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Abstract

Remyelination promoting human IgMs effectively increase the number of myelinated axons in animal models of multiple sclerosis. Hence, they ultimately stimulate myelin production by oligodendrocytes (OLs); however, their exact mechanism of action remains to be elucidated, and in particular, it remains unclear whether they are directly targeting OLs, or their action is mediated by effects on other cell types. We assessed the effect of remyelination promoting antibody rHIgM22 on the proliferative response and on the ceramide/sphingosine 1-phosphate rheostat in mixed glial cell cultures (MGCs). rHIgM22 treatment caused a time-dependent increase in PDGFαR protein in MGCs. Forty-eight hours of treatment with rHIgM22 induced a dose-dependent proliferative response (evaluated as total cell number and as EdU(+) cell number) in MGCs. When the proliferation response of MGCs to rHIgM22 was analyzed as a function of the cell types, the most significant proliferative response was associated with GLAST(+) cells, i.e., astrocytes. In many cell types, the balance between different sphingolipid mediators (the “sphingolipid rheostat”), in particular ceramide and sphingosine 1-phosphate, is critical in determining the cell fate. rHIgM22 treatment in MGCs induced a moderate but significant inhibition of total acidic sphingomyelinase activity (measured in vitro on cell lysates), the main enzyme responsible for the stimulus-mediated production of ceramide, when treatment was performed in serum containing medium, but no significant differences were observed when antibody treatment was performed in the absence of serum. Moreover, rHIgM22 treatment, either in the presence or in absence of serum, had no effects on ceramide levels. On the other hand, rHIgM22 treatment for 24 h induced increased production and release of sphingosine 1-phosphate in the extracellular milieu of MGC. Release of sphingosine 1-phosphate upon rHIgM22 treatment was strongly reduced by a selective inhibitor of PDGFαR. Increased sphingosine 1-phosphate production does not seem to be mediated by regulation of the biosynthetic enzymes, sphingosine kinase 1 and 2, since protein levels of these enzymes and phosphorylation of sphingosine kinase 1 were unchanged upon rHIgM22 treatment. Instead, we observed a significant reduction in the levels of sphingosine 1-phosphate lyase 1, one of the key catabolic enzymes. Remarkably, rHIgM22 treatment under the same experimental conditions did not induce changes in the production and/or release of sphingosine 1-phosphate in pure astrocyte cultures. Taken together, these data suggest that rHIgM22 indirectly influences the proliferation of astrocytes in MGCs, by affecting the ceramide/sphingosine 1-phosphate balance. The specific cell population directly targeted by rHIgM22 remains to be identified, however our study unveils another aspect of the complexity of rHIgM22-induced remyelinating effect.

Keywords (separated by '-')	rHIgM22 - Multiple sclerosis - Remyelination - Sphingolipids
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ORIGINAL PAPER

Human Remyelination Promoting Antibody Stimulates Astrocytes Proliferation Through Modulation of the Sphingolipid Rheostat in Primary Rat Mixed Glial Cultures

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Abstract

Remyelination promoting human IgMs effectively increase the number of myelinated axons in animal models of multiple sclerosis. Hence, they ultimately stimulate myelin production by oligodendrocytes (OLs); however, their exact mechanism of action remains to be elucidated, and in particular, it remains unclear whether they are directly targeting OLs, or their action is mediated by effects on other cell types. We assessed the effect of remyelination promoting antibody rHIgM22 on the proliferative response and on the ceramide/sphingosine 1-phosphate rheostat in mixed glial cell cultures (MGCs). rHIgM22 treatment caused a time-dependent increase in PDGFαR protein in MGCs. Forty-eight hours of treatment with rHIgM22 induced a dose-dependent proliferative response (evaluated as total cell number and as EdU(+) cell number) in MGCs. When the proliferation response of MGCs to rHIgM22 was analyzed as a function of the cell types, the most significant proliferative response was associated with GLAST(+) cells, i.e., astrocytes. In many cell types, the balance between different sphingolipid mediators (the "sphingolipid rheostat"), in particular ceramide and sphingosine 1-phosphate, is critical in determining the cell fate. rHIgM22 treatment in MGCs induced a moderate but significant inhibition of total acidic sphingomyelinase activity (measured in vitro on cell lysates), the main enzyme responsible for the stimulus-mediated production of ceramide, when treatment was performed in serum containing medium, but no significant differences were observed when antibody treatment was performed in the absence of serum. Moreover, rHIgM22 treatment, either in the presence or in absence of serum, had no effects on ceramide levels. On the other hand, rHIgM22 treatment for 24 h induced increased production and release of sphingosine 1-phosphate in the extracellular milieu of MGC. Release of sphingosine 1-phosphate upon rHIgM22 treatment was strongly reduced by a selective inhibitor of PDGFαR. Increased sphingosine 1-phosphate production does not seem to be mediated by regulation of the biosynthetic enzymes, sphingosine kinase 1 and 2, since protein levels of these enzymes and phosphorylation of sphingosine kinase 1 were unchanged upon rHIgM22 treatment. Instead, we observed a significant reduction in the levels of sphingosine 1-phosphate lyase 1, one of the key catabolic enzymes. Remarkably, rHIgM22 treatment under the same experimental conditions did not induce changes in the production and/or release of sphingosine 1-phosphate in pure astrocyte cultures. Taken together, these data suggest that rHIgM22 indirectly influences the proliferation of astrocytes in MGCs, by affecting the ceramide/sphingosine 1-phosphate balance. The specific cell population directly targeted by rHIgM22 remains to be identified, however our study unveils another aspect of the complexity of rHIgM22-induced remyelinating effect.

