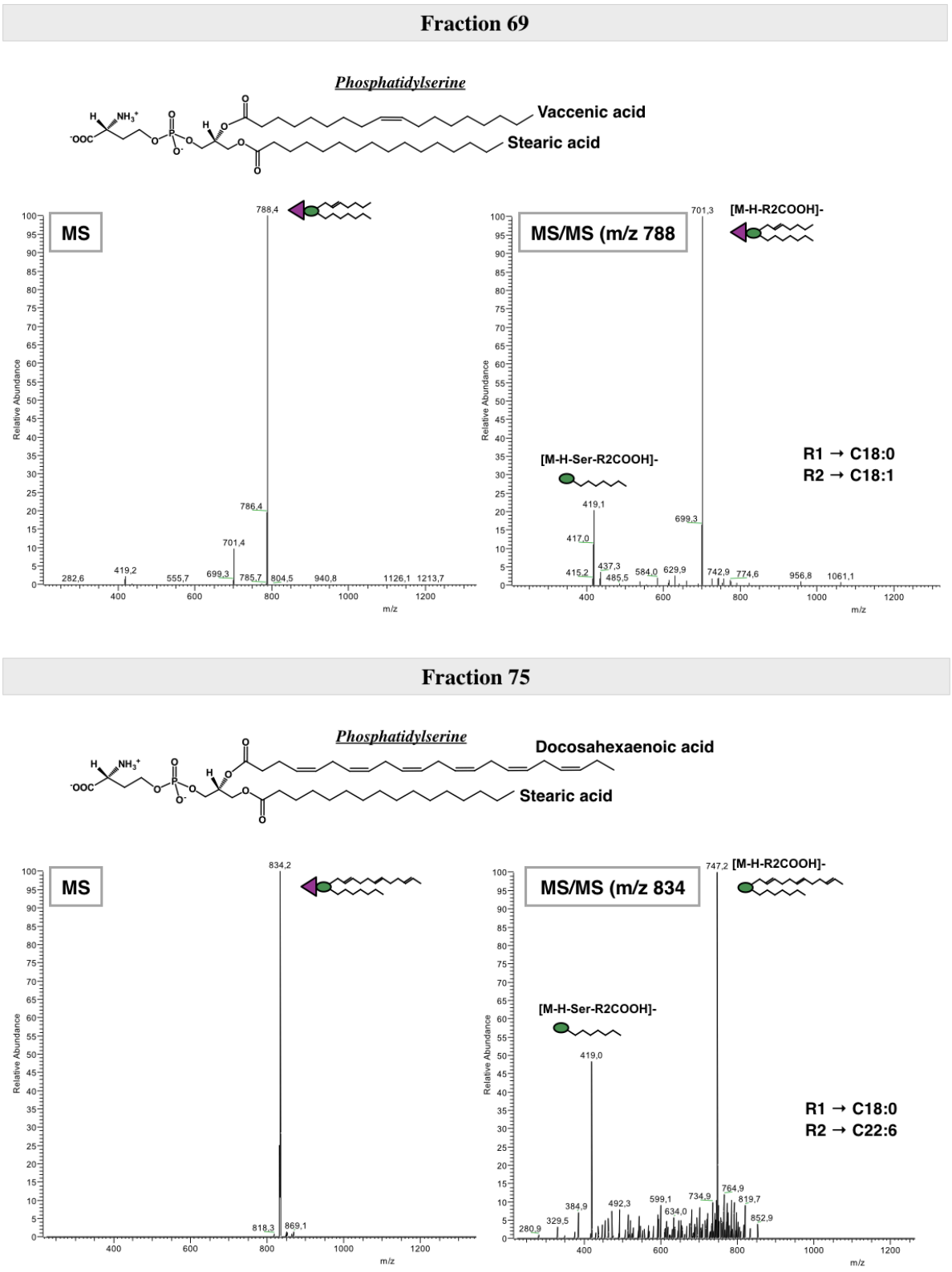


Figure 36. ESI-MS analysis of phosphatidylserine species. After column chromatography separation of aqueous phases from mouse brain, the fractions enriched in phosphatidylserine species were subjected to ESI MS analysis and subsequently to ESI MS/MS.

