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EVALUATION OF rHIgM22 EFFECT ON MIXED GLIAL CELLS CULTURE

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Sommario

ABSTRACT

Multiple sclerosis (MS), considered the lead disease featuring demyelination [316], is the most common cause of non traumatic disability in young people. It is characterized by inflammation, progressive demyelination and gliosis, axonal injury and loss. The pathological hallmarks of all the subtypes of this disease are focal areas, called plaques, of demyelination in the CNS, with surrounding inflammation and neurodegeneration [223,224]. Despite its high prevalence, multiple sclerosis remains a challenging ailment to study. Currently the most widely accepted hypothesis concerning MS pathogenesis is the autoimmune hypothesis: an autoimmune inflammation is proposed to be the cause of demyelination and auto-reactive leukocytes could be the disease initiators [225, 226].

One of the therapeutic approaches that is currently being developed to improve the regenerative outcome involves the use of CNS reactive antibodies to promote remyelination [280]. One of these antibodies, rHIgM22, is able to bind to oligodendrocytes and myelin *in vitro*. Moreover, rHIgM22 is able to enter the CNS, accumulate at lesion site and promote remyelination in mouse models of chronical demyelination [289, 291]. As a matter of fact, this antibody has recently passed a phase I clinical trial for treatment of MS.

Evidence suggests that the binding target of rHIgM22 could be associated with plasma membrane lipid rafts, and that lipid rafts might be involved in the signaling associated with the biological activity of this antibody [294, 295]. Moreover, it has been demonstrated that, isolated OPCs do not respond to rHIgM22 treatment, instead mixed glial cultures consisting of astrocytes, OPCs and microglial cells demonstrate observable rHIgM22-mediated OPC proliferation [299].

The aim of this study was to analyze the plasma membrane lipid rafts composition in MGC in order to evaluate the effects exerted by rHIgM22 on these cells after single dose treatment of various duration.

The analysis of the lipids and proteins distribution in MGC fractions, obtained after discontinuous sucrose gradient centrifugation of cells, and the alteration on lipids and protein composition of MGC due to the rHIgM22 treatment have been tested using TLC immunostaining assays and western blot analysis.

The results obtained show that the DRM fraction obtained from MGC was enriched in sphingolipids, in particular sphingomyelin and gangliosides together with Lyn, Caveolin 1 and PrP(SAF32). On the contrary, phospholipids, in particular phosphatididylethanolamine and phosphatidic acid are enriched in the HD fraction, together with integrin αv and Akt. Furthermore we observed that rHIgM22 exerted an

effect on the expression of P-Src(Y416) family and Lyn, that show a significant decrease, and PDGFRα that shows a significant increase. Moreover, the rHIgM22 treatment also induces a decrease in the activity of the aSMase.

We hypothesize that the treatment with IgM22 could elicit biological responses mediated by alterations of lipid-dependent membrane organization which result in a reorganization of Lyn, integrin αvβ3 and PDGFRα at the cell surface to form a signaling complex. The formation of this complex triggers Lyn activation which in turn promotes oligodendrocyte precursor cells (OPCs) survival and proliferation and an inhibition of the pro apoptotic signaling. Moreover, the increased activation of Lyn could determine a decrease in ASMase activity and consequently in ceramide generation, thus inhibiting pro-apoptotic signaling and/or organization of sphingolipid-dependent signaling platforms.

The identification of the binding targets of this antibody, able to promote remyelination in validated mouse models of MS, and the characterization of their membrane microenvironment could significantly contribute to the reveal the signaling mechanisms underlying the biological activity of rHIgM22.

INTRODUCTION

Glycosphingolipids

Eukaryotic cell membranes, whose basic role is that of a physical barrier between the extracellular milieu and the intracellular environment, are composed of a lipid bilayer.
The three major classes of lipids present in eukaryotic cells are sterols, glycerolipids, and sphingolipids. The biochemical and biophysical properties of these classes differ considerably and impact upon their function [1]. An accurate regulation of their composition is crucial for proper growth, division, and responses to environmental stimuli leading to a correct maintenance of the cellular homeostasis [2].

- Glycerophospholipids, a family of amphipathic molecules distributed asymmetrically across the plasma membrane, are the most abundant lipids in eukaryotic cell membranes. They constitute the backbone of cellular membranes and also provide the membrane with a suitable environment, fluidity, and ion permeability[3]. In neural membranes, it is possible to find three major classes of glycerophospholipids: 1,2-diacyl glycerophospholipid, 1-alk-1-enyl-2-acyl glycerophospholipid or plasmalogen, and 1-alkyl-2-acyl glycerophospholipids; all have a glycerol backbone with a fatty acid, and a phosphobase (ethanolamine, inositol, choline, serine) [4].

  The most represented glycerophospholipids in mammal tissues are phosphatidylethanolamine (PE) phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI; PtdIns). Phosphatidic acid (PA; PtdOH) is also present in all cell membranes and it is a precursor of all neural membrane glycerophospholipids. PA also acts as an intracellular second messenger regulating different signaling proteins [5, 6], including kinases, phosphatases, and also the transcription factor mTOR. Diacylglycerol (DAG), arachidonic acid, and phosphoinositides have been implicated as messengers for Ca^{2+} homeostasis and protein phosphorylation too [7-9].
- The sphingolipids structure and contains a sphingoid backbone, usually sphinganine or sphingosine (sphingosine: (2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol [10]. Additions or modifications to the sphingoid base lead to the production of a variety of members of the sphingolipid family, comprising ceramides (N-acylated), phytoceramides (phosphorylation in position 4), dihydroceramides (degree of saturation), sphingomyelins (ceramides with a phosphocholine polar group), cerebrosides (ceramides with an hexose polar group), sulfatides (sulfate esters of cerebrosides), globosides (containing more

than one sugar) and gangliosides (containing sialic acid). Since their discovery, sphingolipids have been found on virtually all eukaryotic biological membranes. Some sphingolipids have been also found in a small number of prokaryotic genera [11]. The contents and composition of sphingolipids modulate the biophysical properties of model membranes [12] and they can be packed in lipid phase separation defining 2D domains. The membrane properties defined by the specific content/composition in sphingolipids, or these defined domains, allow biological membranes to adapt to variations in temperature, pH, membrane tension, etc. Several authors have suggested that the changes in membrane properties promoted by the sphingolipid composition can trigger cell signaling, even though the link between physical properties and cell signaling is complicated due to the large number of components and the dynamic characteristic of the membranes [13]. However, besides their physical properties, sphingolipids have been also found to be bioactive molecules. The first study that promoted this conceptual shift appeared in the mid-eighties, when sphingosine has been shown to inhibit PKC activity [14]. Since then several downstream protein effectors have been described or suggested mainly for sphingosine, ceramide, ceramide-1-phosphate, glycosphingolipids, and sphingosine-1-phosphate [15-17]. Ceramides and sphingosine-1-phosphate are the most studied sphingolipids with regard to cell signaling, and they most often exert opposing biological functions [18]. Briefly, ceramides have been linked to cell death, cell cycle arrest and senescence. Conversely, sphingosine-1-phosphate has been linked to cell survival, proliferation, migration and protection from apoptosis [11]. Another important and complicating aspect of sphingolipid signaling is the compartimentalization of sphingolipid metabolism within the cells. In fact, not only different steps of the sphingolipid metabolic pathway take place in different compartments in the cell, but, at times, the same biochemical reaction takes place in different subcellular compartments thus defining discrete pools of sphingolipids in different organelles [19]. Moreover, to make it even more complicated, not all ceramides are the same. Researchers are realizing that differences in length and level of unsaturation of the acyl chain, additional hydroxylations, or modifications on the sphingoid backbone (e.g., sphingolipids derived from alternative amino acids) can produce concrete species that exert specific functions [19, 20].

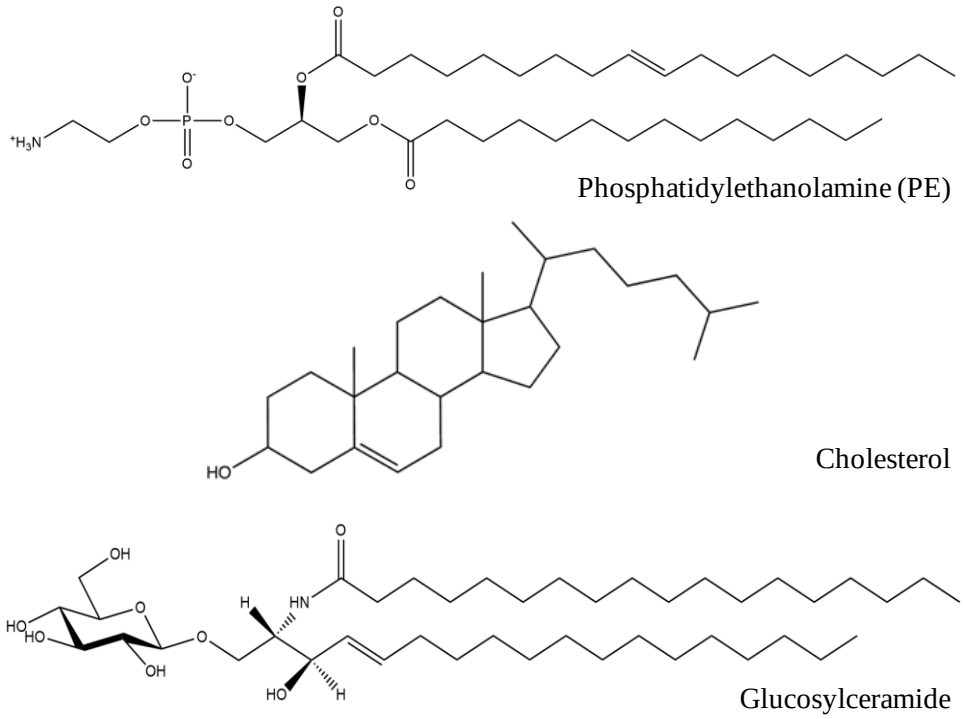

Figure 1. Representatives of the major lipid classes of eukaryotic cell membranes.

Glycosphingolipids biosynthesis, trafficking and degradation

Over the past few decades, the biochemical pathways of glycosphingolipids metabolism and the intracellular sites of synthesis and degradation, respectively in the endoplasmic reticulum/Golgi apparatus and lysosomes, have been extensively characterized [21, 22]. The biosynthesis of canonical glycosphingolipids starts by the condensation of serine and palmitoyl-CoA into 3-ketosphinganine. This reaction occurs in the endoplasmic reticulum (ER) and it is catalyzed by the enzyme serine palmitoyl transferase (SPT), and it is followed by a reduction reaction yielding sphinganine. Other amino acids such as alanine and glycine can be used instead of serine, forming the atypical sphingolipids, leading to deoxy- and deoxymethyl-sphingolipids. It is also worth to mention that fatty acids other than palmitic acid can be found in sphingolipids. Thus, SPT can accept fatty acid-CoA other than palmitoyl-CoA resulting in sphingolipids with different alkyl chain length [23, 24]. Still in the ER, the (dihydro) ceramide synthases (CerS1-6) N-acylate sphinganine with several possible coenzyme A-activated fatty acids resulting in dihydroceramides. The irreversible desaturation of the 4,5-trans double bond leads to the formation of ceramides [25]. Ceramides are an extended family of structurally related molecules, with a growing number of members [19].

In the Golgi, ceramide is converted into sphingomyelin (SM) by sphingomyelin synthase (SMS), or it is incorporated into the glycosphingolipid family by addition of glucosyl- or galactosyl-units, followed by addition of more sugar moieties resulting in more complex glycosphingolipids [26]. Golgi-located ceramides, sphingomyelins and glycosphingolipids are then transported to various locations within the cell, such as the plasma membrane. With phosphatidylcholine (PC), SM is one of the most abundant phospholipid in biological membranes. It is found enriched in the outer leaflet of the plasma membrane, where it plays important structural roles. The presence of SM increases the rigidity and compactness of the plasma membrane (PM). Moreover, SM is found to be associated with cholesterol, further packing the PM, and defining rigid platforms, which have been thought to cluster certain proteins, such as a membrane receptors [27-29].

In the middle of the 1990s, Hannun and colleagues demonstrated that sphingomyelinase activation was required for cell signaling downstream of TNF alpha and other biological effectors [30, 31]. This demonstrated that ceramides produced by hydrolysis of complex sphingolipids (sphingomyelin, cerebrosides, etc.) at the plasma membrane, mitochondria or in the endosome/lysosome compartment could be involved in cell signaling [32]. In the plasma membrane and other cellular compartments, SM can be hydrolyzed to ceramide by alkaline, acid or neutral sphingomyelinases (AlkSMase, aSMase and nSMase respectively). Because the total amount of SM is normally more than 10 times the amount of total ceramide in the cell, hydrolysis of small percentage of SM, is translated to major changes in ceramide levels. Ceramide generated by this pathway is further degraded into sphingosine, which, as we mentioned before, can follow different paths.

A third pathway of ceramide generation involves the catabolism of sphingolipids to sphingosine, which can be reused through N-acylation to produce ceramide. Moreover ceramides can be further broken down into sphingosine by ceramidases (acid, neutral and alkaline). Again, small amounts of ceramide hydrolysis can produce significant increases in sphingosine levels. Sphingosine can be then transported to other cellular compartments and re-acylated into ceramide by CerSs in the ER-Golgi or by reverse activity of ceramidases in the mitochondria [33, 34]. Sphingosine can also be phosphorylated by sphingosine kinases (SKs) to sphingosine-1-phosphate (S1P), a well established bioactive lipid [35]. Consequently, ceramide comprises a family of related structures that are found in different organelles or cellular compartments, even though it is commonly treated as a

unique biological entity. Recently, it has become apparent that the different pools of ceramides (either molecularly different or residing in different organelles) might have great significance as they may exert distinct biological functions [19]. A fundamental approach to start dissecting the complexity of ceramide signaling is to learn about the enzymes that regulate ceramide generation, degradation and turnover, their cellular topology, their related biologies and the diseases arising from their dysregulation. A key tool to study the role, function and regulation of sphingolipid-regulating enzymes in health and disease is the development of chemical inhibitors

While ceramide resides on intracellular organelles such as mitochondria, glycosphingolipids beyond GlcCer are not known to exist on membranes facing the cytoplasm [36, 37]. The biosynthesis of glycosphingolipids in the brain provides an example of how competing biosynthetic pathways can lead to glycan structural diversity [36]. Stepwise biosynthesis of GalCer and sulfatide occurs, in the brain, in oligodendrocytes, the myelinating cells of the central nervous system (CNS). Synthesis of GalCer and sulfatide, in the brain, is switched on at the onset of the terminal differentiation, and remains constant in mature oligodendrocytes [38]. Moreover, in rat brain, their synthesis is maximal at the developmental stage of most rapid myelination [39]. All this suggests that GalCer and sulfatide might represent not only structural components of the myelin sheath but also active players in myelin formation and maintenance [40]. Gangliosides, in contrast, are synthesized by all cells, with concentration of the different forms varying according to cell type. It is well known that during brain development there are marked changes in expression of glycoconjugates ranging from complex proteoglycans to gangliosides. The expression pattern shifts from simple gangliosides, like GM3 and GD3, to complex gangliosides, such as GM1, GD1a, GD1b, and GT1b, and this shift is mainly regulated by the differential expression and intracellular distribution of the enzymes required for the biosynthesis of these glycosphingolipids[41,42]. Moreover, multiple glycosyltransferases can compete for the same precursor. LacCer, for example, can act as a substrate for sialyltransferase I, which forms GM3, or for GalNAc transferase, which forms GA2 [43]. GM3, in turn, can be acted on by N-acetylgalactosaminyltransferase, forming GM2, or by sialyltransferase II, thereby forming GD3, the simplest “b-series” ganglioside [44, 45]. Due to the branch exclusivity, since sialyltransferases cannot convert a-series gangliosides to their b-series correspondent, the relative expression level of the final GSL products is determined by enzyme competition at each branch. The transfer of *N*-acetylgalactosamine to a-, b-, and

c-series gangliosides, converting GM3 into GM2, GD3 into GD2, or GT3 into GT2, is catalyzed by the same N-acetylgalactosaminyltransferase. Likewise, the transfer of galactose to GM2 to form GM1, to GD2 to from GD1b, or to GT2 to form GT1c is accomplished by a single galactosyltransferase [26]. An additional level of regulation may occur via stable association of different glycosphingolipid glycosyltransferases into functional "multiglycosyltransferase" complexes. The enzymes involved are thought to act concertedly on the growing glycosphingolipid without releasing intermediate structures, ensuring progression to the preferred end product [46].

While sphingolipid synthesis occurs in membranes of the secretory pathway, their catabolism occurs predominantly in endosomes and lysosomes. After internalization, GSL-rich membrane portions fuse with early endosomes, where the GSLs destined for degradation are sorted through the formation of multivescicular bodies which reach the lysosomes. Following the fusion, glycosphingolipids are exposed to lysosomal hydrolases and, *in vivo*, they are eventually broken down to their individual components, which are available for reuse [47,48].

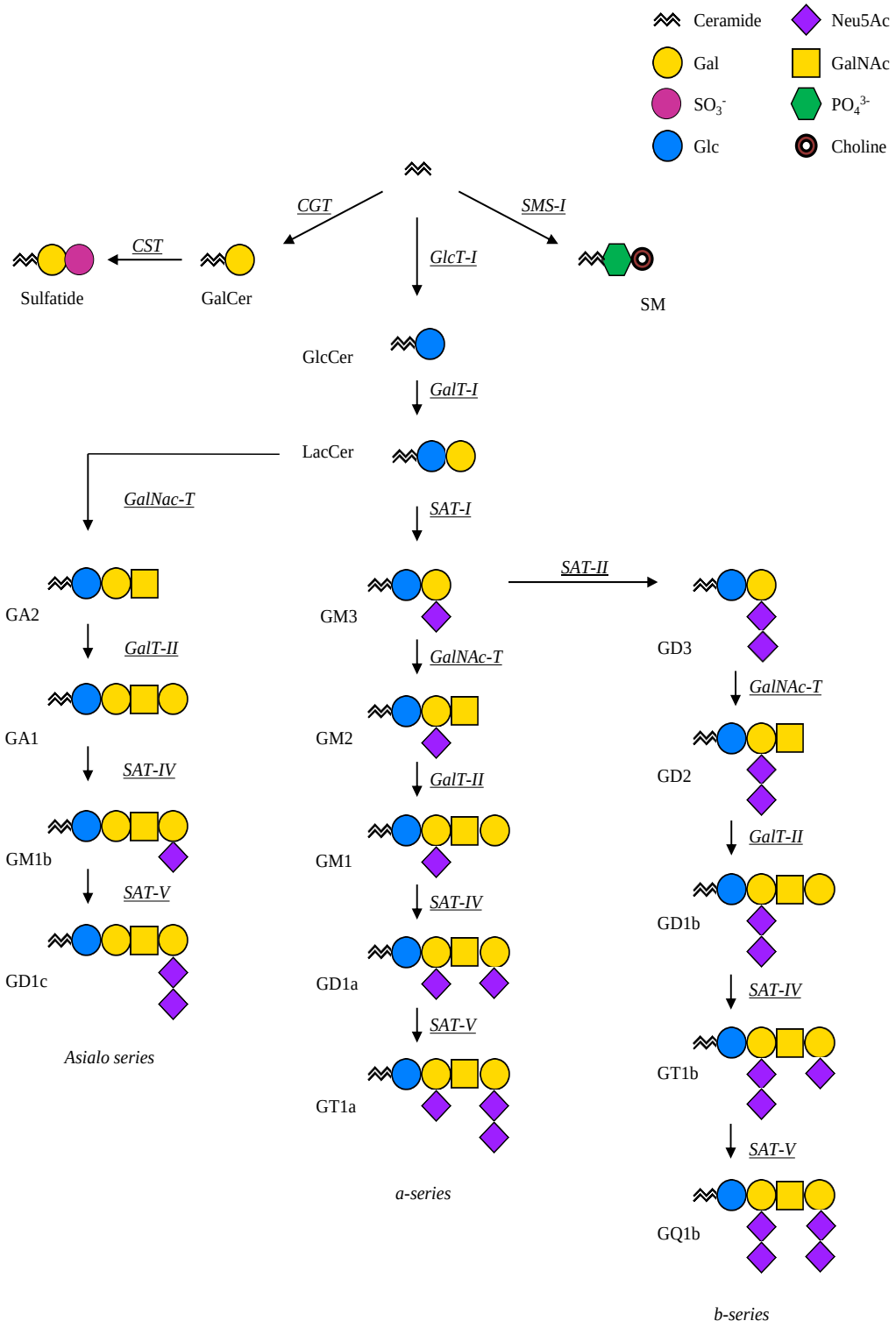

Figure 2. Schematic biosynthetic pathway of glycosphingolipids.

Biological functions glycosphingolipids

GSLs are essential for the proliferation, survival, and differentiation of eukaryotic cells within complex multicellular systems, as established in different studies performed on the role of glycosphingolipids using genetic, biophysical, biochemical and cell biology approaches. Various studies involving the analysis of genetically engineered mice provided a general framework for the understanding of the roles of glycosphingolipids in mammals and their vital importance in the life of cells that are dealing with a multifaceted extracellular reality. These studies revealed that ablation of either GlcCer synthase or of B4GALT-V, responsible for the synthesis of LacCer, leads to embryonic lethality[49, 50]. Moreover, mice lacking all ganglioside, as a result of the knockout of both GalNAcT and SAT-I genes, suffer severe lethality. These mice show enhanced cell apoptosis, perturbed axon-glia interactions and axonal degeneration [51], though, it still remains to be elucidated whether these phenotypes are the result of a functional deficiency or a consequence of the accumulation of substrate precursors.