Keywords rHIgM22 · Multiple sclerosis · Remyelination · Sphingolipids

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Sara Grassi and Paola Giussani have equally contributed to this work.

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Abbreviations

ASM	Acid sphingomyelinase
BSA	Bovine serum albumin
Cer	Ceramide
CNS	Central nervous system
CSF	Cerebrospinal fluid
MGCs	Mixed glial cultures
MS	Multiple sclerosis

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OPCs	Oligodendrocyte precursor cells
SGPL1	Sphingosine 1-phosphate lyase 1
SK	Sphingosine kinase
SK1	Sphingosine kinase 1
SK2	Sphingosine kinase 2
SL	Sphingolipids
SM	Sphingomyelin
SMase	Sphingomyelinase
SPP1	Sphingosine 1-phosphate phosphatase 1
S1P	Sphingosine 1-phosphate

Introduction

Multiple sclerosis (MS) is primarily considered an autoimmune disease, i.e. a disease caused by an adaptive immune response to self-antigens, implying the activation and recruitment of myelin-reactive immune cells (T-cells) from the periphery to the CNS. However, oligodendrocyte death and myelin loss is clearly observed in early lesions of MS even in the absence of T- or B-cells infiltrates, and activation and proliferation of microglia and astrocytes is consistently observed in demyelinating lesions, suggesting that innate immune system contribution by these CNS-resident cells might play a relevant role in the disease [1].

Astrocytes and microglia play dual roles in the initiation and progression of most neurological disorders, and in MS lesion development. Both cell types are key players in driving CNS inflammation and are directly implicated in the pathophysiology of MS, as suggested from studies on patients' tissues, in animal models of the disease and in vitro (reviewed in [2, 3]).

Despite the prevailing view of reactive astrocytes and of activated ("classically activated") microglia as impeding regenerative processes in the CNS, "activated" (cytokine-activated) astrocytes within lesions might also limit the detrimental effects of pro-inflammatory factors, thus providing support and protection for oligodendrocytes and neurons and creating a permissive environment for remyelination. In particular, astrocytes play important roles in maintaining the homeostasis and spatial distribution of different secreted factors that determine oligodendrocyte precursor cells (OPCs) proliferation, migration and differentiation [4]. Pro-inflammatory cytokines (IL-1β and TGFβ1) induced the production of IL-11 in cultured astrocytes, and IL-11 production was upregulated in astrocytes in MS lesions. IL-11 enhanced oligodendrocyte survival and maturation, and increased myelin formation in rodent CNS co-cultures [5]. IL-1β also stimulated the production of the chemokine CXCL1 (GRO-alpha), and CXCL1 produced by hypertrophic astrocytes in MS lesions seems to represent a mechanism for recruitment of oligodendrocytes to damaged area, a prerequisite for remyelination [6]. In the murine cuprizone model of toxic demyelination, IGF-1 and CNTF were elevated in astrocytes in lesion areas, while GDNF, IGF-1 and FGF were upregulated in microglia [7]. Similar to astrocytes, "alternatively activated" microglia within MS lesions show a beneficial, neuroprotective profile. In particular, activation of microglial phagocytosis at the lesion site seems to be crucial in order to remove damaged myelin debris [8], which can inhibit myelin repair. In this sense, an important role of rHIgM22, the remyelination-promoting antibody object of this study, has been recently described [9]. rHIgM22 was indeed able to stimulate myelin phagocytosis in a mouse microglial cell line and primary rat microglia, in a complement receptor-dependent manner that was fully inhibited by use of compstatin to block complement factor 3 (C3) cleavage by C3 convertase. Remarkably, astrocyte and microglia functions in this sense are interdependent and coordinated.