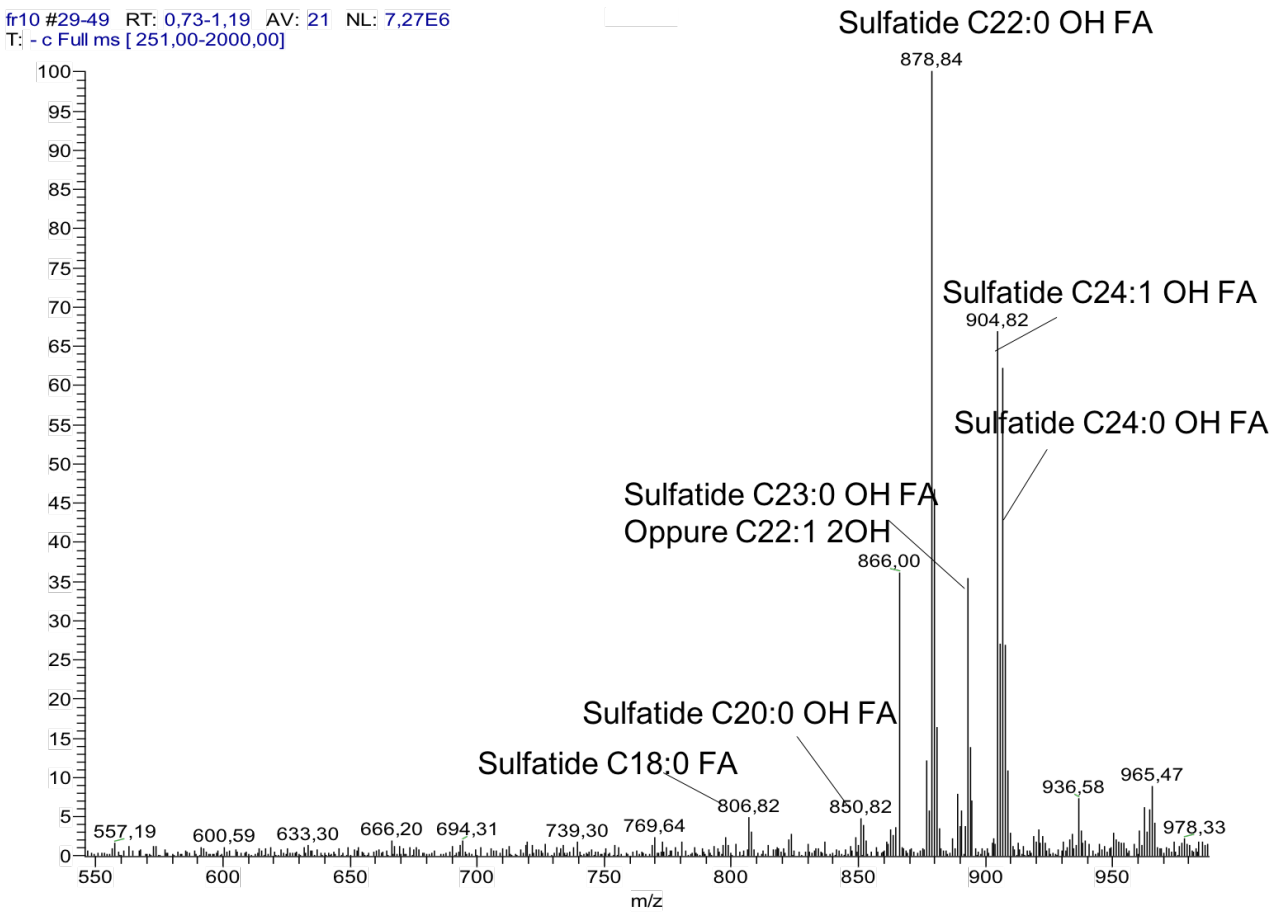


Figure 37. ESI-MS analysis of sulfatide species. After column chromatography separation of aqueous phases from mouse brain, the fractions enriched in sulfatide species were subjected to ESI MS analysis and subsequently to ESI MS/MS.

CONCLUSIONS

Multiple sclerosis (MS) is the most common demyelinating disease of the central nervous system. MS is an autoimmune disease, characterized by immune-mediated myelin damage eventually resulting in axonal loss attributable to the absence of myelin sheaths [179]. T cell subsets (Th1, Th2, Th17, CD8+, NKT, CD4+CD25+ T regulatory cells) and B cells are involved in this disorder, thus the currently available MS therapies are predominantly immune-modulating and are aimed at reducing the number and rate of lesion formation but do not directly promote repair. The stimulation of remyelination, which allows to repair the damaged regions of the central nervous system, is a new and promising potential approach to treating MS.

In adult human brain, the normal response to demyelination is spontaneous remyelination involving the generation of new oligodendrocytes. The myelin sheaths that are generated in remyelination are typically thinner and shorter than those generated during developmental myelination; nevertheless, they are associated with recovery of function [272, 273].

In some circumstances, however, and notably in MS, remyelination occurs but becomes increasingly incomplete/inadequate and eventually fails, leaving the axons and even the entire neuron vulnerable to degeneration, that largely accounts for the progressive clinical decline associated with this disease [199].