Glycosphingolipids are highly segregated, together with cholesterol, in lipid domains with specialized signaling functions [52]. Within the cell, they are highly asymmetrically enriched in the external leaflet of plasma membranes, with the oligosaccharide chain protruding toward the extracellular space, where the sugar residues can engage *cis* and *trans* interactions with a wide variety of cell surface and extracellular molecules [53]. The GSLs local concentration in the membrane affects these interactions. Direct lateral interactions (*cis* interactions) with plasma membrane proteins are strongly favored within a sphingolipid-enriched membrane domain [54], whereas in the case of *trans* interactions, it has been shown that recognition of lipid-bound oligosaccharides by soluble ligands (for example antibodies or toxins) or by complementary carbohydrates and by carbohydrate binding proteins (such as selectins or lectins) belonging to the interfacing membrane of adjacent cells is strongly affected by their degree of segregation (or dispersion)[55].

This variety of interactions is reflected in the variety of roles of glycosphingolipids. GSLs, for example, act as receptors for bacteria and viruses. As a matter of fact, GM1 acts as a receptor for cholera toxin B subunit [56, 57], Gb3 acts as a receptor for verotoxins [58,59], whereas two of the surface proteins of influenza virus are aimed against the terminal Neu5Ac group on glycosphingolipids and glycoproteins of the human host [60]. Some pieces of evidence also suggest a receptor role for glycosphingolipids in HIV infections. The HIV adhesin gp120, in fact, binds to several GSLs, including GalCer, sulfatide [61, 62], GM3, GD3 and also Gb3 [63,64].

Glycosphingolipids have a role in the modulation of the immune response too. For example, the interdigitation of the acyl chains of long chain LacCer with the cytosolic leaflet of the bilayer, leading to Lyn kinase activation, has been strongly implicated in ligand activation of human neutrophils phagocytosis [65]. Moreover, LacCer has also been found to play a role in the induction of proinflammatory cytokines in both glial cells and neutrophils [66, 67], while gangliosides play a role in the modulation of the cytotoxicity of natural killer cells [68].

Glycosphingolipids are also known to interact with growth factor receptors, to modulate cell growth, and, in many cases, to inhibit receptor-associated tyrosine kinases. An example of this is the interaction of ganglioside GM3 with the insulin receptor (IR). Accumulation of GM3 upon acquisition of insulin resistance leads not only to the displacement of IR from caveolin-1 complexes, required for insulin signaling leading to the translocation of GLUT4 at the surface of normal adipocytes [69], but also to its sequestration in a complex with GM3 [70]. Another example is the epidermal growth factor receptor (EGFR) interaction with GM3. GM3 negatively regulates ligand-stimulated autophosphorylation and dimerization of EGFR [71-73], and cross-talk of EGFR with integrin receptors [74] and PKCα [75], inhibiting EGFR-dependent cell proliferation and survival.

Glycosphingolipids also play important roles in modulating several properties of tumor cells. Most tumor cells show altered glycosphingolipid patterns on their surface as well as abnormal sphingolipid signaling and increased glycosphingolipid biosynthesis, which together play a major role in tumor growth, angiogenesis, and metastasis [76, 77].

Glycosphingolipids are also known to play a role in the regulation of axonal growth. For example, gangliosides GD1a and GT1b, enriched in axonal rafts, act as MAG receptors in MAG-induced inhibition of axonal growth [78, 79].

Glial cells in the central nervous system

Glial cells in the mammalian adult central nervous system (CNS), are constituted by oligodendrocytes, astrocytes and microglia. These cells, together, are by far the most abundant cells in the nervous system, comprising about 60-90% of all cells in the human brain [80, 81]. The name glia derives from the Greek word meaning glue, reflecting the property of these cells to hold together the nervous system and, for a long time, they were thought only to support neurons passively. However, in the past few decades, evidence has shown that glia function as master regulators of the nervous system, providing

valuable support in synaptic plasticity, axonal function, and acting as integral mediators of neuronal connectivity. Moreover, in addition to development and aging, these cells also play important roles in repair and remyelination in CNS disease [82-86].

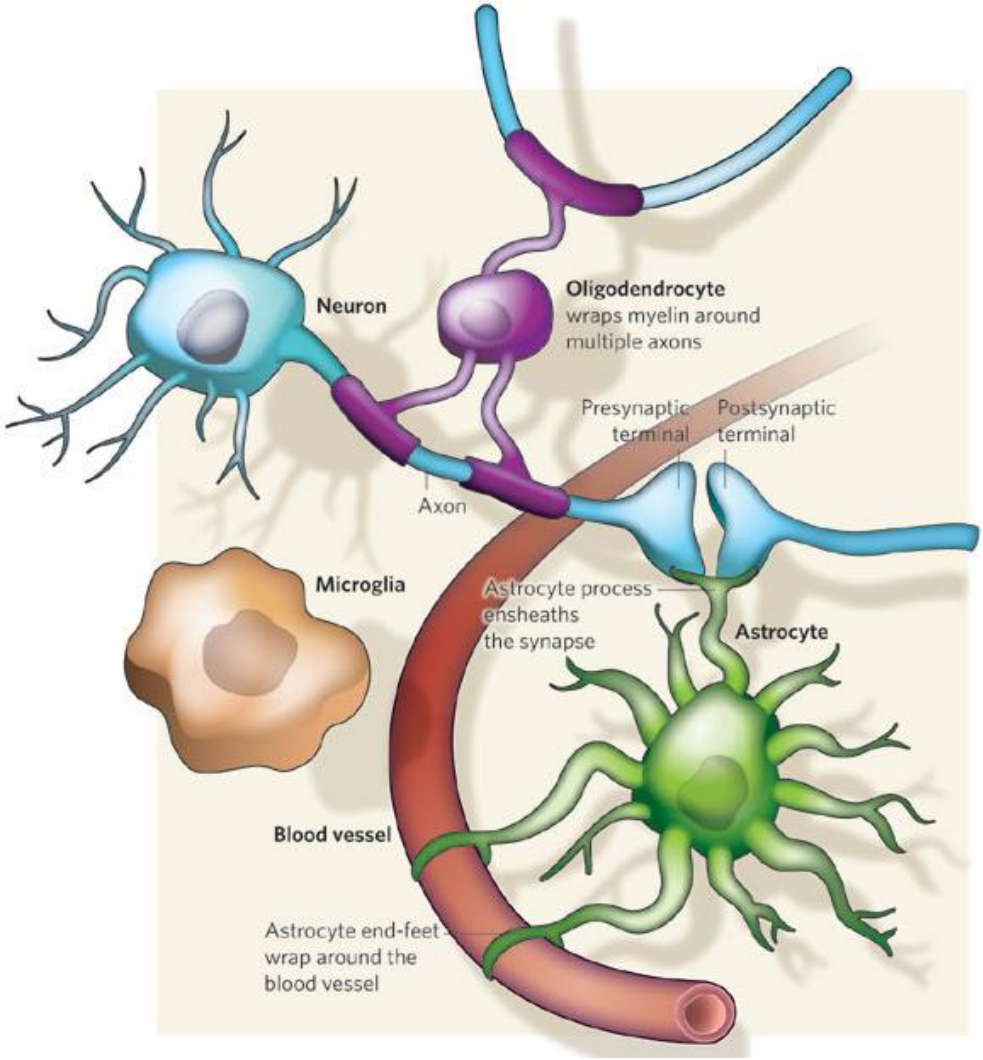

Figure 3. The major types of glia interacting with a neuron. *Adapted from Allen and Barres 2009[80]*.

Oligodendrocytes

In the CNS, the oligodendrocyte is the resident cell type responsible for the production of myelin that consists of oligodendroglial plasma membrane loops tightly wound concentrically around the axon. They arise from oligodendrocytes progenitor cells (OPCs), which are generated in the ventral neuroepithelium of the neural tube in early embryonic life, more specifically from the motor neuron progenitor (pMN) domain [87, 88], and in the dorsal spinal cord and hindbrain/telencephalon of the brain in late

embryonic development and early post-natal life [89, 90]. These proliferative cells migrate into the developing white matter [91-93], exit the cell cycle, undergo differentiation into mature oligodendrocytes, and begin to express a subset of myelin-associated proteins [94, 95] **(Figure 4)**.

OPCs can form either oligodendrocytes or astrocytes depending on the context [96, 97]. Once an OPC is committed to an oligodendroglial fate, it synthesizes large amounts of plasma membrane and extends multiple processes that individually wrap around axons generating a multilayered stack of membranes tightly attached at their cytosolic and external surfaces [98]. The thus formed myelin membrane provides electric insulation of axons and dictates the clustering of the sodium channels at the nodes of Ranvier and the organization of the node itself, allowing saltatory nerve conduction [99, 100]. In addition to these contributions to neuronal signaling, OLs also provide trophic support to neurons, and to long axons that may not receive adequate support from intra-axonal trafficking alone [85]. During the main phase of myelination, oligodendrocytes generate about 5,000-50,000 μm^2 of myelin surface area per cell per day [101], and this is accomplished by a remarkable synthesis and transport of myelin lipids. Moreover, by the time myelination is completed, oligodendrocytes have synthesized about 40% of the total lipids in the human brain [102].

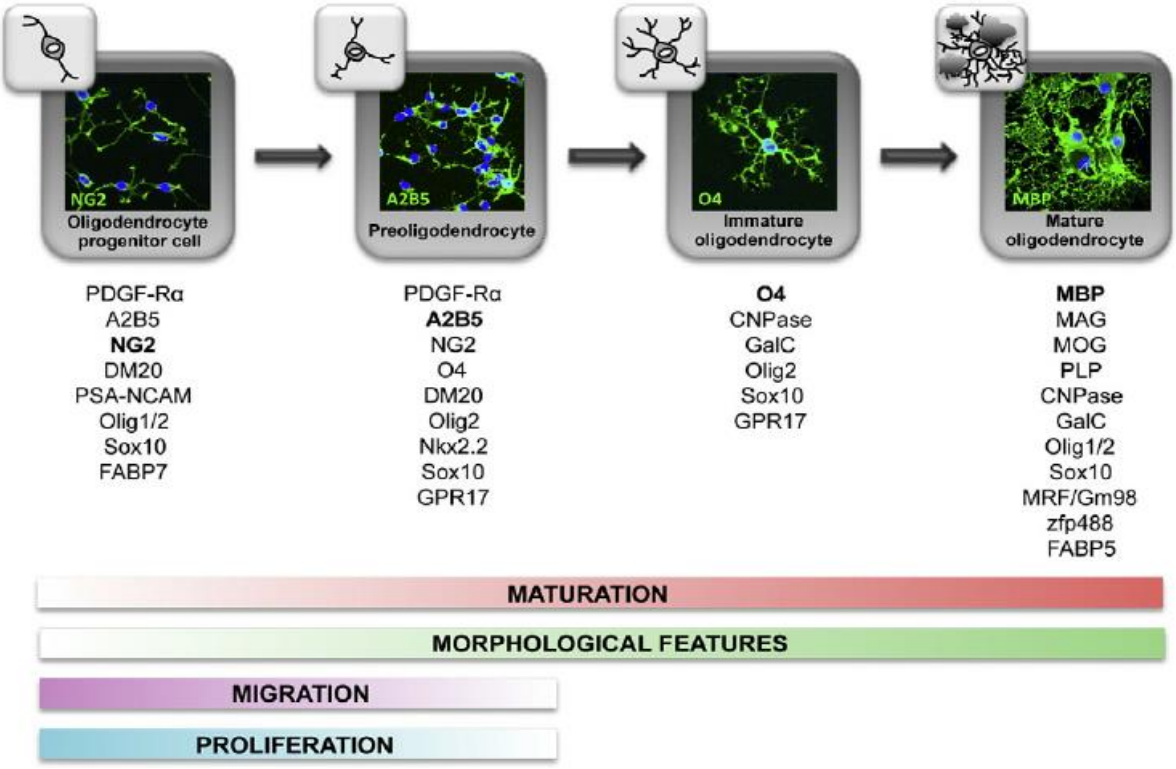

Figure 4. Oligodendrocyte maturation toward oligodendroglial lineage. Each stage is identifiable according to the increasing complexity in morphology, the ability to proliferate, migrate and differentiate, and the expression of specific antigens. *Adapted from Barateiro et al, 2014 [103]*.

Astrocytes

Astrocytes, or astroglia, are the most abundant and heterogeneous glial population in the central nervous system. In fact they outnumber neurons by over five fold [104] . Astrocytes have a plethora of functions, comprising roles as regulators of CNS homeostasis, through control of ion, pH, and neurotransmitter metabolism, and roles in the development and maintenance of the blood brain barrier (BBB), and in synaptogenesis and myelination [105, 106]. They originate from the neural embryonic progenitor cells that line the lumen of the embryonic neural tube, although they can also be formed indirectly via radial glia [107]. Though the heterogeneity of these cells is quite complex, the classification by Cajal, based on morphological differences, is still in use. Based on this classification, two main subtypes of astrocytes exist. Type 1 astrocytes, also named protoplasmic astrocytes, are localized in the gray matter where they ensheath blood vessels and synapses to promote BBB and synapses functions [82]. Type 2 astrocytes, defined fibrous astrocytes, are found in the white matter and have small cell bodies and

processes aligned with myelinated fibers, which gives them an elongated morphology. They also contact the nodes of Ranvier and the blood vessels [82, 105, 108]. In addition to this, other morphologically distinct populations of astrocytes have been described [109]. Moreover, astrocytes can also be diverse in their ability, ranging from inactive (quiescent), which exist in the normal resting CNS, to active (reactive). Astrocytes become activated in response to all form of CNS insults (infection, trauma, ischemia, etc.) through a process defined as reactive astrogliosis [110].

Recent evidence suggests a correlation between astroglial differentiation, during fetal cortical development, and lipid rafts, in particular phosphatidylglucoside-enriched lipid rafts (PGLRs). Phosphatidylglucoside (PtdGlc) is a glycoglycerolipid localized in the outer leaflet of the plasma membrane of several mammalian cell types, in particular in the brain, where it is highly expressed in the two neurogenic regions of the adult brain [111].

Microglia

Microglial cells are widely regarded as the resident immune cells of the brain, constantly scanning through the microenvironment with their long protrusions, readily sensing alterations in tissue homeostasis and integrity [112]. These cells, representing around 10% of the total glial cells in the nervous tissue, are present ubiquitously in the CNS, even though they are more enriched in the grey matter than in the white matter[113]. Under non-pathological conditions, microglia cells are highly ramified. In the diseased brain however their morphology changes, becoming more amoeboid. Upon activation, microglia progressively changes aspect, increasing the size of the cell body, retracting protrusions, and expressing *de novo* or up-regulating distinct profiles of surface phenotypic antigens, leading to an increased motility and to the adoption of immune effectors functions. Moreover, reactive microglia produces pro-inflammatory mediators including nitric oxide, reactive oxygen species (ROS), tumor necrosis factor α (TNFα) and a wide variety of other inflammatory cytokines[114]. Under pathological conditions, microglial functions depend on the stimuli that led to their activation; moderate CNS damage elicits protection by microglia [115-117], while following an intensive acute activation (for example stroke) or chronic activation (typical of neurodegenerative diseases) these cells tend to become neurotoxic, thus impairing neuronal activity [118-120].

The morphology and functions of microglia are highly affected by lipids, and changes in the composition of lipid rafts can lead to a decrease in the release of microparticles, which in turn leads to altered cell-cell communications [121]. Moreover, there is evidence showing that high levels of cholesterol increase the expression of pro-inflammatory genes in microglia [122], while polyunsaturated fatty acids seem to have an anti inflammatory effect on these cells [123]. Sulfatide is also able to induce a rapid activation of microglia. As a matter of fact, sulfatide released at brain lesions sites, following myelin damage, determines not only an increase in the activation of intracellular signaling pathways, including MAPKs and inflammation-associated transcription factors, but also an increase in the production of inflammatory cytokines and chemokines[124].

In addition to contributing to the maintenance of the normal CNS functions and to their role as sensors of altered homeostatic conditions, microglia also regulates apoptosis and survival of developing neurons [125]. Moreover, microglia provides trophic support and promotes differentiation of astrocytes and oligodendrocytes. Furthermore, recent evidence suggests a possible role of microglia as multipotent stem cells able to differentiate into neurons, astrocytes and oligodendrocytes [126].

Myelin: development, damage and repair

Myelin composition

The myelin membrane has a unique composition, characterized by an high lipid content, ranging between 73 and 81% of its total dry weight and a ratio of protein to lipid around 1 to 186 [105] [127,128].

All the major lipid classes are found in myelin, like in other membranes, however myelin still has a remarkably characteristic composition. In fact, while in most cell membranes the molar ratio of cholesterol, phospholipids and glycosphingolipids is 25:65:10, these ratios in myelin are in the range of 40:40:20 [129] which allows the close packing and tight organization of molecules within the membrane.

The myelin membrane has a high level of cholesterol, at least 26% by weight [127, 130,131], which, in contrast with other cell types, is synthesized using mainly ketone bodies as precursors instead of glucose [132]. The high level of cholesterol not only is necessary for myelin growth and compaction, but also provides stability to this membrane through the regulation of fluidity and permeability [132, 133]. Cholesterol is also necessary for correct myelin assembly, and the supply/synthesis of this molecule dictates

this process, suggesting that upstream signaling systems which drive myelin biogenesis could be coupled to cholesterol metabolism [133].

The myelin membrane also has a high amount of ethanolamine plasmalogens, whose levels correlate with the degree of myelination and reach the highest point between 30 and 40 years of age, when myelination is complete [134, 135]. The structural features of these ethanolamine plasmalogens, like the perpendicular orientation of the *sn*-2 acyl chain instead of the bent orientation it has in phosphatidylethanolamine, favors a closer alignment of both acyl chains in plasmalogens, thus decreasing the fluidity of the membrane. Moreover, the absence of a carbonyl oxygen at the *sn*-1 position determines an increased hydrophilicity, resulting in stronger intermolecular hydrogen bonding between the head groups [136, 137]. Considering this, one of the function of the high level of plasmalogens in myelin could be to increase the packing density and with it the stability of the membrane. There is also evidence suggesting that plasmalogens could have a role in protecting unsaturated membrane lipids against oxidation by singlet oxygen [138] and in providing lipid mediators for inflammatory reactions [139].

Gangliosides, brain-enriched sialic acid-containing glycosphingolipids, increase not only in amounts but also in complexity as the brain develops [42]. The most prominent shift in gangliosides levels occurs in the late stages of fetal development and extends into the first two years of human development, a period that coincides with the most active phase of myelination, and, while only small amounts of gangliosides are present in myelin (~1% of total lipids), they represent a major fraction of the neuronal membrane [134]. It is known that gangliosides are able to interact with several growth factor tyrosine kinase receptors, thus regulating their activity[140]. For example, GM3 binding to EGFR determines the inhibition of the receptor tyrosine kinase activity [71-73], and several gangliosides inhibit the dimerization of PDGFR [141]. In addition, FGF-2 is able to interact with several gangliosides [142]. Specific gangliosides in the local environment could therefore modulate the activity of receptors such as PDGFR and FGFR, which are known to influence proliferation and differentiation of oligodendrocyte progenitors[101, 143]. Furthermore, two of the major axonal gangliosides, GD1a and GT1b, are involved in long term myelin stability via their *trans* interaction with the myelin-associated glycoprotein (MAG), leading to MAG-induced inhibition of axon outgrowth [78, 79, 144].

Another distinguishing feature of myelin lipid composition, perhaps the most striking, is the enrichment in galactolipids. Galactosylceramide and 3-*O*-sulfogalactosylceramide

(sulfatide), with long chain fatty acids, account for approximately 20% and 5% of myelin lipids respectively [145,146]. Their biosynthesis involves two sequential steps. The enzyme UDP-galactose:ceramide galactosyltransferase (CGT), localized in the luminal side of the ER, catalyzes the transfer of a galactose from UDP-galactose to ceramide, thus forming GalCer [147]. A subpopulation of GalCer is then transported to the Golgi apparatus where the 3'-phosphoadenosine-5'-phosphosulfate:cerebroside sulfotransferase (CST) enzyme catalyzes the addition of the sulfate group, to obtain sulfatide [40, 146, 148] .

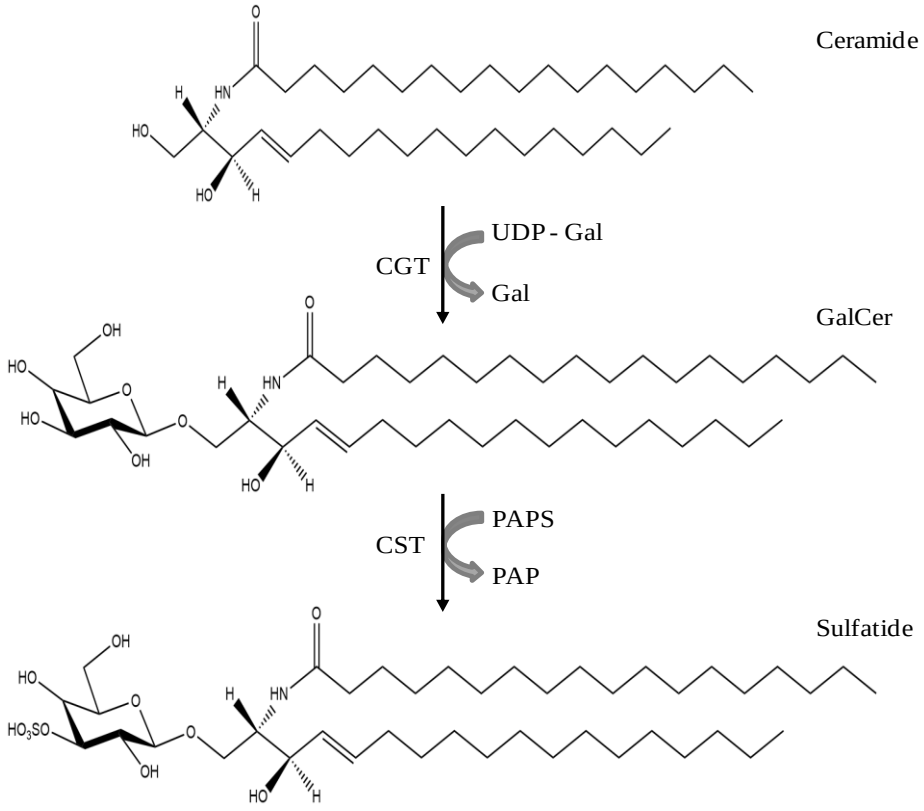

Figure 5. Structure and biosynthetic pathway of sulfatide, the major sulfoglycolipid in the nervous system. 3-O-sulfogalactosylceramide is highly heterogeneous in its fatty acid composition. The main fatty acids found in mature CNS myelin are long chain fatty acids (24:0 and 24:1), including a significant 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).