Some convincing lines of evidence point out that the switch between detrimental versus protective phenotype of astrocytes in MS can be due to the opposing effect of sphingolipid mediators, and that sphingolipid metabolism and sphingolipid-dependent signaling might be the target of factors able to modify astrocyte phenotype in a protective way for MS.

Increased production of the pro-apoptotic sphingolipid ceramide might contribute to oligodendrocyte damage in MS. Ceramide synergistically with TNF was able to induce apoptosis in cultured oligodendrocytes [10]. Ceramide accumulated in reactive astrocytes in active lesions of MS in humans, and in the cuprizone mouse model of demyelination. Concomitantly, sphingosine had accumulated and sphingosine 1-phosphate (S1P) levels were decreased [11]. Sphingosine kinase 1 (SK1) and the $S1P_3$ receptor are upregulated in reactive astrocytes in MS lesions, or in cultured rat astrocytes treated with the pro-inflammatory molecule LPS. On the other hand, S1P induced secretion of CXCL1 in astrocytes. Thus, the SK1/$S1P_3$ pathway seems to be relevant for astrocyte activation. However, S1P-dependent astrocyte activation could play a dual role in the context of MS. On one hand, it could represent a detrimental event, enhancing astrogliosis, on the other hand, it could be beneficial, through increased remyelination sustained by the release of CXCL1 or other trophic factors from activated astrocytes [12–14]. Fingolimod, the only approved oral disease-modifying therapy for relapsing remitting MS (RR-MS), is phosphorylated in vivo to fingolimod-P, a structural analog of S1P. Fingolimod is effective in MS by blocking the migration of immune cells and preventing the invasion of auto-aggressive T-cells into the CNS.

However, emerging evidence indicates that Fingolimod has direct effects in the CNS in MS, and points out the importance of astrocytes in direct CNS effects of Fingolimod. In vitro, fingolimod stimulates astrocyte migration, while in vivo it acts as functional antagonist of astrocyte

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$S1P_1$. In EAE, Fingolimod is highly effective but its efficacy is lost in animals selectively deficient for $S1P_1$ in astrocytes (while still having normal immunological receptor expression and functions) [13, 14].

Strategies aimed at enhancing endogenous myelin repair by stimulating the resident myelin-producing cells seem to be a promising approach to prevent or slow down the progression of MS. Among novel reagents under development to this aim, remyelination-promoting human IgMs are very attractive. rHIgM22 is the recombinant form of a human IgM identified from a patient with Waldenström macroglobulinemia. rHIgM22 was able to bind to myelin and to the surface of mature, O4-positive oligodendrocytes in vitro [15], and to enhance remyelination in three different mouse models of demyelination, i.e. Theiler's murine encephalomyelitis virus (TMEV) [15, 16], lysolecithin- [17] and cuprizone-induced demyelination models [18, 19]. A 16-site phase 1 clinical trial in patients with MS was completed in 2015 (NCT0183867), showing no dose-limiting toxicities, no serious treatment-emergent adverse events, and detectable levels of rHIgM22 in the CSF in all patients. Another phase 1 clinical trial in patients with acute relapses (NCT02398461) is ongoing. Despite these encouraging results, the exact mechanism of action of rHIgM22 remains to be elucidated, and some evidence suggests that its effect on myelin repair by OLs might be mediated by the involvement of other cell types in the lesion niche. Indeed, rHIgM22 induced OPC proliferation by activating PDGFαR in mixed glial cultures, but not in isolated OPCs, suggesting that the stimulation of OPC proliferation by rHIgM22 requires factors produced by astrocytes and/or microglia [20]. Here we demonstrate that rHIgM22 treatment was able to induce astrocyte proliferation and S1P production in mixed glial cultures. rHIgM22-induced release of S1P in mixed glial cultures was reduced in the presence of a selective inhibitor of PDGFαR. On the other hand, rHIgM22 had no effect on S1P production in pure astrocyte cultures, suggesting that a complex cross talk between different cell types is underlying the ultimate myelin repair effect elicited by this antibody.