The normal adult white matter contains astrocytes, microglia and OPCs, in addition to myelinating oligodendrocytes [274]. Following demyelination, characterized by loss of oligodendrocytes and myelin, the microglia and astrocytes become activated [275]; the result is the activation of any OPCs [276], in the vicinity, which respond to mitogens and pro-migratory factors that are generated predominantly by reactive astrocytes and inflammatory cells [277]. The activated OPCs proliferate and migrate in the demyelinated area becoming populated by an abundance of OPCs [200]. Macrophages are also recruited and start to remove the myelin debris [278], a process essential for the generation of

new myelin. In the final phase of remyelination, the recruited OPCs differentiate into mature oligodendrocytes with the formation of a novel myelin sheath [199]. There are a multitude of hypotheses as to why remyelination fails in MS, which may reflect either changes in environmental inputs or intrinsic pathways regulating OPCs functions [279, 280]. Theoretically remyelination can be blocked at any point in the remyelination process: oligodendrocyte recruitment, survival, proliferation, migration, maturation, and/or myelin sheath formation. In late stage MS, oligodendrocyte recruitment is deficient (which could be a product of impaired survival, proliferation, and/or migration of oligodendrocytes) and appears to be the primary reason for poor remyelination [204]; in this case, therapeutic targets should be recruitment factors and not differentiation factors, which would inhibit remyelination. Otherwise, in lesions containing more oligodendrocytes, remyelination could fail due to an irregular differentiation, in which case differentiation promoters are desired and recruitment promoters would counteract remyelination. In addition, the formed glial scars, the glia-secreted factors and the presence of myelin-associated debris within the lesion may inhibit OPCs migration into the scar and subsequent differentiation. Experimental and clinical data suggest that differentiation is the most vulnerable phase of remyelination, and it is during this phase that remyelination generally fails [199].

The strategies that are currently being developed to increase efficiency of remyelination can be divide in three groups: 1) cell transplant, involving the transplantation of myelination-competent cells directly into lesion sites [281, 282]; 2) promotion of repair by the resident CNS stem- and precursor-cell populations, through the administration of growth, trophic, and neuroprotective factors [283]; 3) use of CNS reactive antibodies to induce remyelination [227]. One of these remyelination promoting antibodies is rHIgM22, which has recently been concluded 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). rHIgM22, the recombinant form of an antibody identified from a patient with Waldenström macroglobulinemia, has been shown to enter the central nervous system, accumulate in the demyelinated lesions, and promote remyelination in mouse models of chronical demyelination [214, 223] and to bind selectively to myelin and to the surface of oligodendrocytes *in vitro* [214, 284].

Despite these promising capabilities, both the signaling mechanisms stimulated by rHIgM22 and the antigen recognized are still unclear. Watzlawik et al. data suggest an rHIgM22-membrane mediated reorganization of a signaling complex which includes Lyn, integrin αvβ3 and PDGFαR [231, 246], which leads to inhibition of OPCs apoptotic pathway and differentiation and promotion of OPCs proliferation [219, 233].

Data published by Rodriguez et al. demonstrated that rHIgM22 is able to bind to myelin and to the surface of oligodendrocytes *in vitro* [214, 246] and that it binds to CNS tissue sections with a pattern similar to that of O4, an anti-sulfatide antibody [245], suggesting that sulfatide could be one of the antigens recognized by this remyelination-promoting antibody.

Starting from this observation, we have tested the binding of rHIgM22 to purified lipids and to lipid extracts prepared from several samples using two different experimental approaches: TLC immunostaining and surface plasmon resonance assays.

The data collected show that rHIgM22 binds *in vitro* to sulfatide and its deacylated form, lysosulfatide, (**Fig. 24, 26**) but it does not bind to other myelin sphingolipids, such as galactosylceramide, lactosylceramide and sphingomyelin (**Fig. 27**). Moreover, SPR experiments (**Fig. 25**), where antigen presentation is more physiological respect to TLC immunostaining, showed that the binding of rHIgM22 to sulfatide is specific, suggesting that sulfatide could actually be one of the molecular targets of rHIgM22. In addition, SPR analysis revealed that the binding response of rHIgM22 to sulfatide is modulated by the presence of other lipids; while the presence of GalCer and cholesterol significantly reduce the binding of rHIgM22 to sulfatide, the presence of

sphingomyelin is able to increase the binding of rHIgM22 to sulfatide (**Fig. 30**). Therefore, the addition of other membrane lipids can positively or negatively affect the *in vitro* binding of rHIgM22 to sulfatide, suggesting that, in myelin and oligodendrocytes, the membrane lipid microenvironment containing sulfatide might play a relevant role in the recognition of the antigen by rHIgM22 in intact cell membranes.