Following their synthesis, both lipids are transported to the outer leaflet of the plasma membrane [130], and, although they are not myelin-specific lipids, their enrichment in myelin, which is common feature of both CNS and peripheral nervous system (PNS) across species, is much higher than in any other tissue [40]. The abundance of galactosylceramide and sulfatide has led to the hypothesis that they could be involved in myelin formation and stabilization, and in oligodendrocyte development [149]. Therefore, to gain a better understanding of the role of GalCer and sulfatide, genetically altered animal models, CGT knock-out and CST knock-out respectively, have been established and analyzed.

The CGT enzyme, responsible for the synthesis of galactosylceramide, is highly expressed in the actively myelinating CNS ad PNS [150, 151]. Even though the CGT knock-out mice cannot synthesize GalCer and sulfatide, they are still able to form myelin with an apparently normal structure, which could be due to a partial compensation of the loss of these galactolipids by synthesizing 2-hydroxylated glucosylceramide, usually not present in myelin. These animals, however, display a neuropathological phenotype, characterized by splaying of the hind limbs, tremors, and ataxic locomotion that progressively worsens, leading to death of most animals by the third month of age [149, 152]. This phenotype is consistent with nerve conduction disruption despite the presence of compact myelin and, as a matter of fact, the action potential measured in the spinal cord of these mice is smaller and has a longer latency respect to wild type animals [149]. Moreover, while CGT (-/-) myelin is apparently normal, several ultrastructural abnormalities associated with myelination in the CNS have been identified. CNS myelin thickness is reduced, while nodal length is increased and lateral loops are widely spaced. The disorganization of the lateral loops suggests a disruption in the formation of the tight junctions, unsurprisingly since sulfatide is a prominent constituent of myelin tight junctions and the formation of these junctions may be dependent on the presence of sulfatide. Furthermore, at least one-third of the myelin processes in CGT knock-out mice retains oligodendrocyte cytoplasm, indicating presence of immature myelin in regions that appear as structurally mature myelin, and profiles of compact myelin frequently show more than two oligodendrocytic loops in a single internodal segment [153, 154]. Despite the presence of all these anomalies in CNS myelin, PNS myelin appears normal suggesting that galactolipids, less abundant in the PNS, might not be as essential in the formation and maintenance of the PNS myelin sheath structure [153, 155, 156].

The CGT (-/-) model, however, does not allow discrimination between the specific functions of GalCer and sulfatide, considering that in these mice they are both absent. To overcome this limitation, another model, the CST knock-out mice, was developed [157]. The CST knock-out mice are completely devoid of sulfatide, whereas other glycolipids in the brain, galactosylceramide included, are not significantly altered [158]. CST-deficient mice are born healthy but, around 6 weeks of age, they start exhibiting hind limb weakness, followed by pronounced tremor and progressive ataxia. The phenotype of these mice is in fact similar to that of CGT knock-out mice, even though it is milder in terms of age of onset, life span and severity of symptoms which allows these mice to survive for more than one year[157]. CST knock-out mice produce compact myelin, even though its thickness is reduced, compared to that of wild type mice, and paranodal structure displays alterations similar to those of the CGT (-/-) mice. Whereas in young mice the myelin sheaths are rather stable, the node/paranode structure only moderately altered, and axon size is comparable to that of wild type mice, as they age these mice show nodal structure deterioration, myelin vacuolar degeneration and also reduction of axon caliber [159]. Furthermore, electron microscopy analysis of myelinated nerve fiber revealed disorganized termination of the lateral loops at the node of Ranvier [160]. CST (-/-) mice also exhibit a deterioration in the clustering of Na^+ and K^+ channels at the node[161]. In mutant mice Na^+ channels concentrate in small regions, presumptive nodes of Ranvier, and the lengths of the clusters are occasionally higher than the ones present in the wild type mice. The K^+ channels clusters instead accumulate in regions adjacent to the Na^+ channels clusters in presumptive paranodal regions, whereas in normal CNS axons Na^+ channels cluster at the node of Ranvier and the K^+ channels concentrate in juxtaparanodal regions [161]. These alterations in localization and clustering of ion channels are present in both PNS and CNS and are accompanied by an altered distribution and expression of axonal proteins such as Caspr, contactin and NF155, suggesting that sulfatide might play a role in the trafficking or stabilization of this protein at paranodal level [161-163].

The loss of GalCer and sulfatide affects the proliferation and survival rate of oligodendrocyte precursors. CGT knock-out mice show a significant increase in cellularity in the spinal cord [156], while CST (-/-) mice exhibit an increased number of oligodendrocytes, which mature earlier and in higher number [164]. In CGT (-/-) mice this increase seems to be due to an increased terminal differentiation [164]. This is in contrast with the observation that in CST (-/-) mice the increase in oligodendrocyte population seems to be determined by an increased proliferation and by a reduced

apoptosis [165]. Taken together, the discrepancy in phenotype between CST- and CGT-null mice suggests that GalCer does not only act as a precursor for sulfatide synthesis, but also has a distinct function. GalCer appears to be primarily involved in myelin formation and maturation, while sulfatide contributes to the long term stability of myelin structure, in particular affecting the integrity and stability of the nodal and paranodal regions.

GalCer and sulfatide, or more precisely, GalCer- and sulfatide-rich domains in the oligodendrocyte membrane also regulate the co-clustering and lateral distribution of several myelin proteins, thus affecting the proliferation, differentiation and survival of oligodendrocytes [166]. In the early stages of myelin formation, only a few typical myelin proteins are associated with lipid rafts. However, by the mid-myelination stage, when GalCer and sulfatide are synthesized at detectable levels, the myelin/oligodendrocyte glycoprotein (MOG) and the proteolipid protein (PLP) tend to localize in lipid rafts, and subsequently, in the final stages of myelination MAG and myelin basic protein (MBP) are also translocated into lipid rafts [167-169]. In particular, sulfatide seems to be essential for the transport of PLP to myelin membranes, which is consistent with the observation that association of PLP is reduced in CGT (-/-) mice [170]. In addition, oligosaccharide-oligosaccharide trans interactions between GalCer and sulfatide present in the extracellular surfaces of the multilayered myelin membrane form a specialized "glycosynapse" which stabilizes the myelin sheath [171-174].

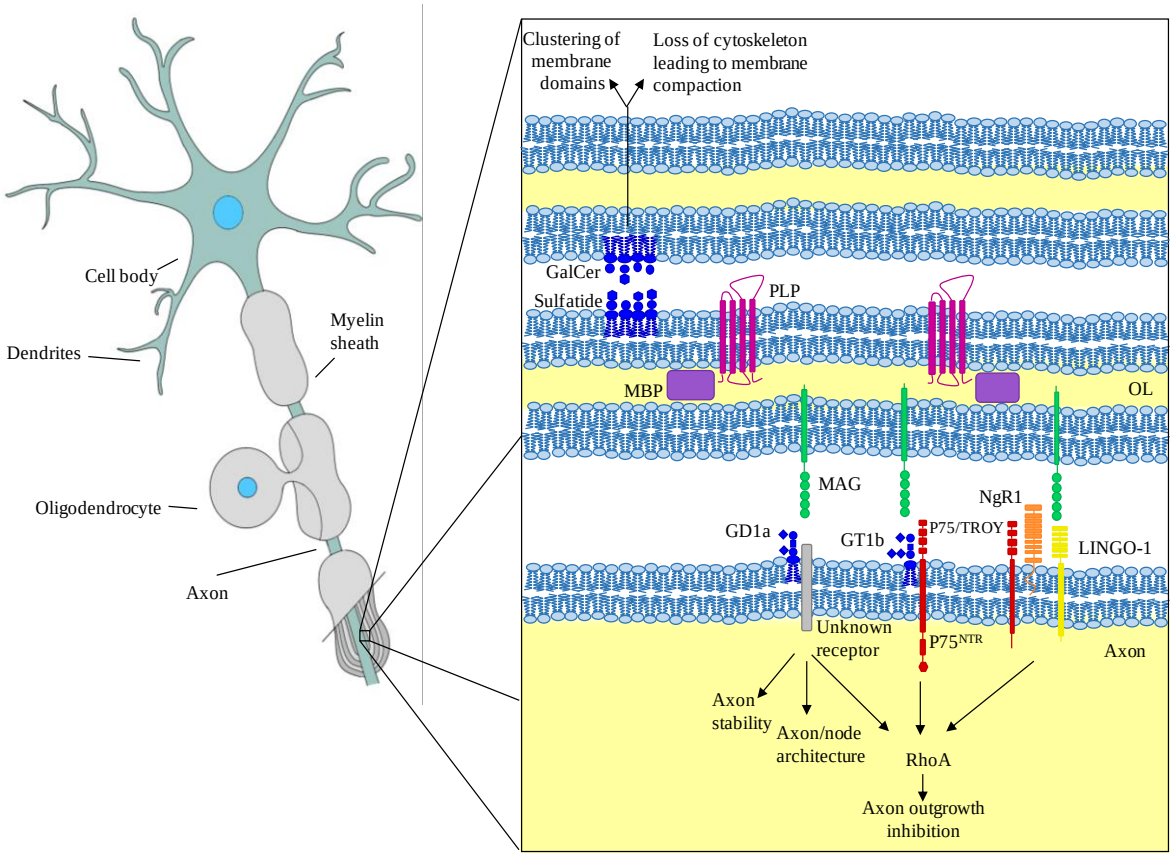

Figure 6. Glycolipid-enriched membrane domains in myelin. Glycolipid–glycolipid and glycolipid–protein interactions play multiple roles in myelin formation, maintenance and functioning but also in axon-myelin stability and communication. GalCer and sulfatide on opposing surfaces of the myelin wrap interact 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 [175]*.

Glial cells interaction in CNS (re)myelination and demyelination

The synthesis of myelin and the consequent ensheathment of axons by this multilamellar membrane restrict action potentials to short unmyelinated segments, namely the nodes of Ranvier. This provides the structural basis for saltatory action potential propagation, which in turn speeds up nerve conduction 10-20 times compared to non myelinated axons [176]. Myelin however is also important for axon maintenance and function [177].

Moreover, the cross talk between oligodendrocytes and axons is necessary to maintain metabolic function of axons, cytoskeletal arrangement, axonal transport, trophic support, and ion channel organization [85, 177-181].

Whereas the early postnatal human brain is mostly non-myelinated, CNS myelination increases progressively in a defined temporal and topographic sequence within the first two decades of life [182]. During development, oligodendrocytes progenitor cells (OPCs), highly proliferative, motile, bipolar cells, are the main source for mature oligodendrocytes and myelin. These cells originate in sequential waves in specific regions of the ventral and dorsal neuroepithelium of the spinal cord and brain before migrating and dispersing into the CNS [183, 184]. The majority of these cells undergo a series of changes triggered by first contact with the axonal membrane and characterized by a rapid increase in morphological complexity and expansion of uncompacted myelin membrane, ultimately leading to their differentiation into myelinating oligodendrocytes [185]. A small pool of OPCs, characterized by the expression of the surface antigens platelet derived growth factor α receptor (PDGFRα) and neural/glial antigen 2 (NG2), instead, remains undifferentiated and quiescent in the adult CNS [184] where they are involved in myelin repair in the injured or diseased CNS [186]. A number of CNS diseases damage or destroy myelin and oligodendrocytes, leading to demyelination. This pathological process is typically a consequence of either a direct insult aimed at the oligodendrocytes, or of primary axonal loss. The first, commonly referred to as primary demyelination, can be further divided in two categories from a clinical point of view: genetic abnormalities affecting glia (leukodystrophies), and inflammatory damage to myelin and oligodendrocytes (multiple sclerosis being the most representative) [187]. Following the loss of the myelin sheath, axons undergo several molecular reorganizations and physiological changes that ultimately result in axonal dysfunction, degeneration, and loss of sensory and motor function [188] and, regardless of causes or underlying mechanisms, the adult CNS has only a limited capability to repair damaged tissue. This limitation does not only involve neurons and their axons but also mature oligodendrocytes, unable to compensate for the myelin loss as they usually degenerate [189, 190]. However, demyelination often triggers a spontaneous myelin repair process, defined remyelination [187]. This process, mediated by OPCs that are recruited to differentiate and replace the lost oligodendrocytes [191], leads to myelin sheath restoration, reinstatement of saltatory conduction and functional recovery, and, ideally, it should recapitulate developmental myelination and tissue reconstruction should be complete. The myelin sheaths generated

during this process however are thinner and exhibit shorter internodes than the developmental ones [192, 193]. On the other hand, recent data suggests that at late time points of recovery newly remyelinated fibers have comparable internode length and thickness compared to their developmental counterparts [194].

As previously stated, the remyelination process is mediated by OPCs. In particular, NG2/PDGFRα-expressing adult progenitors are recruited to lesion sites and differentiate into mature oligodendrocytes able to remyelinate axons, thus restoring nerve conduction [195]. The efficacy of this process however is compromised and limited by the inflammatory and activated milieu surrounding the demyelinated lesions [196]. In multiple sclerosis, for example, changes in the CNS microenvironment during the progression of the pathology cause OPCs to gradually lose their ability to respond to myelin damage, thus limiting their remyelination capacity [197]. All the steps of the remyelination process (OPC activation, recruitment, differentiation and myelination) are tightly regulated by a plethora of extrinsic and intrinsic factors acting either as activators or inhibitors [198, 199]. In response to injury, adult OPCs undergo a switch from a quiescent state to an active one, corresponding to a regenerative phenotype. During this activation step, the progenitor cells become responsive to growth factors, cytokines and chemokines, enhancing their proliferation and recruitment to the demyelinated area. Moreover, several genes involved in oligodendrocyte development and differentiation are upregulated [200-203]. Astrocytes and microglia, both activated by injury, are the main source of the factors that induce the rapid activation of OPCs during demyelination. Astrocytes, for example, secret several soluble factors implicated in enhancing myelination, including platelet derived growth factor (PDGF) and basic fibroblast growth factor (FGF2) [185, 204-206], whereas microglia is able to induce chemotaxis of OPCs through the secretion of hepatocyte growth factor [207]. Following activation, OPCs, in addition to the ongoing proliferation, migrate to demyelinated areas. Concomitantly, macrophages and microglia begin the removal of the myelin debris, whose presence impairs remyelination [208,209]. Astrocytes also play a role in this clearance, by inducing the recruitment of microglia to the lesion site, a process regulated by the chemokine CXCL10 [210]. Once OPCs reach the demyelinated area, they must differentiate into remyelinating oligodendrocytes. To do so, they need to establish contact with the demyelinated axon, synthesize the myelin membrane and subsequently form the myelin sheath, a process that presents many similarities with the developmental myelination.

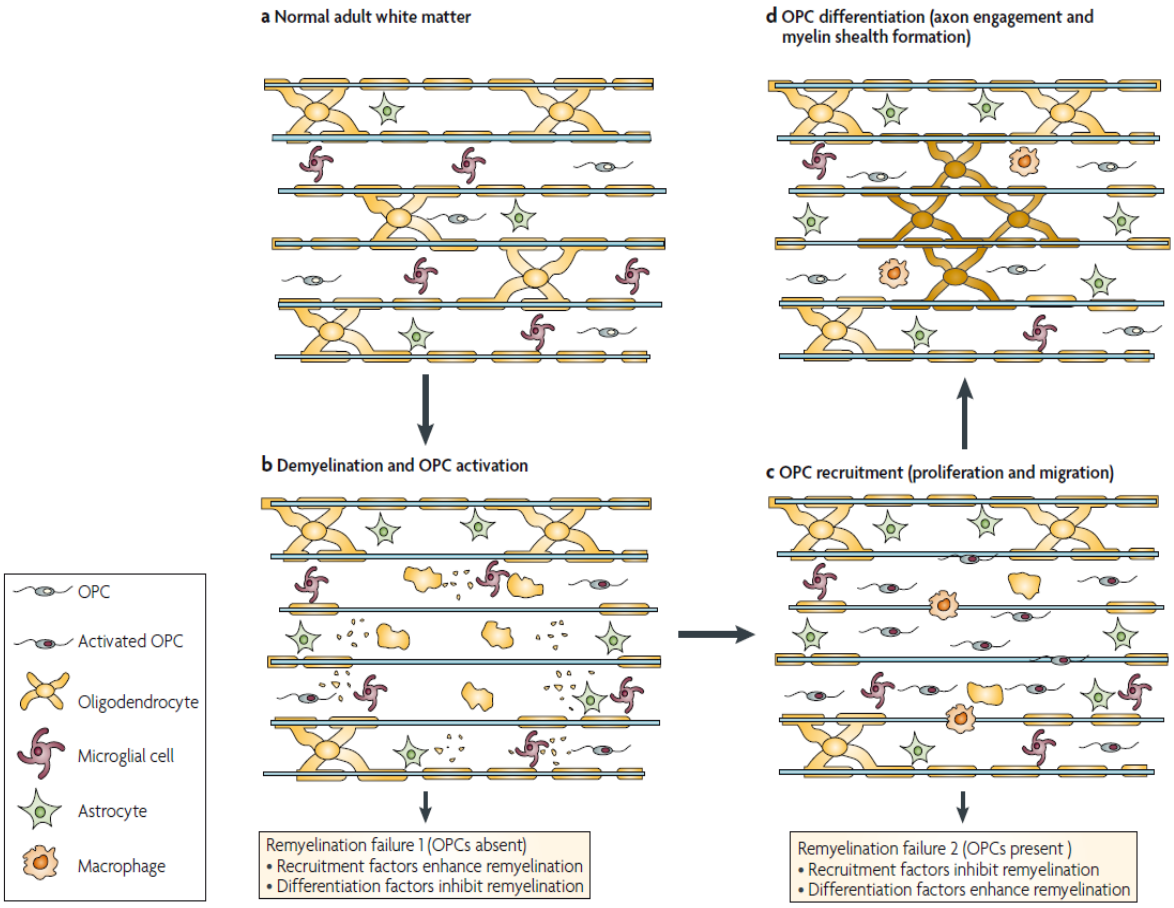

Figure 7. Phases of remyelination. Following demyelination, astrocytes and microglia activate, consequently leading to OPC activation. These activated OPC are then recruited and migrate toward the lesion area, while macrophages and microglia start to remove the myelin debris. In the final phase of remyelination, the recruited OPCs differentiate into mature oligodendrocytes, leading to the formation of a new myelin sheath. *Adapted from Franklin et al, 2008 [187].*

FGF and IGF, secreted respectively by astrocytes and microglia, play a role both in developmental myelination and in remyelination [211, 212]. However, differences between the regulation of development and regeneration of myelin do occur. OLIG1, for example, which is essential for developmental myelination, has a less redundant role during remyelination [213]. Another example is represented by the Notch signaling pathway, which is redundant during remyelination [214]. While remyelination can be quite efficient in experimental models, its efficiency remains generally low, leading to permanent deficits and dysfunctions. To a certain degree this decline is due to failure to the recruit OPC and to the failure of these cells to differentiate [187]. The underlying reasons for this are not completely understood but several pieces of evidence suggest the involvement of different factors, including age [215], gender [216], genetic background

[217], and also the presence of a variety of differentiation inhibitors that affect the glial regeneration potential [199, 218].

Myelin damage in multiple sclerosis

Several neurological diseases are characterized by loss of myelin sheath and destruction of oligodendrocytes. As previously stated, primary demyelination in the CNS can be caused by genetic abnormalities affecting glial cells. The diseases associated with this kind of abnormalities, though rare, usually present during childhood with generalized neurological symptoms. They can be divided into those due to defects of lysosomal function, like metachromatic leukodystrophy, those resulting from defects in astrocytes providing trophic support for myelinating cells, like Alexander's disease, and those due to deficiencies or misfolding of myelin proteins which in turn lead to abnormal myelinogenesis, like hypomyelinating leukodystrophies [219]. Primary CNS demyelination can also be caused by inflammation damage. The diseases associated with this kind of damage, characterized by myelin loss that occurs on a background of inflammation, include pathologies such as multiple sclerosis (MS), Marburg disease, neuromyelitis optica (NMO), Balo's concentric sclerosis, acute disseminated encephalomyelitis (ADEM) and its hyperacute variant, acute hemorrhagic leukoencephalitis (AHL) [220].

Among these, MS has been considered the lead disease featuring demyelination as a result of the scientific effort invested into its description and of its high prevalence.

Multiple sclerosis is the most common cause of non traumatic disability in young people, with an onset usually between 20-40 years of age [221], affecting 2.5 million people throughout the world [222]. MS is characterized by inflammation, progressive demyelination and gliosis, axonal injury and loss. The pathological hallmarks of all the subtypes of this disease are focal areas, called plaques, of demyelination in the CNS, with surrounding inflammation and neurodegeneration [223, 224]. MS etiology however remains to be defined. Currently the most widely accepted hypothesis concerning MS pathogenesis is the autoimmune hypothesis. This hypothesis proposes autoimmune inflammation as the cause of demyelination, and auto-reactive leukocytes as disease initiators. The process begins when naïve myelin specific $CD4^+$ T cells are primed in the lymph nodes by dendritic cells presenting either myelin or myelin cross-reactive epitopes. These cells differentiate into Th17 cells following stimulation by interleukin 23 (IL-23),

likely to play a central role in CNS autoimmunity [225, 226]. As these cells enter the CNS via subarachnoid space (SAS), together with activated macrophages, microglia and astrocytes, they secrete cytotoxic cytokines leading to demyelination [227-229]. A second hypothesis regarding MS onset is that the disease might be triggered by viral infection. This hypothesis does not exclude the autoimmune hypothesis, considering that virus could trigger the autoimmunity. Moreover, the viral infection might occur several years before the development of the MS lesions [230, 231]. A large amount of evidence supporting these hypothesis however was obtained using the most frequently used MS animal model, experimental autoimmune encephalomyelitis (EAE) and, while there are similarities between EAE and MS, there are still major differences between the two [232]. Therefore, EAE, while useful and suitable to study CNS-immune relationships and to test drugs targeting the CNS, might not represent a complete model of MS and the autoimmune hypothesis remains unproven.

There is, however, a third hypothesis, defined as the oligodendrogliopathy hypothesis, which is based on neuropathological studies on MS. Through histopathological examination of MS lesions Lucchinetti et al. defined four major immunopatterns, on the basis of specific myelin protein loss, plaque extent and topography, immunoglobulin deposition, oligodendrocyte destruction, and complement activation [220, 233], each possibly reflecting a different pathogenesis. Whereas in the first two types, type 1 (T cell-mediated autoimmune encephalomyelitis) and type 2 (T cell plus antibody-mediated autoimmune encephalomyelitis), demyelinated lesions are associated with inflammation consisting mainly of T cells and macrophages, in the other two types, type 3 (primary oligodendroglial apoptosis) and type 4 (primary oligodendroglial dystrophy), oligodendroglial death is a prominent feature in active and inactive lesions. The four types of patterns identified by Lucchinetti were further analyzed first by Barnett and Prineas [234] and later on by Henderson[235]. These studies, based on a thorough analysis of oligodendroglial apoptosis and inflammatory cell distribution in the various lesions, provided evidence suggesting that oligodendrocyte death and microglial activation are the initial event in MS lesion formation, followed by immune responses to scavenge dead myelin. Moreover, these immune responses seem to be permissive for oligodendroglial regeneration and remyelination, consistently with the observation that removal of myelin debris is necessary for remyelination [208]. The pathological heterogeneity observed in the four patterns is reflected in the clinical spectrum of MS. MS extends from an asymptomatic phase, of unknown duration starting at an unknown age, to clinically

symptomatic phases commonly known as radiologically isolated syndrome, clinically isolated syndrome (CIS), single-attack MS (SAMS), relapsing–remitting MS (RRMS), single-attack progressive MS (SAPMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS) [220]. These different phases of MS are characterized by an interplay between different levels of inflammation-demyelination, remyelination and axonal loss [236]. Moreover, remission of the disease symptoms in the initial stages is most likely due to a combination of resolution of inflammation, axonal plasticity, and remyelination. Furthermore, in early MS lesions remyelination is a frequent phenomenon while the majority of chronic MS lesions is characterized by limited remyelination [237]. The efficiency of this remyelination process seems to be influenced by factors such as anatomical localization, disease course, lesion size, and other patient dependent factors [237-240]. Astrocytes also seem to play a role. Following injury, these cells are activated and form a glial scar, composed of a dense network of hypertrophic cells, whose formation is crucial for restoring the blood brain barrier (BBB) normal function and integrity. In demyelinating conditions, however, the glial scar also represents a physical barrier hindering OPC entry into the demyelinated area to interact with neurons [177, 241-243]. Moreover, the scar also poses a biochemical obstacle for remyelination. The reactive astrocytes have marked changes in the expression levels of several molecules, including adhesion molecules, antigen presentation molecules, growth factors, receptor, cytokines, and protease inhibitors able to modify the composition of the extracellular matrix and to directly affect remyelination [244].

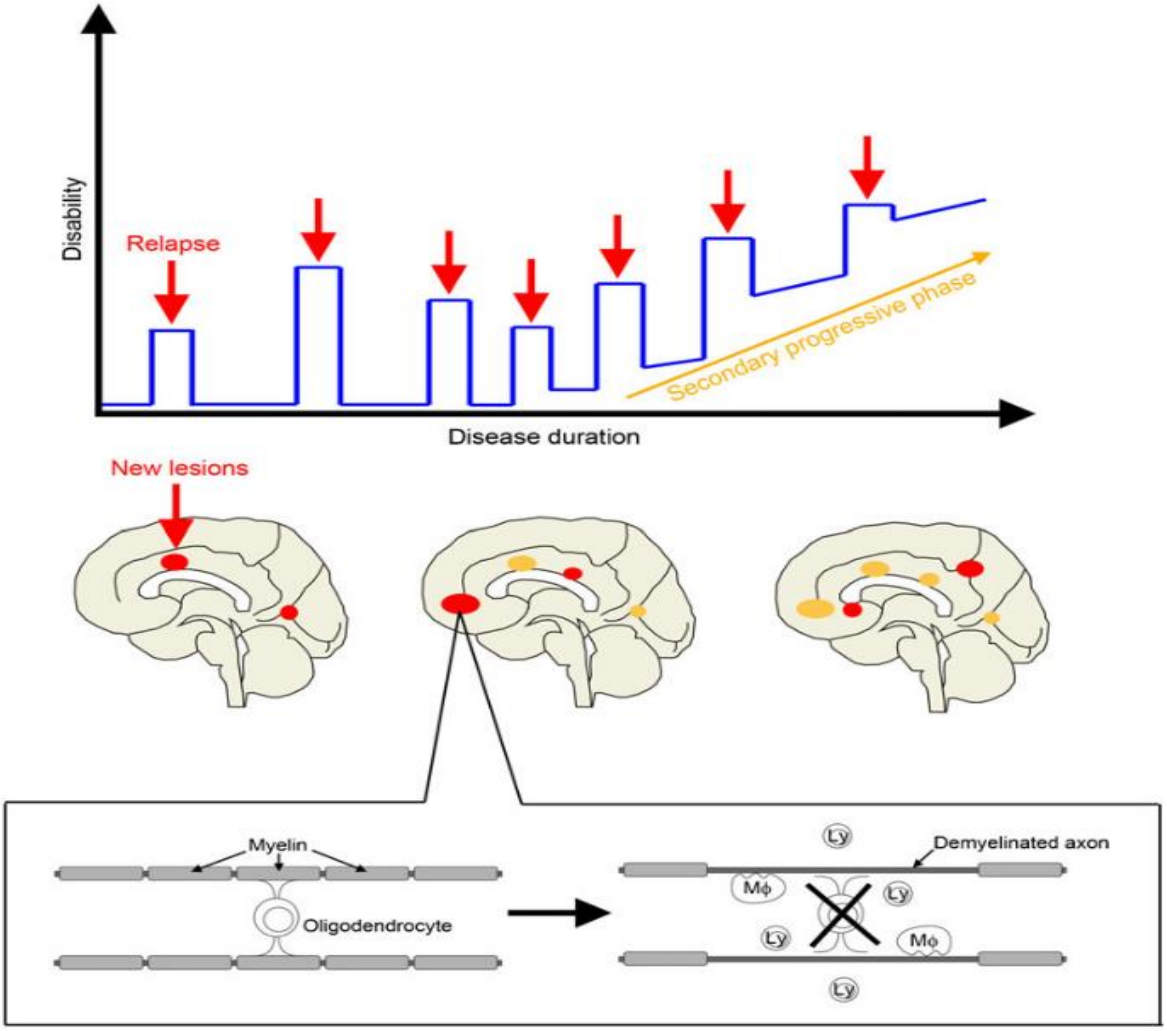

Figure 8. Clinical course of multiple sclerosis. The majority of MS patients initially develop RRMS and, usually, during the remittance there is a spontaneous neurological recovery. Later, these patients progress to SPMS, and the functional recovery is abolished. The hallmark of MS lesions is inflammatory demyelination. Loss of mature oligodendrocytes is often associated with this demyelinating process. *Adapted from Nakahara et al, 2012 [229]*.

Whether the immune response is the cause of the pathogenesis or simply a consequence of the oligodendroglial cell death, the failure of the immune system to discriminate myelin components from foreign antigens plays a critical role in the pathophysiology of MS. Several CNS myelin proteins, including MBP, PLP, MAG and MOG, have been described as targets for autoantibodies in MS [245-251]. Recent evidence however also suggests possible roles for myelin lipids in MS. In fact, in MS patients increased serum levels of glycolipids and anti glycolipid antibodies have been reported [252-256]. For example, it has been hypothesized that anti GD1a antibodies, increased in sera of MS

patients, could have a role in the impairment of OPC maturation [257]. Moreover, it has been observed that MS patients exhibit an enhanced antibody response against sulfatides [258, 259]. Furthermore, recent evidence has shown that increased levels of serum and CSF sulfatides are found in MS patients and in their healthy siblings, with stage-specific accumulation of different molecular species [260-262], suggesting that the presence of sulfatide in these biological fluids could represent a risk/prognostic factor for the onset and progression of MS.

Studies have also shown how anti-sulfatide antibodies can interact with the surface of cultured oligodendrocytes and affect the lateral organization of sulfatide with myelin proteins with opposite consequences (demyelination versus stimulation of myelin formation), depending on the type of ECM protein prevalent in the culture environment [167]. Hence, these antibodies might play a role in the onset of the disease, but they might also represent an important immunological tool for the treatment of demyelinating diseases [263].

Therapeutic approaches and remyelination promotion

The adult mammalian CNS is usually regarded as a regeneration incompetent organ, as opposed to the PNS, where axonal connections and myelin sheaths can be restored more easily [264]. The development of therapies aimed to promote remyelination within the demyelinated lesion in the CNS is an important therapeutic goal. In MS, naturally occurring remyelination is an overall inefficient process that fails to successfully counteract the accumulation of lasting axonal damage and increasing brain atrophy, thus resulting in motor and neurological deficits [265, 266]. Considering OPCs have been detected in chronic MS lesions [267], other determinants such as factors affecting migration and differentiation of these cells might be involved in the failure to remyelinate. Treatment able to modulate these factors could be clinically valuable. One of the FDA approved immunomodulatory drugs for MS, Fingolimod, is a sphingosine-1-phosphate (S1P) receptor modulator able to control lymphocyte trafficking [268]. However, it was also found to modulate resident glial cells, including oligodendrocytes, and to increase remyelination efficiency [269-272]. It is however still unclear whether this remyelination-promoting effect is simply due to a modulation of the inflammatory microenvironment or if it is due to a direct modulation of oligodendrocytes. Currently two major approaches involving a more direct stimulation of the remyelination process are being tested in animal models of demyelination. The first approach involves the transplantation of cells capable

of remyelination and is based on the evidence, gathered from multiple studies, that transplanted glial cells myelinate in the CNS following their introduction in the developing CNS of rodents with myelin mutations or with toxin-induced demyelination [273,274]. This approach however has limitations. There is little benefit to be gained by transplanting OPCs into lesions that already contain abundant cells with the ability to generate new oligodendrocyte. In these lesions the environment is inhibiting differentiation and regeneration and it would likely do the same for the exogenous cells. Moreover, the method of delivery also represents a problem. In fact, while for focal lesions a single injection might be enough, for diffuse disease multiple injections at different sites, each carrying a risk of intracerebral hemorrhage, would be necessary [275]. The second approach being tested in animal models of demyelination involves the promotion of repair by the resident stem- and precursor-cell populations in the adult CNS, through the administration of growth, trophic, and neuroprotective factors [276]. This approach is based on the idea that if the mechanisms of remyelination can be understood, and non-redundant pathways described, the causes of remyelination failure and consequently possible therapeutic targets, will be identified. As discussed in previous sections, remyelination failure is likely associated with either failed recruitment or differentiation of OPCs. However, different and mutually exclusive biologies underlie these two phases or remyelination. For example, PDGF promotes OPCs proliferation and migration but there is also evidence showing its inhibitory effect in the final stages of differentiation when the myelin sheath is formed [277]. Therefore, therapies aimed to recruit OPCs might not promote remyelination in situations where the main problem is OPC differentiation, and vice versa [278, 279].

An alternative therapeutic approach is the use of CNS reactive antibodies to promote remyelination [280]. So far, all identified remyelination promoting antibodies have natural autoreactive antibodies (NAbs) features and are of the IgM isotype, with one exception. This exception is represented by the high affinity anti-LINGO IgG antibodies, which are able to promote remyelination but do not have NAb features. One of these anti-LINGO antibodies, BIIB033, a monoclonal antibody, is currently being tested for its efficiency as a remyelinating drug. Neutralization of LINGO-1, an axonal protein involved in the regulation of axonal growth and in OPC differentiation, has been found to promote remyelination in several animal models [281], and has fueled high expectations regarding its potential effect in MS. While a study investigating the effect

of BIIB033 in optic neuritis (ClinicalTrial.gov: NCT01721161) has not reached its endpoint, a Phase II study in RRMS is still underway (ClinicalTrial.gov: NCT01864148). Excluding the anti-LINGO IgGs, all remyelination promoting antibodies have NAbs features and are of the IgM isotype. These antibodies react to self antigens and, compared to conventional antibodies, they have a relatively low affinity. 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 multiple sphingolipids. For example, the ganglioside-binding antibody A2B5 is able to recognize several GSLs due to their similar carbohydrate epitope [282, 283]. O4 recognizes sulfatide, seminolipid and also the unknown proligodendroblast antigen (POA) [284, 285], while HNK-1 targets MAG and also 3-sulfoglucuronyl paragloboside (SGPG) [286, 287]. All remyelination promoting IgMs produce a calcium influx in astrocytes, oligodendrocytes precursor cells, and immature oligodendrocytes [288]. The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptor has been shown to mediate the calcium influx into oligodendrocytes (both OPC and immature OL), while this influx, after antibody stimulation, in astrocytes is mediated by inositol triphosphate-sensitive channels[288]. Another feature common to all remyelination promoting IgMs is their ability to access demyelinated lesions within the CNS. Direct evidence of this was obtained through a magnetic resonance imaging-based study, however, accumulation seems to occur only in models in which the BBB integrity is compromised, and not in animals without demyelination [289].

One of these remyelination promoting IgMs, recombinant human IgM22 (rHIgM22), is able to bind to myelin and to the surface of oligodendrocytes *in vitro* and has successfully completed a phase I clinical trial, aimed to evaluate safety, tolerability, pharmacokinetics and immunogenicity of a single intravenous dose of rHIgM22 in patients with MS (ClinicalTrial.gov: NCT01803867). In addition, a second phase I trial aimed to evaluate safety and tolerability in relapsing MS patients is now recruiting (ClinicalTrial.gov: NCT02398461). This antibody was first identified through the screening of human serum of a patient with Waldenström macroglobulinemia, a rare, low grade malignancy, characterized by the presence of IgM-secreting clonal cells in the bone marrow [290]. The serum of this patient was screened to identify antibodies able to bind to myelin, and, out of the six antibodies satisfying this criteria, two IgMs (sHIgM22 and sHIgM46)

promoted significant remyelination *in vivo* [291]. sHIgM22, in particular, was able to bind to the surface of rat, mouse, and human oligodendrocytes [291, 263]. Further characterization of sHIgM22 led to the production of its recombinantly expressed version, rHIgM22 [292], which was found able to promote myelin repair in Theiler's virus infection-induced (TMEV) and lysolecithin-demyelinated models of multiple sclerosis [293]. The actual target and mechanisms of rHIgM22 are still under investigation however several pieces of evidence suggest that the antigen recognized by this antibody might be a plasma membrane lipid, possibly sulfatide, and that lipid raft might be involved in the signaling associated with rHIgM22 remyelinating activity. 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 [294], and that binding of rHIgM22 is abolished in CNS tissue sections from CST (-/-) mice [295]. Moreover, existing literature suggests that the binding target of rHIgM22 could be associated with detergent-resistant membranes (DRM)/lipid rafts and that rHIgM22 biological activity depends on lipid raft organization[294, 295]. rHIgM22 exerts its biological activity by inhibiting apoptotic signaling in OPCs and also by inhibiting the differentiation of these cells [294, 296]. The inhibition of the apoptotic signaling pathways is achieved via reduction of caspase-3 and caspase-9 cleavage and alteration of the caspase gene expressions in TMEV mice and in primary rat oligodendrocytes [294, 296], and this is dependent on calcium influx, through CNQX-sensitive AMPA channels [294]. Moreover, literature strongly suggests that rHIgM22 biological activity, responsible for its myelin-repair promoting activity, could require a multimolecular complex organizing Lyn and the cell surface molecules integrin αvβ3 and PDGFRα[296, 297]. Taken together, these observations have led to hypothesize that rHIgM22, through its pentameric structure, could mediate the clustering of a lipid antigen and stabilize lipid rafts domains. Moreover, it could determine the reorganization of Lyn, integrin αvβ3 and PDGFRα to form a signaling complex which, in turn, promotes OPC survival and proliferation [295, 296]. Signaling through this complex determines Lyn activation, and subsequent activation of the ERK 1/2 MAPK cascade, leading to the inhibition of caspase-3/9, to inhibition of OPCs differentiation and promotion of OPCs proliferation [297, 298].

In isolated OPCs, PDGF is required for rHIgM22-mediated inhibition of apoptotic signaling and differentiation. PDGF is produced by neurons and astrocytes and stimulates OPC proliferation and promotes OPC survival both *in vivo* and *in vitro*. Indeed, IgM-mediated OPC proliferation is detectable only in cultures containing substantial amounts

of astrocytes, microglia and OPCs (mixed glial cultures) but not in highly enriched OPC population[297].

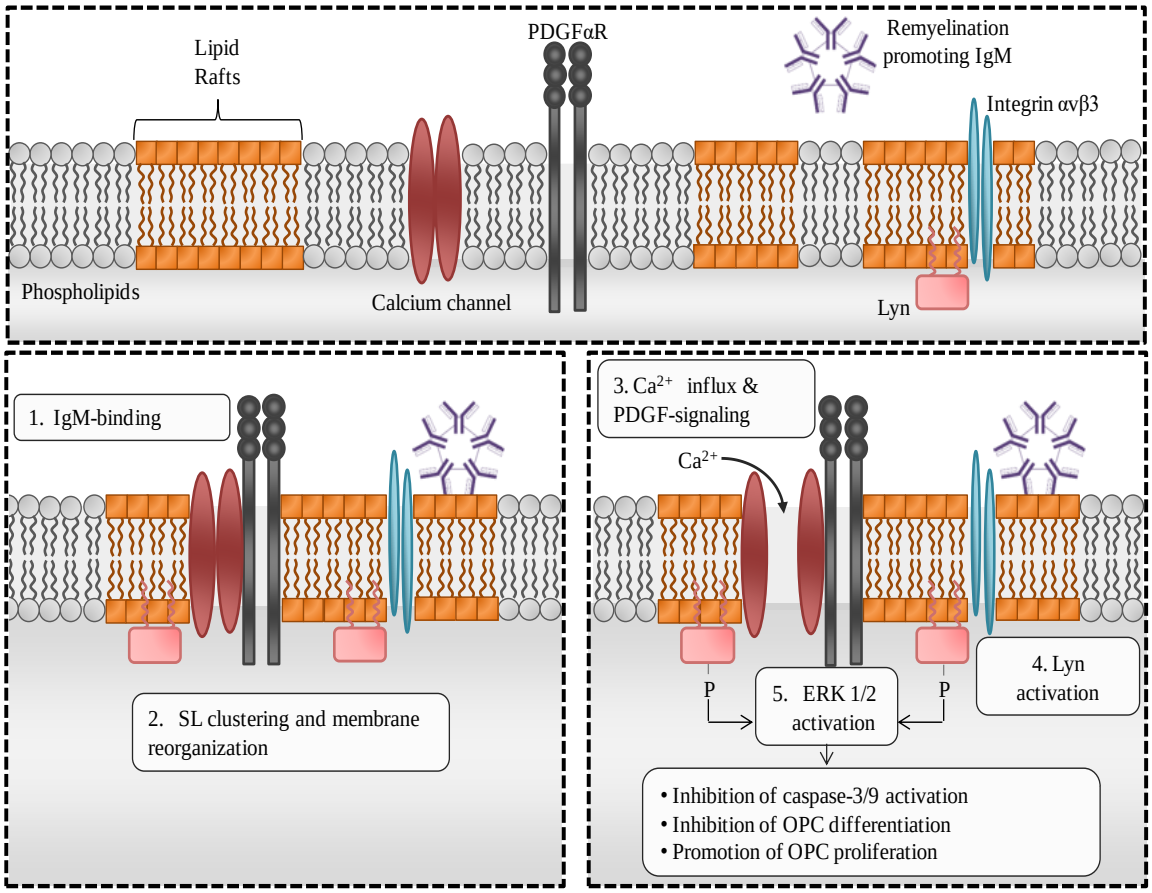

Figure 9. Proposed mechanism of action of rHIgM22. Binding of rHIgM22 to the surface of oligodendrocyte determines a reorganization of the membrane, favoring the interaction of Lyn, integrin αvβ3 and PDGFαR. IgM-stimulated activation of Lyn, with consequent activation of ERK 1/2 determines the inhibition 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 [298]*.