Using the TLC immunostaining experimental setting, other potential lipid targets have been screened. rHIgM22 weakly binds also phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidyinositol (PI), but TLC experiments using an isotype IgM control allowed to confirm that while rHIgM22 binding to PA, PS and PI is specific, rHIgM22 binding to PE is not. Moreover, further analysis, with different techniques, might be required to confirm this. In addition, this set of experiments revealed no significant binding of rHIgM22 to PC, the most abundant phospholipid in any biological membrane.

To verify whether rHIgM22 can bind sulfatide or other lipids in myelin extracts and other CNS cells, the binding of rHIgM22 has been tested not only to purified lipids, but also to partially purified lipid extracts obtained from mice and rat brain, cerebellum, brain myelin, mixed glial cultures, microglial cells and cultured oligodendrocytes. In TLC immunostaining experiments for phospholipid-void methanolized organic phases (**Fig. 32**), it was possible clearly to detected a double band co-migrating with the sulfatide standard in samples from wt and ASMKO mouse brain, myelin and rHIgM22-positive cultured oligodendrocytes. All these samples showed a weak reactivity with the control IgM, however the ratio of the rHIgM22 signal and the control IgM signal was comparable to that observed for pure sulfatide.

In TLC immunostaining experiments for aqueous phases (**Fig. 33**), we observed rHIgM22-immunoreactive bands co-migrating with the pure sulfatide standard and, unexpectedly, a second rHIgM22-immunoreactive band migrating below sulfated, co-migrating with the pure PI and PS standard, confirming the previously observation that the antibody is able to bind sulfatide, PI and PS. In order to better understand the characteristics of these two glycerophospholipids, (**Fig. 34**), they were partially purified and were analyzed by ESI mass spectrometry. The results obtained led to hypothesize that the unknown antigens could be phosphatidylinositol (18:0/20:4-PI)

and two different phosphatidylserine species, 18:0/22:6-PS and 18:0/18:1-PS (**Fig. 35, 36**). In addition, MS analysis for the fractions enriched in sulfatide (**Fig. 37**), shows that rHIgM22 can bind different sulfated species, with a wide variety of acyl chains of different lengths and degree of hydroxylation, suggesting that this binding is not fatty acid species-specific for the sulfatide (in agreement with the observation that the antibody is as well able to bind to lysosulfatide). This point of view is supported by the fact that only the hydrophilic carbohydrate moiety of the sphingolipids is exposed to the cell surface and, therefore, detectable by antibody. This emphasizes the carbohydrate moiety and neglects the lipid backbone as the essential part of the antigen.

The data collected in our laboratories demonstrate that rHIgM22 binds to sulfatide *in vitro*, according to 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, which lacking sulfatide. The binding to sulfatide is specific, even if the binding affinity is low, and can be modulated by the presence of other lipids. This confirm the hypothesis that there may be a possible role of the membrane microenvironment in the recognition of the antigen by rHIgM22. Likely, the binding of rHIgM22 to the cells could require a complex molecular arrangement, and, in particular, sulfatide might be part of the functional rHIgM22 antigen localized at the cell surface.

In addition, rHIgM22 also reacts with phosphatidic acid, phosphatidylserine and phosphatidylinositol present in lipid extracts from various sources, including CST knock-out mice brains, MGC, and isolated astrocytes and microglia. This suggests that not only sulfatide, but also other membrane lipids might play a role in the binding of rHIgM22 to oligodendrocytes and suggests an involvement of other cell types. Indeed, as reported in literature, activation and proliferation of astrocytes and microglia (that is present in mixed glial cells too) is observed in demyelinating lesions, suggesting that immune response might be important in both oligodendrocyte injury and axonal degeneration [285]. In MS astrocytes promote the degeneration of myelin, but at the same time, create a permissive environment promoting remyelination through the secretion of molecules like cytokines [286]. In addition, data in literature show that rHIgM22 induced OPC proliferation in mixed glial cultures, but not in isolated OPC.

This suggest that the stimulation of OPC proliferation requires factors produced by astrocytes and/or microglia [233].

So, our hypothesis is that rHIgM22 does not activate myelination only binding to OPCs, but it also recruits in some way other cell types within the lesion niche to exert its function. An interaction between OPCs, astrocytes and microglia is necessary to the process of remyelination. This is an important step to understand the process of remyelination in general, however, other experiments are necessary to go deeper in the comprehension. Although rHIgM22 antigens seem to be identified, the molecular process and pathways activated are still not clear. However, we hypothesize that rHIgM22 needs to bind sulfatide in a particular membrane environment containing these lipid antigens, probably a lipid rafts organization. On the other hand, lipid rafts not containing sulfatide might represent the target of the antibody in cell population not expressing significant amounts of sulfatide, such as astrocytes and microglia.

The identification of the binding targets of rHIgM22, the characterization of their membrane microenvironment and the identity of the cell types involved could significantly contribute to understand 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.

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