AIM OF THE STUDY

A number of CNS diseases are characterized by the damage or loss of myelin and oligodendrocytes, leading to demyelination. This pathological process is typically a consequence of either a direct insult aimed at the oligodendrocytes, or of primary axonal loss, and ultimately leads to the loss of the myelin sheath. Following demyelination in the central nervous system, a demyelinated axon has two possible fates. The normal response to demyelination, at least in most experimental models, is spontaneous remyelination, a process mediated by oligodendrocyte precursor cells. These cells are activated and recruited by the other glial cells in response to CNS injury and their proliferation and subsequent differentiation leads to the formation of new myelin sheaths, typically thinner and shorter than their developmental counterparts but nevertheless associated with functional improvement or recovery. However, even though in experimental models this process is efficient, remyelination is often inadequate in human demyelinating diseases such as multiple sclerosis. If remyelination fails, the axon, devoid of its myelin sheath, undergoes deep molecular reorganization and physiological changes that ultimately result in axonal dysfunction, degeneration, and loss of sensory and motor function [188]. For this reason, therapies that increase the chances of the regenerative outcome of demyelination are keenly sought.

One of the therapeutic approaches that is currently being developed to improve the regenerative outcome involves the use of CNS reactive antibodies to promote remyelination [280]. One of these antibodies, rHIgM22, which is able to bind to myelin and to the surface of oligodendrocytes *in vitro*, has successfully completed a phase I clinical trial for the treatment of MS (ClinicalTrial.gov: NCT01803867). Moreover, a second phase I trial aimed to evaluate safety and tolerability in relapsing MS patients is now recruiting (ClinicalTrial.gov: NCT02398461). This antibody is able to enter the CNS, accumulate at lesion site and promote remyelination in mouse models of chronical demyelination [289, 291]. The antigen recognized by this antibody and the molecular mechanism underlying the remyelinating activity of rHIgM22 however are yet to be elucidated. Evidence suggests that the binding target of rHIgM22 could be associated with plasma membrane lipid rafts, and that lipid rafts might be involved in the signaling associated with the biological activity of this antibody [294, 295]. Moreover, a signaling complex in OPCs responsible for rHIgM22-mediated actions including platelet-derived growth factor (PDGF)Rα, integrin αvβ3 and the Src family kinases (SFK) Lyn has been identified [295, 296]. Despite this finding, isolated OPCs do not respond to rHIgM22

treatment, instead mixed glial cultures consisting of astrocytes, OPCs and microglial cells demonstrate observable rHIgM22-mediated OPC proliferation [299].

In the light of previous statements, the aim of this study is to analyze the plasma membrane lipid rafts composition in MGC and the effects exerted by rHIgM22 on these cells after single dose treatment of various duration. The characterization of the membrane microenvironment necessary for the activity of this antibody, able to promote remyelination in validated mouse models of MS, and the analysis of alteration induced by its action could contribute to understand the signaling mechanisms underlying the biological activity of rHIgM22. Moreover, this could allow to understand the molecular mechanisms involved in the MS and to define new potential therapeutic targets.

MATERIALS AND METHODS

Materials

Commercial chemicals were the purest available and, unless otherwise stated, were purchased from Sigma Aldrich Srl.

Ca^{2+} and Mg^{2+}-free HBSS, D-Glucose, BSA fraction V, HEPES, trypsin, sodium pyruvate, poly-D-lysine, PBS, Na_3VO_4, KCl, NaOH, methanol, chloroform, hexane, NaCl, PMSF, aprotinin, sucrose, and EDTA were purchased by Sigma Aldrich; penicillin/streptomycin, bovine fetal serum, DMEM high glucose, and glutamine from Euroclone Spa; $MgSO_4$, $CaCl_2$, and HPTLC plates from Merck; DNaseI from Roche Spa; acetic acid from Fluka; HCl from VWR International PBI Srl.

Chrompure Human IgM (#009-000-012) has been purchased from *Jackson Immuno Research, Inc*.; Human purified IgM (#I8260) and anti-GAPDH (#G9545) were purchased from *Sigma Aldrich;* anti-Integrin αv (#611012) was purchased from *Becton, Dickinson and Company;* anti-Akt (#9272), anti-Caveolin 1(#3267), anti-P-Src(Y416)family (#2101), anti-PDGFRα (#3164) and anti-Lyn (#2732) were purchased from *Cell Signaling Technology;* anti-PrP(SAF32)(#189710) was purchased from *Cayman Chemical*.

HRP-conjugated anti-mouse IgG antibody (#31430) has been purchased from *Thermo Fisher Scientific, Inc*.; HRP-conjugated anti-rabbit IgG antibody (#7074) was purchased from *Cell Signaling Technology.*

The rHIgM22 antibody has been kindly provided by *Acorda Therapeutics, Inc. (Ardsley, NY).*

Pure galactosylceramide (GalCer), and sulfatides were purchased from *Avanti Polar Lipids*; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), and phosphatidic acid (PA) were purchased from *Sigma Aldrich*. Lysosulfatide was purchased from *Matreya*. Ceramide, gangliosides (GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b), glucosylceramide (GlcCer), 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, used for preparation of mixed glial cultures.

Cell culture

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 [297, 300]. 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 [297]. Briefly, the hemispheres from P3 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.

Treatment of cell cultures with [1-^{3}H]sphingosine

24 h after seeding, cells were incubated in the presence of 3.68×10^{-8} M [1-^{3}H]sphingosine [1-^{3}H]sphingosine (5 ml/dish) in culture medium for 2 h (pulse). After the pulse, the medium was replaced with fresh medium without radioactive sphingosine, and cells were further incubated for up to 48 hours (chase). Under these conditions, all sphingolipids (including ceramide, SM, neutral glycolipids, and gangliosides) and phospholipids (obtained by recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine) were metabolically radiolabeled.

Preparation of DRM fractions by sucrose gradient centrifugation

Cells were subjected to homogenization and to ultracentrifugation on discontinuous sucrose gradient, as previously described [301]. Briefly, cells were harvested, lysed in 1% Triton X-100 in TNEV (10 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA) in the presence of 1 mM Na_3VO_4, 1 mM PMSF, and 75 mU/ml aprotinin, and *Dounce homogenized* (10 strokes, tight). Cell lysate was centrifuged for 5 min at 1300 g to remove nuclei and cellular debris. The postnuclear supernatant (PNS) was mixed with an equal volume of 85% sucrose (w/v) in TNEV, placed at the bottom of a discontinuous sucrose gradient (30% - 5%), and centrifuged for 17 h at 200,000 g at 4°C with ultra-centrifuge Beckman Coulter optima L-90K. After ultracentrifugation, eleven fractions were collected starting from the top of the tube. The entire procedure was performed at 0-4°C in ice immersion. The protein levels and lipid distribution were analyzed individually in each fraction. A part of each fraction (about 50%) was subjected to dialysis, with the end to eliminate the excess of sucrose due to the fractioning process, snap frozen and lyophilized. The other part of each fractions was directly mixed with *Laemmli buffer* (1x: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% Bromophenol blue, 10% glycerol) for protein analysis, as described further. To investigate the metabolism of sphingolipids and phospholipids, cells were previously labeled with [1-^{3}H]sphingosine.

Lipid analysis

Sample preparation

Cultured mouse mixed glial cells 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 snap frozen and stored at -80°C. Frozen samples were then thawed at room temperature (RT), resuspended in ice-cold water, snap frozen and lyophilized.

Total lipid extraction, phase partitioning and alkali treatment

Lipids from the lyophilized samples were extracted with chloroform/methanol/water 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.) [4]. Briefly, 1550 µL of the solvent system were added to the lyophilized samples. The samples were then mixed at 1100 rpm,

RT for 15 minutes and centrifuged at 13200 rpm, RT for 15 minutes. The supernatant was collected as Total lipid extract (TLE) and the extraction was repeated again twice by adding the 1550 µL of the solvent system to the pellets. The pellets were air dried and resuspended in 1N NaOH and incubated overnight at RT before being with water to 0.05N NaOH to allow the determination of the protein content with DC assay. Aliquots of the TLE were then subjected to phase partitioning adding 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) was 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 aqueous phases were dried under N_2 flux, and resuspended in water before undergoing dialysis and lyophilization. The organic phases were dried under N_2 flux and resupended in a known volume of cholesterol/methanol 2:1. Aliquots of the organic phases were then subjected to alkali treatment to remove glycerophospholipids [302].

Thin layer chromatography

To determine lipid content, the various samples were analyzed by mono-dimensional silica gel HPTLC using different solvent systems. The total lipid extracts were analyzed using chloroform/methanol/0.2% aqueous 60:35:8 (v/v/v) as a solvent system, the aqueous phases were analyzed with chloroform/methanol/0.2% aqueous $CaCl_2$ 50:42:11 (v/v/v), whereas the organic phases and the methanolyzed organic phases were analyzed using chloroform/methanol/water 110:40:6 (v/v/v). The organic phases were also subjected to HPTLC separation with chloroform/methanol/acetic acid/water 30:20:2:1 (v/v/v/v) to analyze the glycerophospholipid content. After separation, radioactive lipids were detected and quantified by radioactivity imaging performed with a Beta-Imager 2000 instrument (Biospace Lab, Paris, France) using an acquisition time of about 48 h. The radioactivity associated with individual lipids was determined with the specific M3Vision software provided by Biospace Lab. Identification of lipids after separation was assessed by co-migration with lipid standards. Cholesterol was separated by monodimensional HPTLC using the solvent system Hexane/Hetyl Acetate 3:2 (v/v) and visualized by spraying the plate with 15% solution of concentrated sulfuric acid in 1-butanol.

Protein analysis

Protein quantification

The protein quantification was performed through DC assay (Bio-Rad). The assay was performed in 96 well plates following the protocol supplied with the Bio-Rad DC assay kit. The samples were analyzed in triple, like the protein standard, bovine serum albumin (BSA), at different concentrations. 25 μL of reagent A and 200 μL of reagent B, both supplied with the kit, were added to each well. After 15 minutes of incubation, the absorbance at 750 nm was measured with the spectrophotometer. The samples reading were compared with the ones of the standard. The assay is linear between 1.5 and 7.5 μg of protein amount.

Electrophoresis and Western Blotting

The samples were analyzed using electrophoresis on a polyacrilamide gel with denaturing conditions. The samples were resuspended in *Laemmli buffer* (1x: 62.5 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 0.01% Bromophenol blue, 10% glycerol) and boiled for 5 minutes at 100°C before being analyzed.

The electrophoresis run was performed using a Miniprotean II unit, produced by Bio-Rad. To obtain optimal resolution, a stacking gel is polymerized on top of the resolving gel. A solution of 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 was used as *running buffer*. The proteins were separated using 10% polyacrylamide gels.

After electrophoresis separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilion P-Membrane #IPVH00005 Merkck Millipore), at 200 mA for 3 hours at 4°C with a wet blotting (Mini Transblot Biorad). The transfer buffer used is Blotting buffer 1x (25 mM Tris-HCl, 192 mM glycine, 15 % methanol, pH 8.0-8.5).

After the transfer, the PVDF membranes were immunoblotted using Integrin αv, Akt, Caveolin 1, Lyn, PDGFRα, GAPDH, P-Src(Y426) family and PrP(SAF32). Briefly, after the transfer the membrane was incubated in 5% milk in TBS-T 0.05% (1 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween) to block the non-specific binding sites of the membrane. The membrane was then washed three times with TBS-T 0.05% and incubated with a specific antibody (primary antibody) for time depending on the antibody used. The primary antibody was diluted in a solution of TBS-T 0.05% containing 1% bovine serum albumin (BSA). The membrane was washed again with TBS-T 0.05% for four times, to

get rid of the antibody excess, before being incubated with the secondary antibody conjugated with horseradish peroxidase (HRP) at room temperature for 45 minutes. For membranes previously incubated with either Integrin αv or PrP(SAF32) primary antibodies, an anti-mouse IgG (Thermo, #31430) was used. For membranes incubated with Akt, Caveolin 1, PDGFRα, GAPDH and P-Src(Y416) family and Lyn antibodies, an anti rabbitIgG-HRP (Cell Signaling, #7074) was used. The membrane was then washed again for six times and the peroxidase activity was assessed through incubation with a non-radioactive light emitting substrate for the detection of immobilized specific antigens conjugated with horseradish peroxidase-linked antibodies (LiteAbLot Plus, Euroclone) for 2 minutes. The luminescent compound generated following the reaction can be detected through exposition to the Alliance 9.7 Western Blot Imaging System fom UVItec Limited and analyzed by the Alliance 1D software.

Treatment of MGC cultures with rHIgM22

Treatment with rHIgM22 and Human IgMs

After 24 hours from the beginning of the chase, MGC, labeled with [1-^{3}H]sphingosine, were incubated in the presence of a MGC complete medium, described above, added with 10μg/mL of either rHIgM22 (lot 1-FIN-1223), Human IgM, Human IgM directly in the petri dishes. The morphological effect of this treatment was evaluated at different time (6, 24 and 48 hours) after treatment by observation at the inverted fluorescence microscope (IX50 Olympus).

The following experiments were conducted after the treatment.

ASMase activity assay

Sample preparation

MGC after 6, 24 and 48h of treatment with rHIgM22 (lot 1-FIN-1223), Human IgM (Jackson #009-000-012) and Human IgM (Sigma #I8260), have been washed twice and scraped twice in PBS containing 1 mM Na_3VO_4. Then, cells were centrifuged at 3000 rpm for 10 minutes at 4°C, the supernatant was discarded and pellet was resuspended in 100 µL 0.2% Triton X-100 in H_2O. After 10 minutes on ice, samples were sonicated (3 times; 10 second “on”, 30 second “off”) and the protein content was determined using DC assay. Each sample was, then, diluted to obtain a concentration of 1 µg/µL.

Substrate preparation

500 pmol of SM have been mixed with 10000 dpm (about 12 pmol) of ^{3}H-SM and added with 25 μL of 0.2% Triton X-100 in $CHCl_3$:CH_3OH 2:1 (v/v) for each sample to analyze. The mixture obtained was vortexed, sonicated (3 times; 10 second "on", 30 second "off") and dried under N_2 flux. After, 25 μL of 250 mM sodium acetate, pH 5.1 for each sample were added.

The substrated prepared has been mixed at 300 rpm, 37°C for 1h to allow micelles formation.

ASMase activity reaction

25 μg of protein of cell lysates were added to 25 μL of reaction substrate and samples (final SM concentration 10 μM) were incubated for 2 h at 37°C. The reaction was stopped by the addition of 200 μL of CHCl3:CH3OH 2:1 (v/v) at RT for 20 min, followed by centrifugation at 13200 rpm for 20 min. Organic and aqueous phases were, then, separated and the radioactivity associated with each phase was determined by liquid scintillation counting. For samples labelled as "Blank", 25 μL of 0.2% Triton X-100 in H2O were added to the substrate instead of the cell lysate. Each reaction was run in triplicate. The amount of SM hydrolyzed was determined through autoradiography.

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

The actual target and mechanisms of rHIgM22 are still under investigation however existing literature suggests that the antigen recognized by rHIgM22 might be a plasma membrane lipid, possibly sulfatide, that the binding target of rHIgM22 could be associated with detergent-resistant membranes (DRM)/lipid rafts, and that rHIgM22 biological activity depends on lipid raft organization [294, 295].

Moreover, Wootla et al., suggested an involvement of the astroglial growth factor PDGF in rHIgM22-mediated actions in OPCs; in fact they identified a signaling complex in OPCs responsible for rHIgM22-mediated actions including platelet-derived growth factor (PDGF)αR, integrin αvβ3 and the Src family kinases (SFK) Lyn113. Further, they observed that isolated OPCs do not respond to rHIgM22 treatment, instead mixed glial cultures consisting of astrocytes, OPCs and microglial cells demonstrate observable rHIgM22-mediated OPC proliferation [299].

Therefore, analysis of lipids and proteins distribution in MGC gradient fractions and the effect of rHIgM22 treatment on MGC were performed.

Lipid distribution in MGC gradient fractions

In order to investigate the possible target of rHIgM22 in plasma membrane lipid rafts, MGC gradient fractioning was performed. To do this, cells, previous labeled with [1-^{3}H]sphingosine, were lysed in the presence of Triton X-100 and subjected to discontinuous sucrose gradient centrifugation as described in Materials and Methods section. To determine how lipids distributed in the various fractions, radioactivity associated with the single fractions was determined by liquid scintillation counting.

The radioactivity distribution, as shown in in **Figure 1**, allowed to define three major fractions: a low density, detergent resistant membrane fraction (DRM, fractions 4 and 5), an intermediate fraction (INT., fractions 6, 7 and 8) and an high density fraction (HD, fractions 9, 10 and 11).

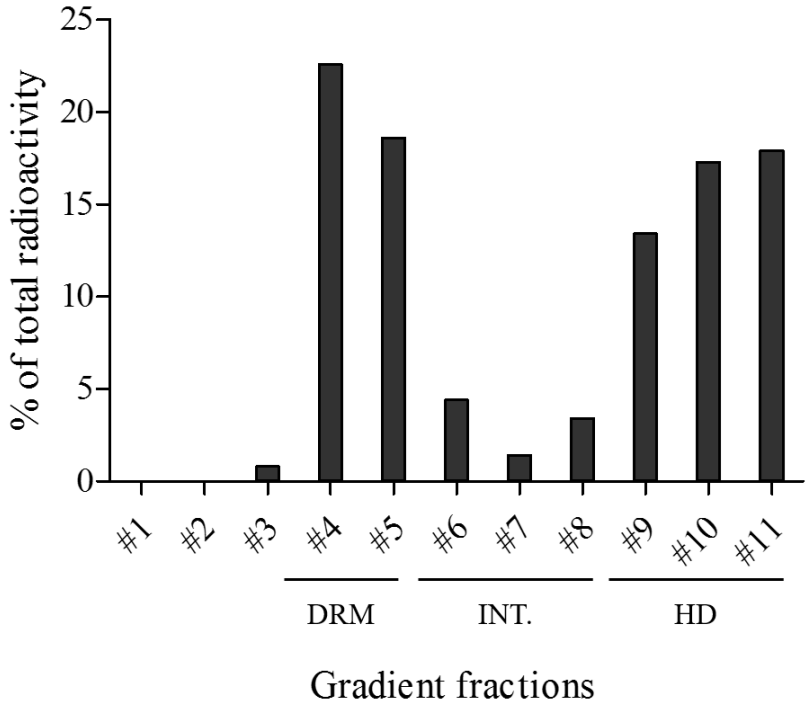

Figure 1. Radioactivity distribution in MGC gradient fractions. Cells were subjected to metabolic labeling with [1-^{3}H]sphingosine, homogenization and to ultracentrifugation on discontinuous sucrose gradient, as previously described in Materials and Methods section. Briefly, cells were harvested, lysed in 1% Triton X-100 in TNEV and Dounce homogenized. Cell lysate was centrifugated to remove nuclei and cellular debris and the postnuclear supernatant (PNS) was mixed with an equal volume of 85% sucrose (w/v) in TNEV, placed at the bottom of a discontinuous sucrose gradient (30% - 5%), and ultracentrifugated. Eleven fractions were collected starting from the top of the tube. Radioactivity distribution was analyzed individually in each fraction by liquid scintillation counting.

TLC separations of lipid extracts from MGC gradient fractions

In order to evaluate the lipid pattern of the different MGC gradient fractions , total lipids, have been extracted using chloroform/methanol/water 20:10:1 (v/v/v) and partitioned as described in Materials and Methods section. Then, TLC analysis of total lipids, organic and aqueous phases obtained were performed.

Sphingomyelin (SM), phosphatidyletanolamine (PE) and gangliosides were the most represented lipids in the analyzed fractions, as shown in **Figure 2**.

Lipid separation of the organic phases, performed using HPTLC, revealed the presence mainly of SM, PE and ceramide (Cer) (**Figure 3).** In particular, SM was enriched in DRM fraction and decreased both in intermediate fraction and in HD fraction; PE, was

enriched in HD fraction and decreased both in intermediate fraction and in DRM fraction; and ceramide was present in all the fractions analyzed.
Moreover, the analysis of the aqueous phases, reported in **Figure 4**, showed the presence of various gangliosides, namely GM1, GM2, GM3 GD3, GD1a and GT1b, which were found to be enriched in the DRM fraction.

Summarizing, the data just described allowed to compare the distribution of different classes of lipids among the MGC gradient fractions.
In details, sphingolipids (SL), including ceramide (Cer), glucosylceramide (GlcCer), sphingomyelin (SM) and gangliosides, were more represented in the DRM fraction and slightly represented in the HD fraction; differently, phospholipids (PL), including phosphatididylethanolamine (PE) and phosphatidic acid (PA), took an opposite distribution, as shown in **Figure 5**.

Moreover, **Figure 5** showed that both SM and gangliosides were distributed, mainly, in the DRM fraction and very poorly represented in the other fractions. So, as expected, the DRM fraction was highly enriched in sphingolipids, and relatively depleted of glycerophospholipids. The major amount of glycerophospholipids existed in HD fraction.

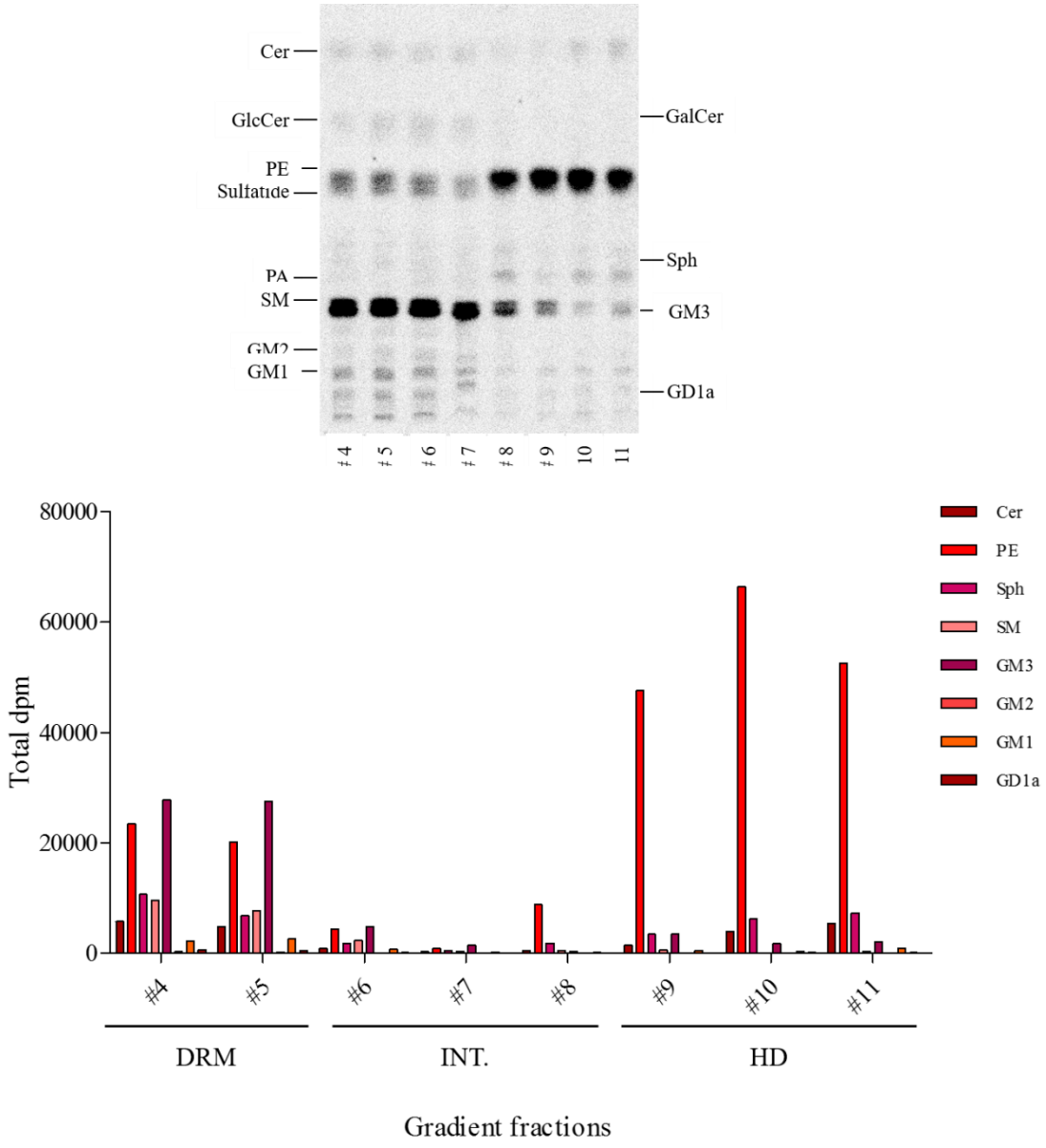

Figure 2. TLC separation of total lipids extract from MGC gradient fractions. After metabolic labeling of MGC lipids with [1-^{3}H]sphingosine, cell gradient fractions were prepared by sucrose gradient centrifugation after lysis in the presence of 1% Triton X-100 as described under "Materials and Methods". The radioactive lipids extract were separated by HPTLC using chloroform/methanol/$CaCl_2$ 0.2% 60:35:8 by volume as solvent system, and the plates were acquired under Beta-Imager 2000 instrument. The radioactivity image was quantified with the specific M3Vision software provided by Biospace Lab. *Right panel* relative quantities of components present in each sucrose gradient fraction 4–11 as in the *left panel.* The relative quantities of each lipid in DRM (fractions 4-5), Intermediate (fractions 6-7-8) and HD (fractions 9-10-11) fractions were calculated by densitometry and were expressed as percentage of total signal assessed in the histogram.

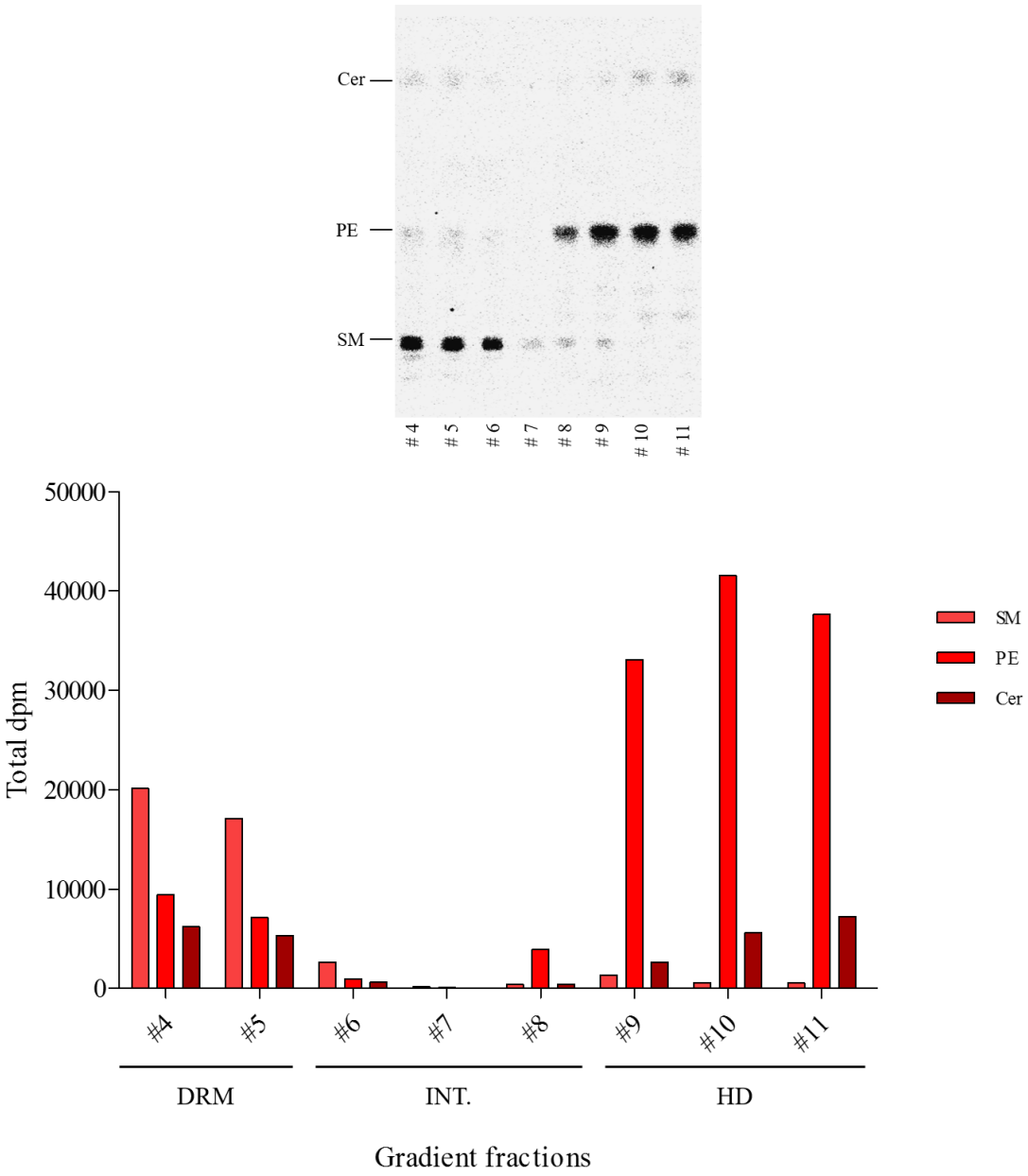

Figure 3. TLC separation of organic phases obtained from MGC gradient fractions. After metabolic labeling of MGC lipids with [1-^{3}H]sphingosine, cell gradient fractions were prepared by sucrose gradient centrifugation after lysis in the presence of 1% Triton X-100 as described under "Materials and Methods". The lipids of different fractions were extracted with chloroform/methanol/water 20:10:1 by volume. The total lipid extract of different fractions were also subjected to a modified two-phase Folch's and were divided into aqueous phase and organic phase. The radioactive lipids extract were separated by HPTLC using chloroform/methanol/water 110:40:6 by volume as solvent system, and the plates were acquired under Beta-Imager 2000 instrument. The radioactivity image was quantified with the specific M3Vision software provided by Biospace Lab. *Right panel* relative quantities of components present in each sucrose gradient fraction 4–11 as in the *left panel.* The relative quantities of each lipid in DRM (fractions 4-5), Intermediate (fractions 6-7-8) and HD (fractions 9-10-11) fractions were calculated by densitometry and were expressed as percentage of total signal assessed in the histogram

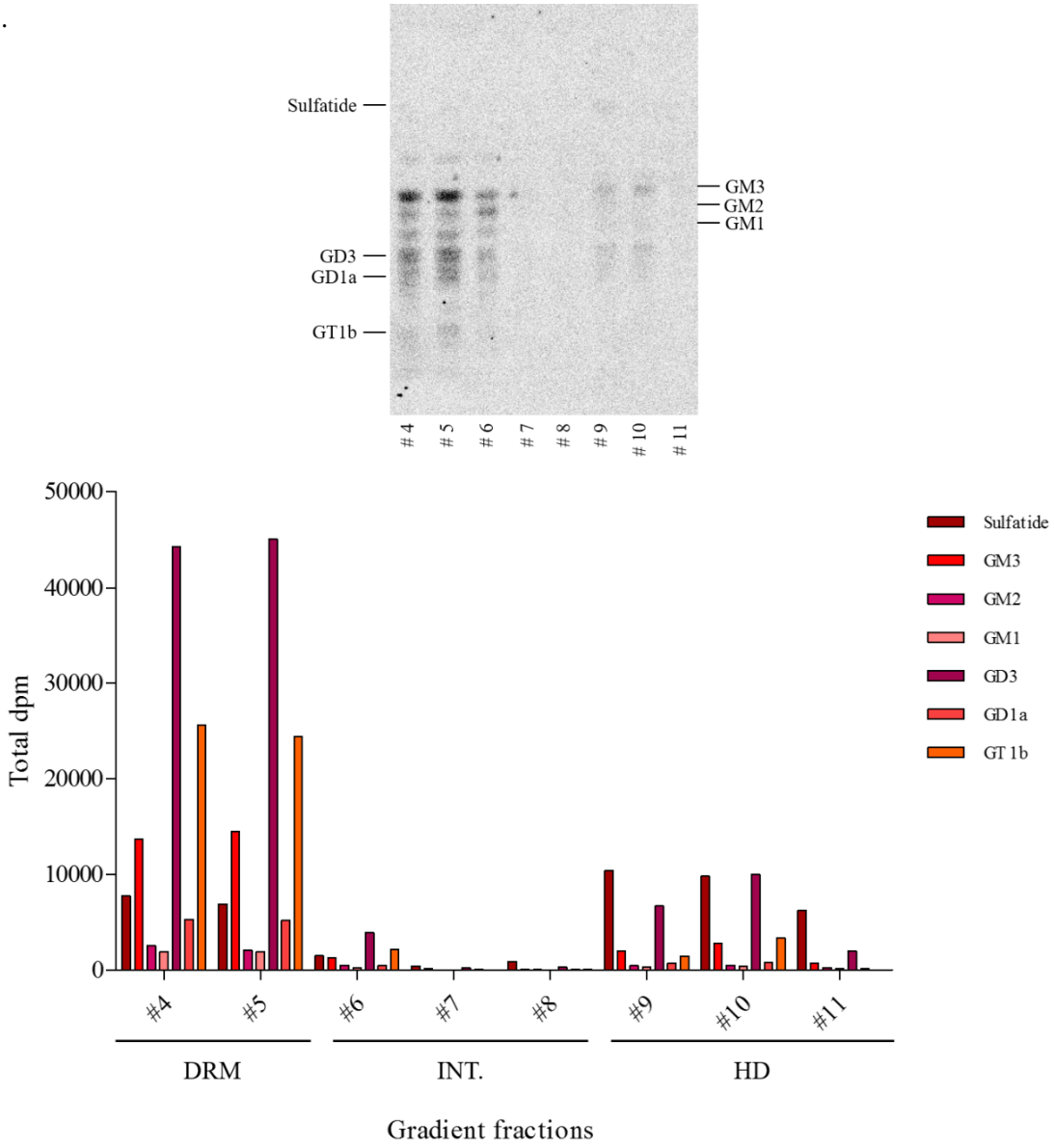

Figure 4. TLC separation of aqueous phases obtaned from MGC gradient fractions. After metabolic labeling of MGC lipids with [1-^{3}H]sphingosine, cell gradient fractions were prepared by sucrose gradient centrifugation after lysis in the presence of 1% Triton X-100 as described under "Materials and Methods". The lipids of different fractions were extracted with chloroform/methanol/water 20:10:1 by volume. The total lipid extract of different fractions were also subjected to a modified two-phase Folch's and were divided into aqueous phase and organic phase. The radioactive lipids extract were separated by HPTLC using chloroform/methanol/ $CaCl_2$ 0.2% 50:42:11 by volume as solvent system, and the plates were acquired under Beta-Imager 2000 instrument. The radioactivity image was quantified with the specific M3Vision software provided by Biospace Lab. *Right panel* relative quantities of components present in each sucrose gradient fraction 4–11 as in the *left panel.* The relative quantities of each lipid in DRM (fractions 4-5), Intermediate (fractions 6-7-8) and HD (fractions 9-10-11) fractions were calculated by densitometry and were expressed as percentage of total signal assessed in the histogram.

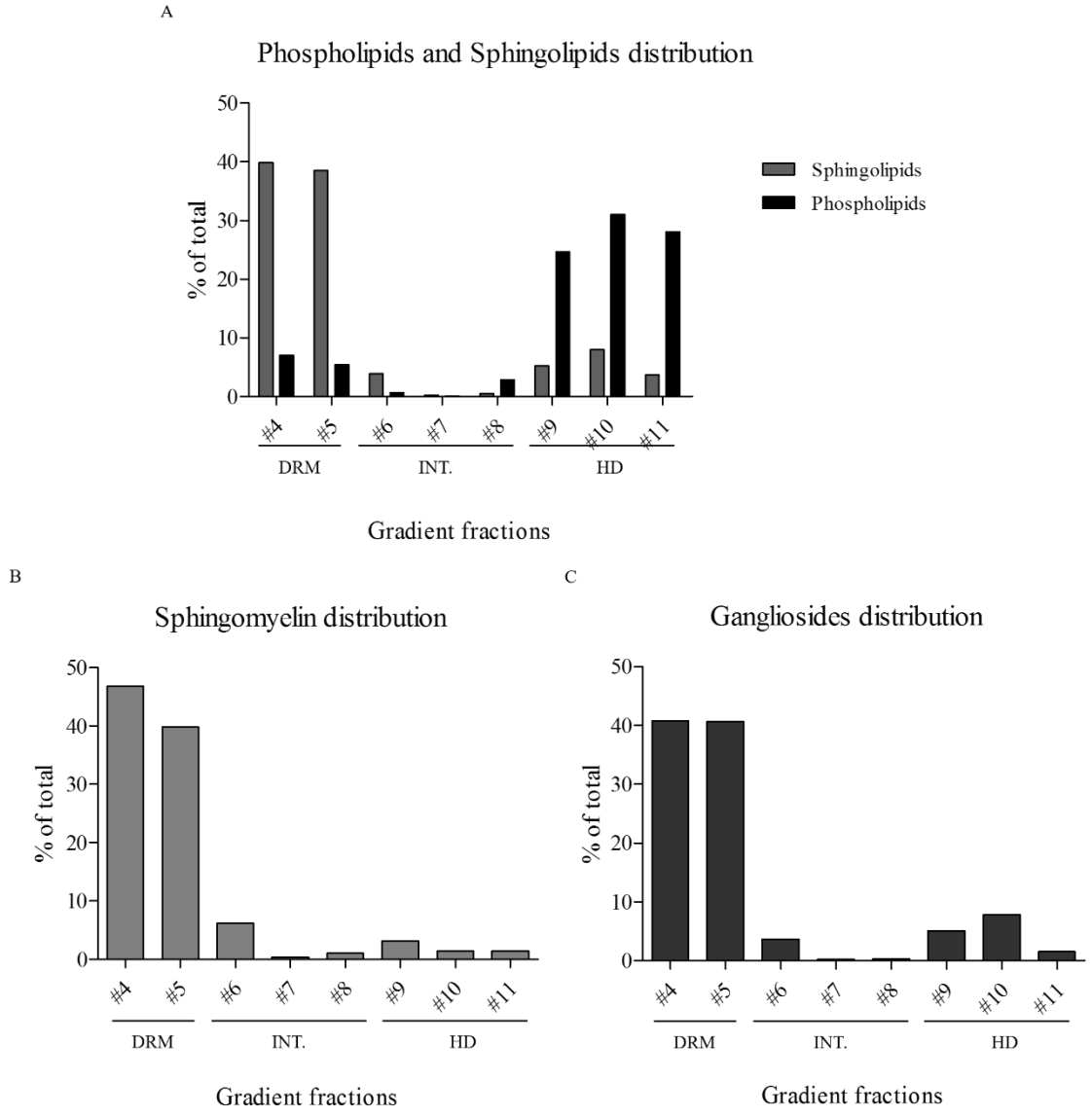

Figure 3. Lipid distribution in MGC gradient fractions. HPTLC analysis of total lipids extracts, aqueous and organic phases of [1-^{3}H]sphingosine labeled MGC gradient fractions were performed. **Panel A** shows the sphingolipids and phospholipids distribution in the different fractions. **Panel B** shows the sphingomyelin (SM) distribution in the different fractions. **Panel C** shows the gangliosides distribution in the different fractions.

Analysis of cholesterol content in MGC gradient fractions

Sphingolipid-enriched domains, that are reported to be enriched in gangliosides, sphingomyelin, and cholesterol [301], are emerging as membrane compartments with relevant biological functions. The distribution of cholesterol in MGC fractions prepared by sucrose gradient centrifugation is shown in **Figure 6**. Unlike what was expected, in MGC cholesterol was more represented in the HD fraction than in DRM fraction. In fact,

about 50% of cellular cholesterol was associated with HD fraction and only about 25% of cellular cholesterol was associated with DRM fraction.

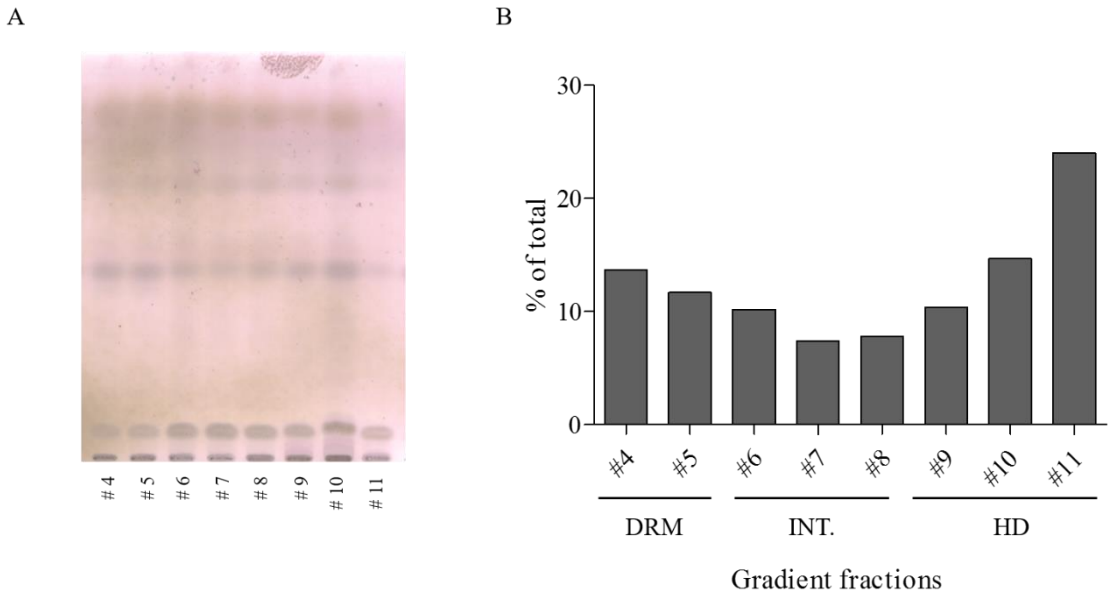

Figure 6. Distribution of cholesterol in sucrose gradient fractions from MGC culture. The amount of cholesterol in gradient fractions was determined after organic phases separation by HPTLC using Hexane/Hetyl Acetate 3:2 (v/v), as solvent system and visualized by sprying with 15% solution of concentrated sulfuric acid in 1-butanol **(Panel A)**. Data are expressed as percentages of total cholesterol present in the total fractions **(Panel B)**.

Protein distribution in MGC gradient fractions

Glycosphingolipids clusters at the cell surface membrane interact with functional membrane proteins such as integrins, growth factor receptors, tetraspanins, and nonreceptor cytoplasmic protein kinases to form "glycosynaptic domains" controlling cell growth, adhesion, and motility.

Therefore, western blotting analysis were performed with the aim to verify the distribution in MGC gradient fractions of particular proteins of interest (Integrin αv, Lyn, Akt, Caveolin 1, PrP(SAF32)).

To do so, half of each fraction obtained from MGC, prepared as described in Material and Methods section, was subjected to SDS PAGE followed by western blot analysis.

In **Figure 8 (Panel A)** the distribution of Integrin αv among MGC gradient fractions was shown. Integrins are α/β heterodimeric cell surface receptors that play a pivotal role in cell adhesion and migration, as well as in growth and survival. Integrins not only transmit signals to cells in response to the extracellular environment (outside-in signaling), but

also sense intracellular cues to alter their interaction with the extracellular environment (inside-out signaling) [303, 304]. Integrin αv was localized in HD fraction.

In **Figure 7 (Panel A)** the distribution of Lyn among MGC gradient fractions was shown. Lyn, one of the Src family members, is predominantly expressed in hematopoietic cells. Two tyrosine residues have been reported to play a crucial role in the regulation of protein tyrosine kinases of the Src family. Tyrosine phosphorylation and activation of Lyn occurs upon association with cell surface receptors [305]. Lyn was localized both in DRM and in HD fractions.

In **Figure 8 (Panel B)** the distribution of Akt among MGC gradient fractions was shown. Akt plays a critical role in controlling survival and apoptosis [306-308]. This protein kinase is activated by insulin and various growth and survival factors to function in a wortmannin-sensitive pathway involving PI3 kinase [307, 308]. It is activated by phospholipid binding [309] and by phosphorylation within the carboxy terminus at Ser473 and promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets. Akt was localized in HD fraction.

In **Figure 7 (Panel B)** the distribution of Caveolin 1 among MGC gradient fractions was shown. Caveolins are the principal structural components of the cholesterol/sphingolipid-enriched plasma membrane microdomain caveolae. Three members of the caveolin family (caveolin-1, -2, and -3) have been identified with different tissue distributions. Caveolins form hetero- and homo-oligomers that interact with cholesterol and other lipids [310]. Caveolins are involved in diverse biological functions, including vesicular trafficking, cholesterol homeostasis, cell adhesion, and apoptosis, and are also implicated in neurodegenerative disease [311]. Caveolins interact with multiple signaling molecules such as Gα subunit, tyrosine kinase receptors, PKCs, Src family tyrosine kinases, and eNOS [310, 311]. It is believed that caveolins serve as scaffolding proteins for the integration of signal transduction. Caveolin 1 was localized mainly in DRM fraction, but was also present in HD fraction.

In **Figure 7 (Panel C)** the distribution of PrP(SAF32) among MGC gradient fractions was shown. Cellular prion protein (PrPC) is a highly conserved protease-sensitive sialoglycoprotein of unknown function which is endogenously expressed in brain. Studies have shown this protein to be anchored to the external surface of the cell membrane by glycosyl-phosphatidylinositol [312]. PrP(SAF32) was localized in DRM fraction.

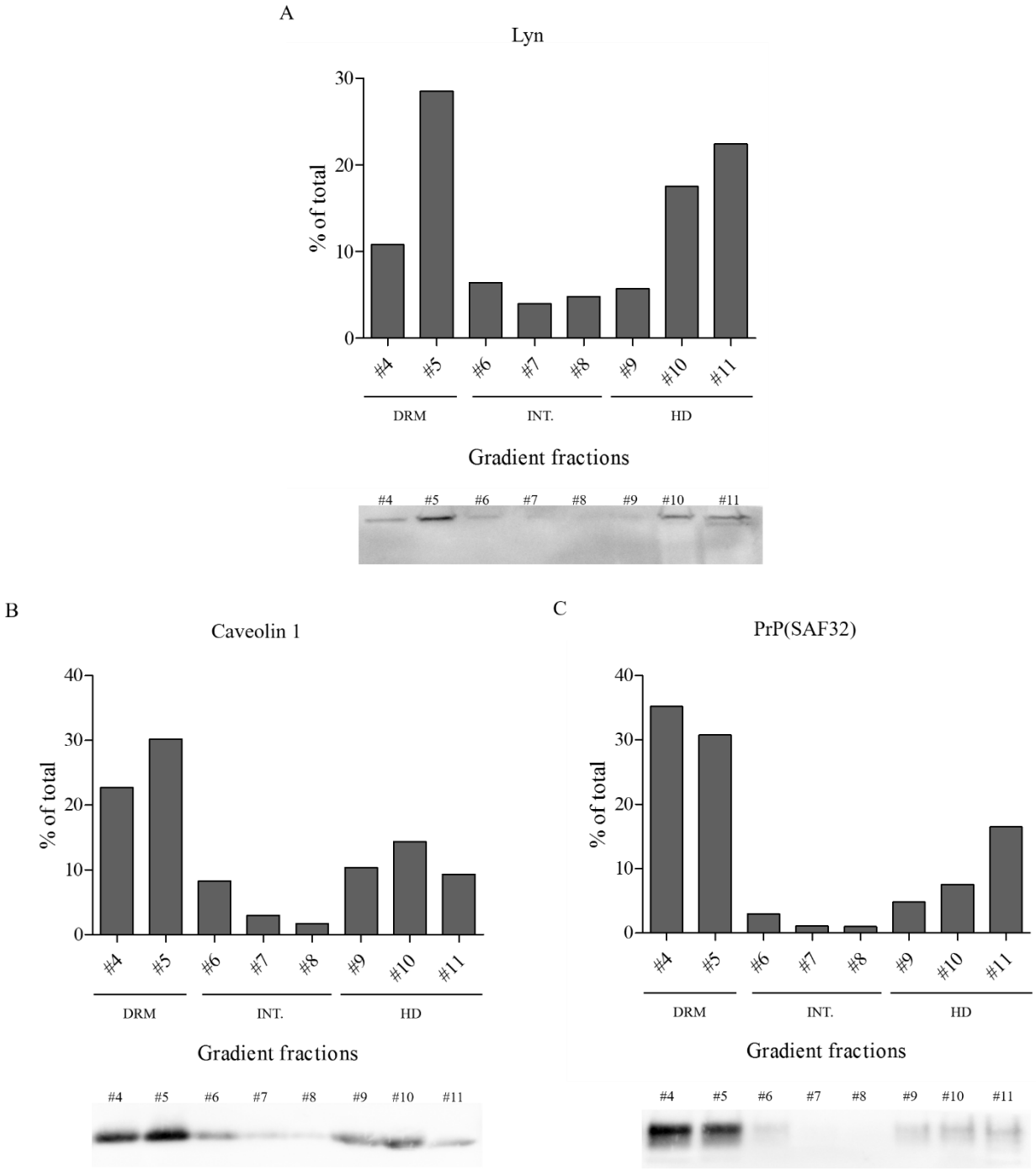

Figure 7. Analysis of different proteins expressed in DRM fraction. Half of each fraction analyzed was mixed with Laemmli buffer and boiled for 5 minutes before analysis. Electrophoresis separation was performed in a 10% polyacrylamide gel. After electrophoresis separation, proteins were transferred to PVDF membranes, subjected to immunoblotting using three different monoclonal primary antibody (Lyn **(Panel A)**, Caveolin 1 **(Panel B)** and PrP(SAF32) **(Panel C**)) and incubated with the secondary antibody conjugated with HRP. The peroxidase activity was assessed through incubation with a non-radioactive light emitting substrate for the detection of immobilized specific antigens conjugated with HRP-linked antibodies and detected through exposition to the Western Blot Imaging System, as described in Material and Methods section.

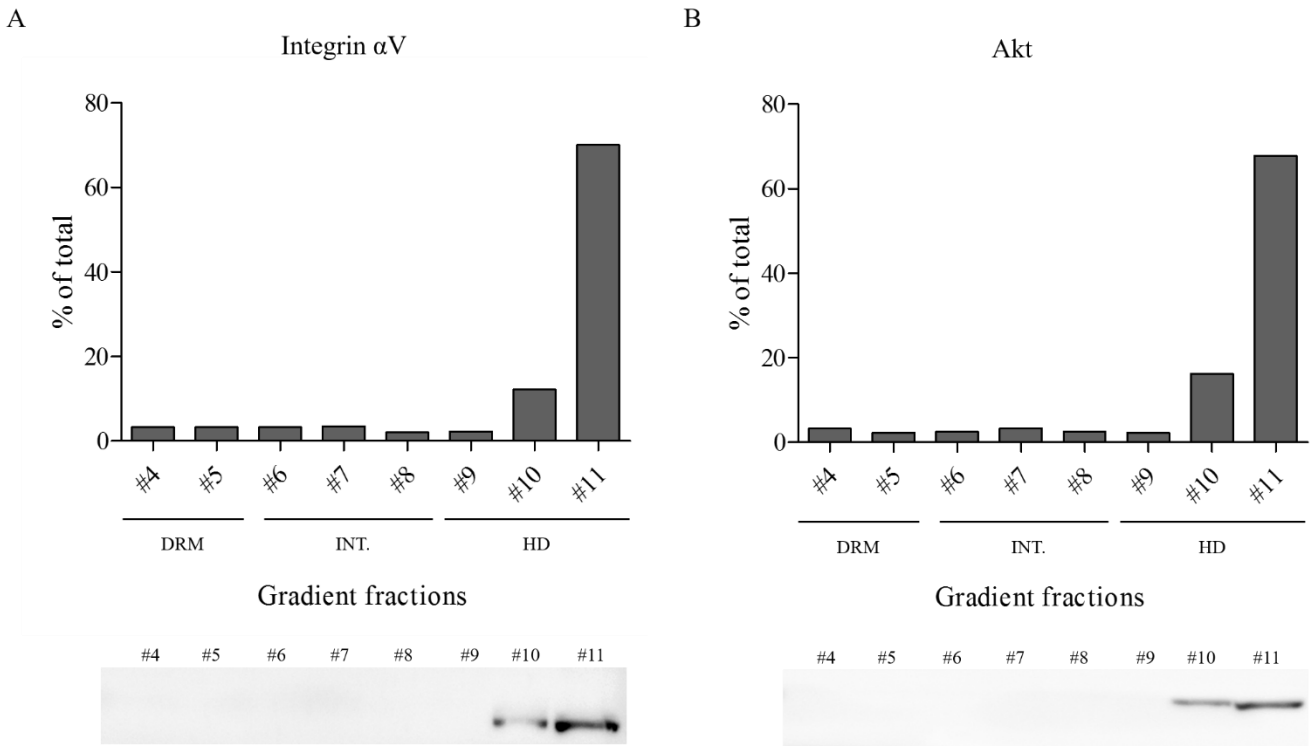

Figure 8. Analysis of different proteins in HD fraction. Half of each fraction analyzed was mixed with Laemmli buffer and boiled for 5 minutes before analysis. Electrophoresis separation was performed in a 10% polyacrylamide gel. After electrophoresis separation, proteins were transferred to PVDF membranes, subjected to immunoblotting using two different monoclonal primary antibody (Integrin αv **(Panel A)** and Akt **(Panel B)**) and incubated with the secondary antibody conjugated with HRP. The peroxidase activity was assessed through incubation with a non-radioactive light emitting substrate for the detection of immobilized specific antigens conjugated with HRP-linked antibodies and detected through exposition to the Western Blot Imaging System, as described in Material and Methods section..

Effect of rHIgM22 on MGC

In order to investigate the possible role of rHIgM22, the effect of rHIgM22 on MGC culture, following a single dose treatment of various duration, was analyzed.

To start, MGC seeded in 100 mm plates were treated with rHIgM22 or with two different non immunogenic Human IgM provided by Sigma-Aldrich and Jackson Immuno-Research., to see if there was any morphological alteration due to the treatment. Photographs were taken at different time points (0, 6, 24 and 48 hours after treatment).

No significant morphological changes in MGC culture were detectable following treatment with either rHIgM22 (**Figure 9, Upper panel**) or Human IgM provided by Sigma (**Figure 9, Lower panel**), whereas treatment with Human IgM provided by Jackson determined an increase in astrocytes content (**Figure 9, Middle panel**).

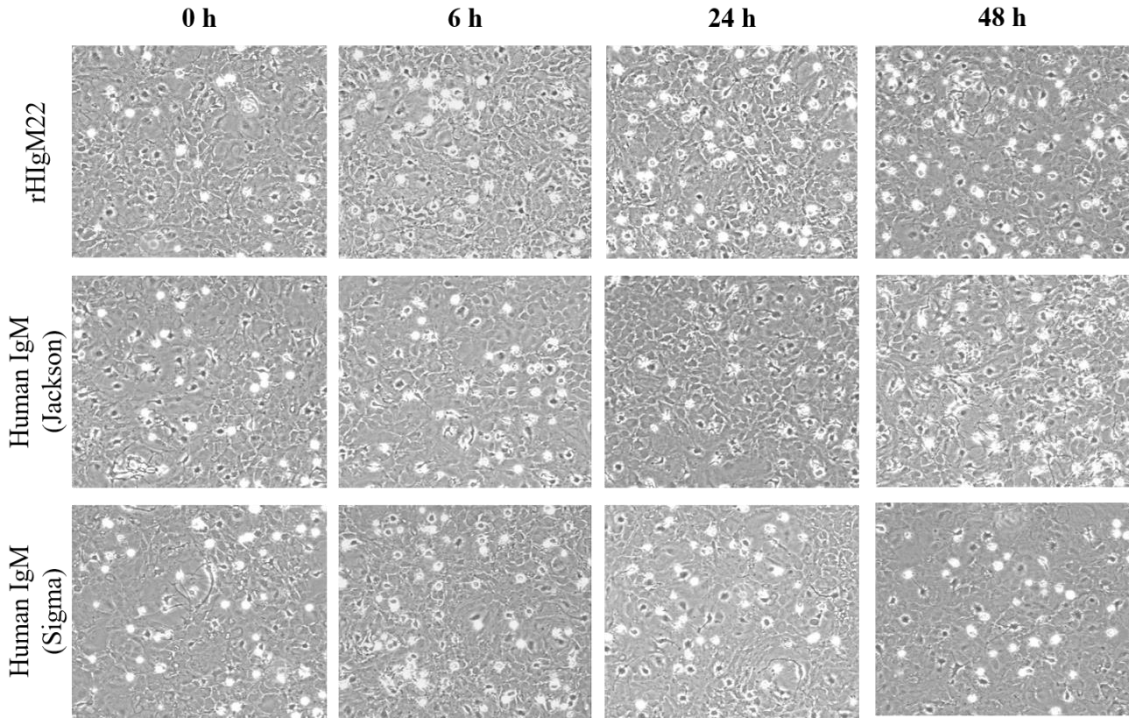

Figure 9. Morphological changes due to rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non immunogenic human IgMs for 6, 24 or 48 hours. The **Upper Panel** shows the MGC culture plate treated with rHIgM22. The **Middle Panel** shows the MGC culture plate treated with Jackson negative control Human IgM. The **Lower Panel** shows the MGC culture plate treated with Sigma negative control Human IgM.

Lipid analysis

Then, the lipid pattern of the cells after 24h of treatment was evaluate. **Figure 10**, shows the HPTLC analysis of lipids obtained as described in Material and Methods section from MGC after single dose treatment with rHIgM22, control Human IgM provided by Sigma and control Human IgM provided by Jackson. **Panel A** shows the HPTLC analysis of organic phases obtained from MGC after treatment. **Panel B** shows the HPTLC analysis of alkali treated organic phases obtained from MGC after treatment. **Figure 11** shows the HPTLC analysis of aqueous phases obtained from MGC after treatment.

No significant difference in the lipid pattern could be observed between cells treated with rHIgM22 and the relative negative controls.

Moreover, the glycerophospholipid content in MGC after 24h of treatment was analyzed in the organic phases, obtained after partitioning of total lipids extract and results are shown in **Figure 12**. Any differences between the glycerophospholipid content in MGC treated with rHIgM22 and the two relative negative controls could be observed.

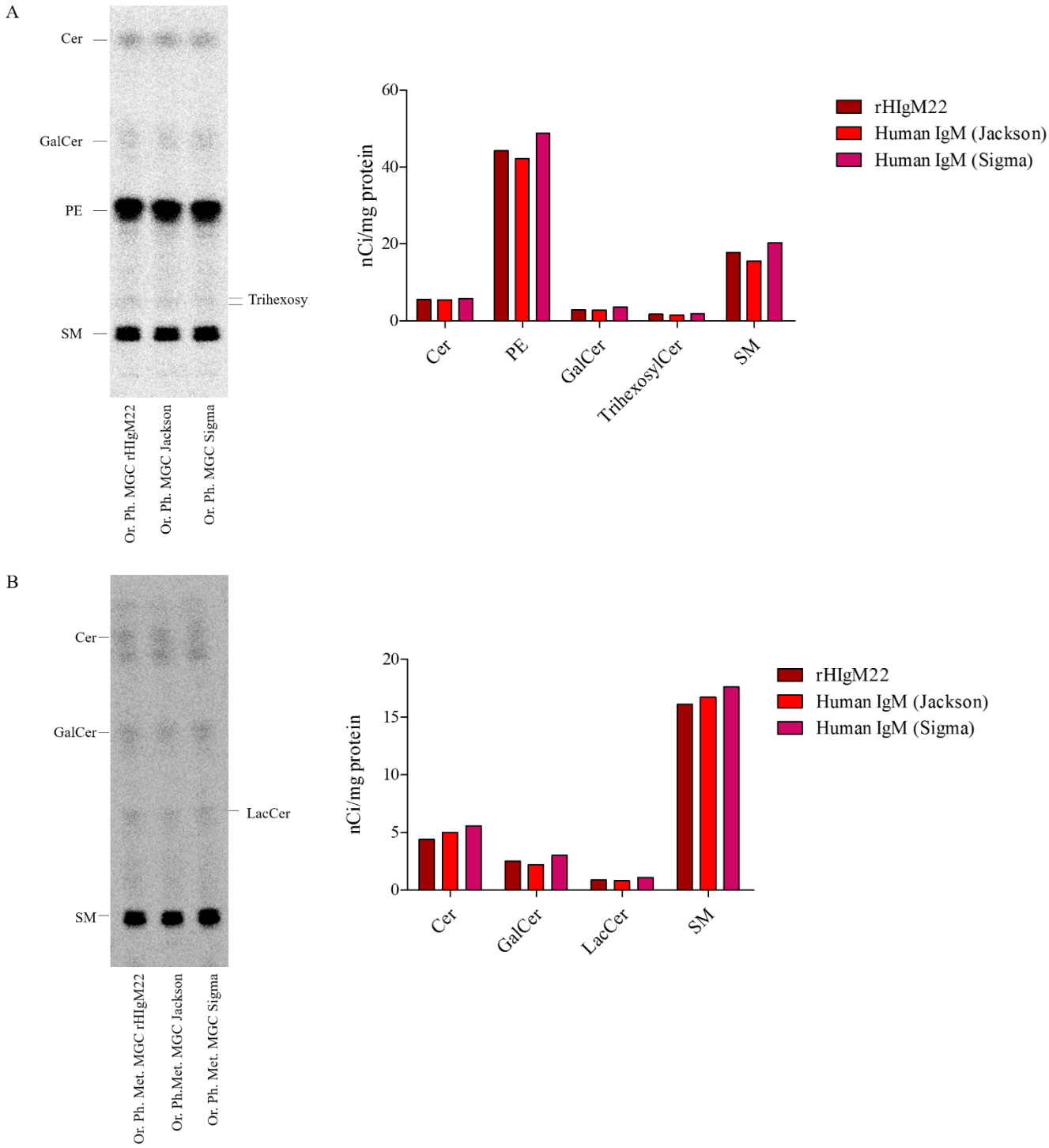

Figure 10. Evaluation of lipid pattern expressed by MGC after rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non-immunogenic human IgMs for 24 hours. Then, total lipids extracted from cells were subjected to a partitioning in aqueous and organic phases and analyzed by HPTLC using chloroform/methanol/water 110:40:6 by volume as solvent system (**Panel A** shows the organic phases). Then, aliquots of organic phases were subjected to alkali treatment to remove glycerophospholipids and analyzed by HPTLC using chloroform/methanol/water 110:40:6 by volume as solvent system (**Panel B**).

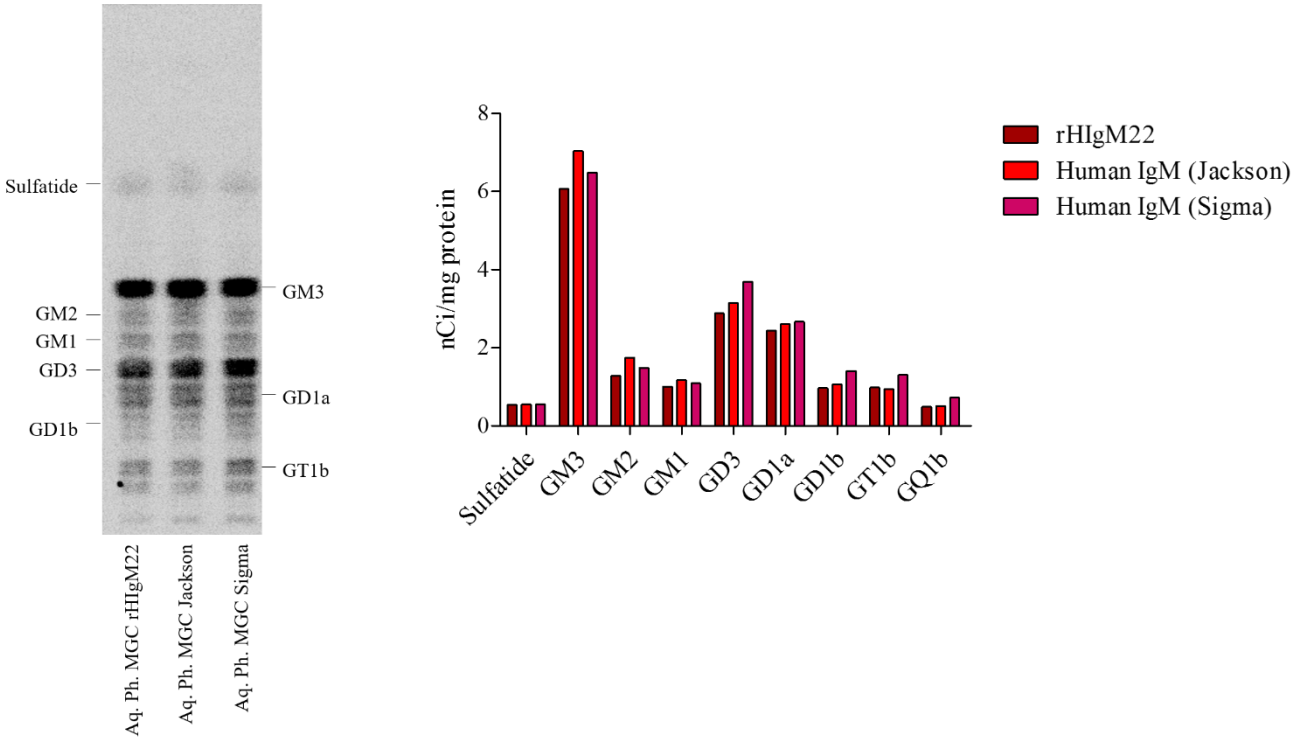

Figure 11. Evaluation of lipid pattern expressed by MGC after rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non-immunogenic human IgMs for 24 hours. Then, total lipids extracted from cells were subjected to a partitioning in aqueous (shows here) and organic phases and analyzed by HPTLC using chloroform/methanol/ $CaCl_2$ 0.2% 50:42:11 by volume as solvent system.

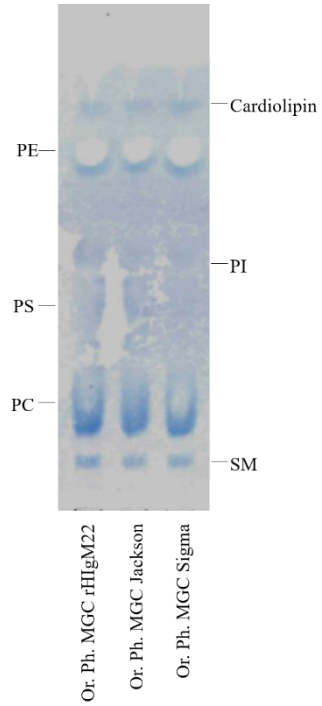

Figure 12. Evaluation of lipid pattern expressed by MGC after rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non-immunogenic human IgMs for 24 hours. Then, total lipids extracted from cells were subjected to a partitioning in aqueous (shows here) and organic phases. The organic phases was analyzed by HPTLC using chloroform/methanol/acetic acid/water 30:20:2:1 as solvent system and visualized by sprying with a reagent specific to identify phosphorus.

Protein analysis

In order to investigate the effect of rHIgM22 treatment on MGC culture, also the protein expression, after 6, 24 and 48 of treatment, was evaluated with specific antibodies.

Proteins expression are shown in **Figure 13**.

The Src family of protein tyrosine kinases, which includes Src, Lyn, Fyn, Yes, Lck, Blk, and Hck, are important in the regulation of growth and differentiation of eukaryotic cells [313]. Src activity is regulated by tyrosine phosphorylation at two sites, but with opposing effects. While phosphorylation at Tyr416 in the activation loop of the kinase domain upregulates enzyme activity, phosphorylation at Tyr527 in the carboxy-terminal tail by Csk renders the enzyme less active [314]. After 24 hours of treatment, the P-Src(Y416) family expression in cells treated with rHIgM22 undergo to a 2-fold decrease compared with its expression in cells treated with Human IgM provided by Sigma. The reduction in the expression rate of P-Src(Y416) family in the cells treated with rHIgM22 was 3 times compared with that observed in Human IgM provided by Jackson (**Figure 13, Panel B**).

After 48 hours of treatment, the P-Src(Y416) family expression in cells treated with rHIgM22 undergo to a 1.5-fold decrease compared with its expression in cells treated with both Human control IgM (**Figure 13, Panel C**).

As previously described, Lyn is one of the Src family members. A decrease in Lyn expression was detected after 24 and 48h of treatment with rHIgM22 compared only with Human control IgM provided by Jackson of 1.5 and 2-fold respectively (**Figure 13, Panel B and C**).

The platelet-derived growth factor (PDGF) family consists of proteins able to regulate diverse cellular functions by binding a receptor named PDGFRα. PDGFRα is a member of the class III subfamily of receptor tyrosine kinases (RTK). All class III RTKs are characterized by the presence of five immunoglobulin-like domains in their extracellular region and a split kinase domain in their intracellular region. Ligand-induced receptor dimerization results in autophosphorylation in trans resulting in the activation of several intracellular signaling pathways that can lead to cell proliferation, cell survival, cytoskeletal rearrangement, and cell migration [315].

MGC treated with rHIgM22 showed an increase in the PDGFRα expression, proportional to the duration of treatment (**Figure 13, Panel A, B and C**).

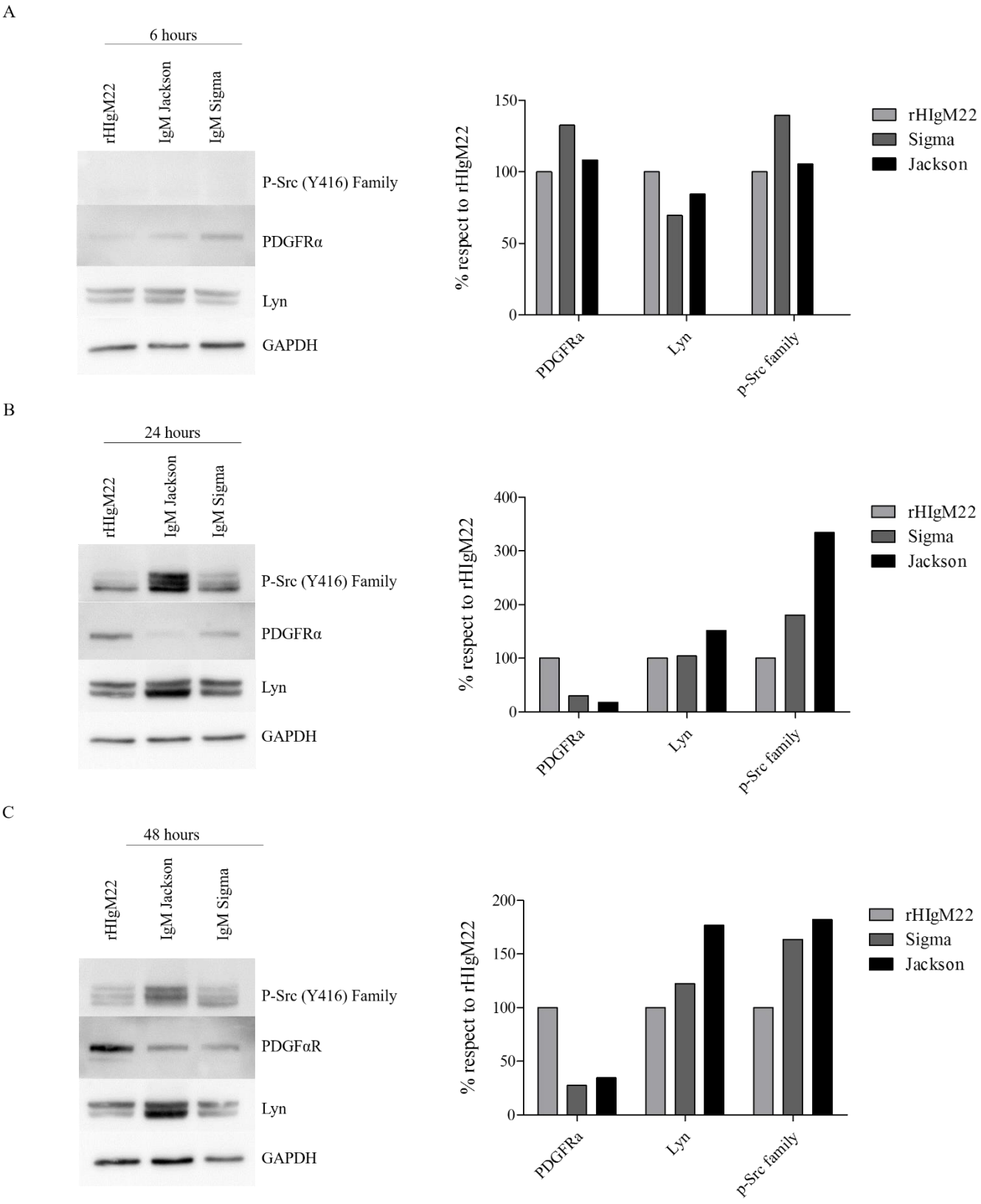

Figure 13. Evaluation of proteins expressed by MGC after rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non-immunogenic human IgMs for 6, 24 and 48 hours. Then, proteins extracted from cells were subjected to gel electrophoresis separation and western blot analysis. As internal control GAPDH was evaluated.

aSMase activity

Further, the effect of rHIgM22 on aSMase activity of MGC culture, following a single dose treatment of various duration, was assessed.

To start, MGC have been labeled with [1-^{3}H]Sphingosine by a pulse and chase experiment, as described in Material and Methods section. After 24 hours from the beginning of the chase, ^{3}H-MGC were treated with rHIgM22 or with two different non-immunogenic Human IgM provided by Sigma-Aldrich and Jackson Immuno-Research., for 6, 24 and 48 hours.

Interestingly, the total aSMase activity in MGC treated with rHIgM22 was significantly reduced, compared to that in both Human control IgM provided by Jackson and Sigma.

These data are shown in **Figure 14.**

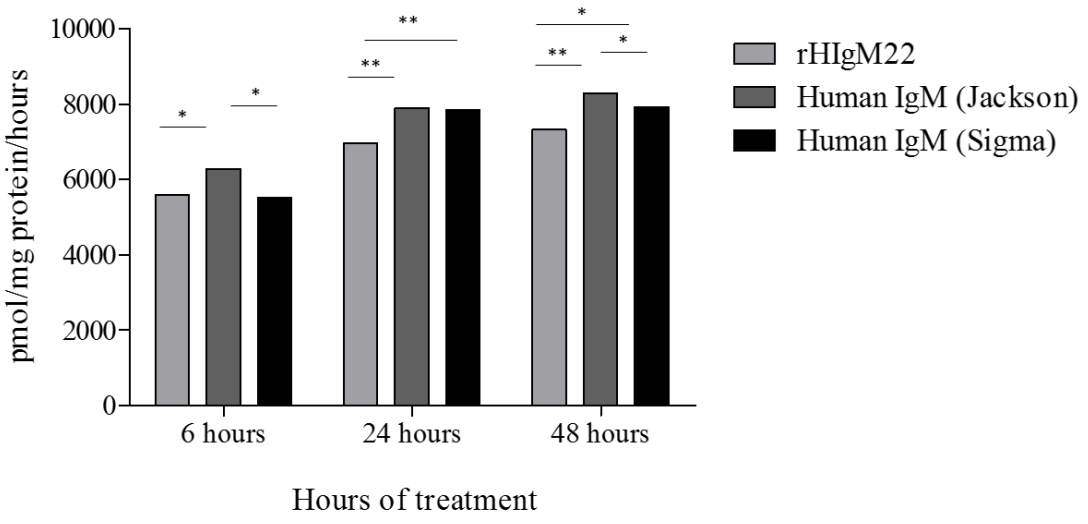

Figure 14. Evaluation of aSMase activity in MGC after rHIgM22 treatment. MGC culture were treated with rHIgM22 or with two different non-immunogenic human IgMs for 6, 24 or 48 hours before assessing aSMase activity. For each timing, the enzymatic activity assessed was significant reduced in MGC treated with rHIgM22 compared to the relative controls. The aSMase activity was expressed as pmol/mg of protein/hours. Data is expressed as mean ± SD of six experiments *, $p<0.05$; **, $p < 0.001$

DISCUSSION

Multiple sclerosis (MS), pathological characteristics has been firs decribed by Charcot, Carswell, Cruveilhier and others more than 100 years ago, is considered the lead disease featuring demyelination [316].

MS is the most common cause of non traumatic disability in young people, with an onset usually between 20-40 years of age [221], affecting 2.5 million people throughout the world [222]. It is characterized by inflammation, progressive demyelination and gliosis, axonal injury and loss. The pathological hallmarks of all the subtypes of this disease are focal areas, called plaques, of demyelination in the CNS, with surrounding inflammation and neurodegeneration [223,224].

Despite its high prevalence, multiple sclerosis remains a challenging ailment to study. The aetiology is unknown, the pathophysiologic mechanisms are various, and the chronic and unpredictable course of the pathology represent a drawback when it comes to defining whether the positive effects of short-term treatment will be sustained [316]. Currently the most widely accepted hypothesis concerning MS pathogenesis is the autoimmune hypothesis: an autoimmune inflammation is proposed to be the cause of demyelination and auto-reactive leukocytes could be the disease initiators [225, 226].

Whether the immune response is the cause of the pathogenesis or simply a consequence of the oligodendroglial cell death, the failure of the immune system to discriminate myelin components from foreign antigens plays a critical role in the pathophysiology of MS.

One of the FDA approved immunomodulatory drugs for MS, Fingolimod, is a sphingosine-1-phosphate (S1P) receptor modulator able to control lymphocyte trafficking [268]. However, it was also found to modulate resident glial cells and to increase remyelination efficiency [269-272]. Currently two major approaches involving a more direct stimulation of the remyelination process are being tested in animal models of demyelination.

- The first approach involves the transplantation of cells capable of remyelination and is based on the evidence that transplanted glial cells myelinate in the CNS following their introduction in the developing CNS of rodents with myelin mutations or with toxin-induced demyelination [273, 274].
- the second approach being tested in animal models of demyelination involves the promotion of repair by the resident stem- and precursor-cell populations in the adult CNS, through the administration of growth, trophic, and neuroprotective factors [276].

An alternative therapeutic approach is the use of CNS reactive antibodies to promote remyelination [280]. Two of these remyelination promoting antibodies are represented by BIIB033 and the recombinant human IgM22 (rHIgM22). The first is an anti-LINGO-1 IgG acting on LINGO-1, a protein known to inhibit remyelination via RhoA activation, and is currently being tested in a Phase II study in RRMS (ClinicalTrial.gov: NCT01864148). The second antibody, instead, is an IgM sharing several features with naturally occurring antibodies and is currently undergoing a phase I clinical trial aimed to evaluate safety and tolerability in relapsing MS patients (ClinicalTrial.gov: NCT02398461), after the first phase I clinical trial in MS patients was completed successfully (ClinicalTrial.gov: NCT01803867).

In particular, rHIgM22 was first identified through the screening of human serum of a patient with Waldenström macroglobulinemia as a myelin-binding antibody and it's able to promote significant remyelination *in vivo* [291]. The actual target and mechanisms of rHIgM22 are still under investigation however several pieces of evidence suggest that the antigen recognized by this antibody might be a plasma membrane lipid, possibly sulfatide [294, 295]. Moreover, existing literature suggests that the binding target of rHIgM22 could be associated with detergent-resistant membranes (DRM)/lipid rafts and that rHIgM22 biological activity depends on lipid raft organization [294, 295]. Furthermore, literature strongly suggests that rHIgM22 biological activity, responsible for its myelin-repair promoting activity, could require a multimolecular complex organizing Lyn and the cell surface molecules integrin αvβ3 and PDGFRα [296, 297]. Taken together, these observations have led to hypothesize that rHIgM22, through its pentameric structure, could mediate the clustering of a lipid antigen and stabilize lipid rafts domains. Moreover, it could determine the reorganization of Lyn, integrin αvβ3 and PDGFRα to form a signaling complex which, in turn, promotes OPC survival and proliferation [295, 296]. In isolated OPCs, PDGF is required for rHIgM22-mediated inhibition of apoptotic signaling and differentiation. PDGF is produced by neurons and astrocytes and stimulates OPC proliferation and promotes OPC survival both *in vivo* and *in vitro*. Indeed, IgM-mediated OPC proliferation is detectable only in cultures containing substantial amounts of astrocytes, microglia and OPCs (mixed glial cultures) but not in highly enriched OPC population[297].

Previous analysis, performed in our laboratory, of the binding of rHIgM22 to sulfatide revealed that rHIgM22 is able to recognize sulfatide *in vitro*, and that this binding is specific. Moreover, our data suggests that the binding of rHIgM22 to sulfatide might be

affected by the composition of the lipid microenvironment (data not shown). These data are in agreement with evidence reported in the literature, which shows that the lipid environment might play a role in the determination of the surface topology of sulfatide [317, 318].

The aim of this study was to analyze the plasma membrane lipid rafts composition in MGC in order to evaluate the effects exerted by rHIgM22 on these cells after single dose treatment of various duration.

The analysis of the lipids and proteins distribution in MGC fractions, obtained after discontinuous sucrose gradient centrifugation of cells previous labeled with [1-^{3}H]sphingosine and lysed in the presence of Triton X-100, revealed that sphingolipids (ceramide, glucosylceramide, sphingomyelin and gangliosides) were more represented in the DRM fraction and slightly represented in the HD fraction; differently, phospholipids (phosphatididylethanolamine and phosphatidic acid) took an opposite distribution. Moreover, both sphingomyelin and gangliosides were enriched in the DRM fraction. So, as expected, the DRM fraction was highly enriched in sphingolipids, and relatively depleted of glycerophospholipids [301]. The major amount of glycerophospholipids existed in HD fraction.

Furthermore, it has been reported that sphingolipid-enriched domains, that are also enriched in cholesterol, are emerging as membrane compartments with relevant biological functions. Unlike what was expected, in MGC cholesterol was more represented in the HD fraction than in DRM fraction. In fact, about 50% of cellular cholesterol was associated with HD fraction and only about 25% of cellular cholesterol was associated with DRM fraction [301].

Glycosphingolipids clusters at the cell surface membrane interact with functional membrane proteins such as integrins, growth factor receptors, tetraspanins, and nonreceptor cytoplasmic protein kinases to form "glycosynaptic domains" controlling cell growth, adhesion, and motility.

Western blotting analysis performed on the MGC gradient fractions samples revealed that integrin (heterodimeric cell surface receptors that play a pivotal role in cell adhesion, growth and survival), in particular integrin αv, was localized in HD fraction. Likewise, Akt (protein kinase activated by insulin and various growth and survival factors, that promotes cell survival by inhibiting apoptosis through phosphorylation and inactivation of several targets), was localized in HD fraction. On the other hand, Lyn (one of the Src family members), Caveolin 1(the principal structural components of the

cholesterol/sphingolipid-enriched plasma membrane microdomain caveolae, involved in diverse biological functions, including vesicular trafficking, cholesterol homeostasis, cell adhesion, and apoptosis) and PrP(SAF32) (highly conserved protease-sensitive sialoglycoprotein of unknown function which is endogenously expressed in brain, anchored to the external surface of the cell membrane by glycosyl-phosphatidylinositol) were mainly localized in DRM fraction.

Furthermore, the morphological evaluation of the effects exerted by treatment with rHIgM22 on MGC culture revealed no significant change compared with two different non immunogenic Human IgM. In the same way, no significant difference in the lipid pattern and glycerophospholipids content could be observed between cells treated with rHIgM22 and the relative negative controls.

On the other hand, the rHIgM22 treatment exerted an effect on the protein expression in MGC culture compared with the two negative control. In particular, the P-Src(Y416) family, tyrosine kinases important in the regulation of growth and differentiation of eukaryotic cells, expression in cells treated with rHIgM22 decreased if compared with its expression in cells treated with Human control IgM, higher after 24 hours of treatment. Moreover, also a decrease in Lyn expression was detected after 24 and 48h of treatment with rHIgM22. On the contrary the PDGFRα expression undergoes to an increase, proportional to the duration of treatment. This is interesting because PDGFRα has been proposed as one of the members of the signaling complex through which rHIgM22 exterts its biological activity. In fact, literature strongly suggests that rHIgM22 biological activity is mediated by the reorganization of Lyn, integrin αvβ3 and PDGFRα at the cell surface to form a signaling complex triggering Lyn activation which, in turn, promotes oligodendrocyte precursor cells (OPCs) survival and proliferation [296, 297].

Finally, the total aSMase activity in MGC treated with rHIgM22 was significantly reduced, compared to that in both Human control IgM, with consequent reduced ceramide generation. In fact it has been reported that, in oligodendrocytes, activation of Lyn promotes cell survival by suppressing acid sphingomyelinase activity, and ceramide generated by the action of ASMase represents not only an important pro-apoptotic signal, but also a signal for the re-arrangement of sphingolipid-rich signaling platforms.

And this effect might also be relevant from the therapeutic point of view, in fact ASMase and its enzymatic product, ceramide, have been shown to be expressed at higher levels in human MS lesions compared to unaffected brain tissue and deficiency or blockade of the ASMase/Cer system significantly improves myelin repair after demyelination [319].

Summarizing, the data so far collected demonstrate that the DRM fraction obtained from MGC was enriched in sphingolipids, in particular sphingomyelin and gangliosides together with Lyn, Caveolin 1 and PrP(SAF32). On the contrary, phospholipids, in particular phosphatididylethanolamine and phosphatidic acid are enriched in the HD fraction, together with integrin αv and Akt.

Furthermore we observed that rHIgM22 exerted an effect on the expression of P-Src(Y416) family and Lyn, that show a significant decrease, and PDGFRα that shows a significant increase. Moreover, the rHIgM22 treatment also induces a decrease in the activity of the aSMase.

In the light of these data we hypothesize that the treatment with IgM22 could elicit biological responses mediated by alterations of lipid-dependent membrane organization which result in a reorganization of Lyn, integrin αvβ3 and PDGFRα at the cell surface to form a signaling complex. The formation of this complex triggers Lyn activation which in turn promotes oligodendrocyte precursor cells (OPCs) survival and proliferation and an inhibition of the pro apoptotic signaling. Based on the data we obtained we hypothesize that the increased activation of Lyn could determine a decrease in ASMase activity and consequently in ceramide generation, thus inhibiting pro-apoptotic signaling and/or organization of sphingolipid-dependent signaling platforms.

Understanding whether rHIgM22 effect on remyelination involves a lipid-organized membrane complex, and the exact identity of the antigen involved and their organization in this complex is of great importance. The identification of the binding targets of this antibody, able to promote remyelination in validated mouse models of MS, and the characterization of their membrane microenvironment could significantly contribute to the reveal the signaling mechanisms underlying the biological activity of rHIgM22. This, in turn, would allow to obtain a better comprehension of the process of (re)myelination, and of the molecular mechanism involved in the pathophysiology of multiple sclerosis, thus allowing to define new potential therapeutic targets.

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