Materials and Methods

Materials

All reagents were of analytical grade. Ca^{2+} and Mg^{2+}-free HBSS, D-Glucose, BSA fraction V, HEPES, trypsin, sodium pyruvate, poly-D-lysine, PBS, Na_3VO_4, $MgSO_4$, DNase I, methanol, chloroform, sphingomyelin (SM) were purchased from Sigma Aldrich (Darmstadt, Germany); penicillin/streptomycin, bovine fetal serum, DMEM high glucose, and glutamine from Euroclone Spa (Pero, Milan, Italy). The antibodies anti-PDGFR, anti-SK1, and goat anti-mouse or goat anti-rabbit horseradish peroxidase-linked secondary antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-SK2, anti-SGPL1 and anti-SPP1 antibodies were from Abcam (Cambridge, UK). Anti-phosphoSK1 (Ser^{225}) antibody was from ECM Biosciences (Versailles, KY, USA). Anti-GAPDH was from Sigma Aldrich (Darmstadt, Germany). LiteABlot Plus and LiteABlot Turbo Chemiluminescent Substrate were purchased from Euroclone Spa (Pero, Milan, Italy). D-Erythro-[3-^{3}H] sphingosine ([^{3}H]Sph) was from Perkin Elmer (Boston, MA, USA). [1-^{3}H]sphingosine (radiochemical purity over 98%; specific radioactivity of 1.36 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[^{3}H] hydride as previously described [21]. High performance thin layer chromatography (HPTLC) silica gel plates and solvents were purchased from Merck (Darmstadt, Germany). [^{3}H]-sphingomyelin, isotopically labelled at the sphingosine moiety, was synthesized and purified in our laboratories [22].

Human IgM from human serum has been purchased from Sigma Aldrich; rHIgM22 antibody was provided by Acorda Therapeutics, Inc. (Ardsley, NY, USA).

Mixed Glial Cells (MGCs) and Astrocytes Cell Culture

MGCs were prepared according to Watzlawik et al. [20]. Briefly, the hemispheres from P1 to P2 C57BL/6N mice or from P1 to P2 Sprague Dawley rats were minced with a surgical blade and incubated for 30 min 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 min and resuspended in modified HBSS. The tissue was further dissociated by trituration through a sterile flame narrowed glass pipette, centrifuged at 200×*g* at 8 °C for 10 min, 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 changed every 3–4 days.

Purified astrocytes were obtained from MGCs using a modified version of the protocol from McCarthy and de Vellis [23]. Briefly, MGCs were cultured for 8–10 days to allow the stratification of astrocytes and oligodendrocytes before being subjected to a shaking procedure (20 h, 200 rpm). This procedure, which removes oligodendrocytes and microglia, was repeated three times, allowing a week to pass between each shaking. Astrocytic cell layers were then detached using trypsin–EDTA, plated on poly-D-lysine coated dishes and

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cultured for two weeks 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.

EdU-Incorporation Assay

MGCs were prepared as described above. For measurements of DNA synthesis, metabolic labeling was performed using the uridine analog 5-ethynyl-2-deoxyuridine (EdU). Cell suspensions were washed by centrifugation for 5 min, 200×*g* and plated at 7×10^4 cells per well on poly-D-lysine-coated 48 well dish (50 µg/mL poly-D-lysine for 1 h at room temperature (RT) and subsequently washed twice with water). The cells were maintained in DMEM/10% FBS for 5 days. After 5 days in culture, cells were washed twice with DMEM/F12 and switched to modified DMEM/F12 media supplemented with N2 supplement (1:100), 2 mM GlutaMax, penicillin/streptomycin and 0.1% BSA. Either rHIgM22 or human IgM isotype control was added at a concentration range of 1–50 µg/mL on day 5. All treatments were performed in triplicate. PDGF and FGF-2 (Growth Factors at 10 ng/mL each) were added on day 6. EdU was added to MGCs at the end of day 6 at a final concentration of 10 µM for an additional 18 h. At the end of the experiment, cells were processed for image analysis or flow cytometric analysis.

MGC Flow Cytometry Methods

Following the 48 h of treatment with IgM22 (1.25, 5.0 or 20 µg/mL), isotype control (20 µg/mL), serum-free media/vehicle (PBS), or growth media, culture supernatants (non-adherent cells) were transferred to collection tubes and adherent cells were lifted from the wells of a 48-well plate by addition of 0.25 mL of prewarmed StemPro™ Accutase™ (#A1110501; Thermo Fisher). Plates were incubated for 5 min at 37 °C and wells were rinsed twice with 0.5 mL of PBS containing 1% BSA, w/v, (1% BSA/PBS). The recovered cells were combined with corresponding culture supernatant and pelleted by centrifuging at 800×*g* for 5 min at 4 °C, washed with 1.0 mL 1%BSA/PBS and resuspended in 200 µL of 1%BSA/PBS. For each labeling condition, 75 µL of cells was transferred to clean tube for triple labeling with A2B5/CD11bc/GLAST or O4/CD11bc/GLAST antibodies (Anti-A2B5-PE, #130-093-581; Anti-O4-PE, #130-095-887; Anti-GLAST(ACSA-1)-APC, #130-095-814; Anti-CD11b/c-PE-Vio770, 130-105-276; Miltenyi Biotec, Germany).

Labeling antibodies were added to the recovered live cells for a final dilution of 1 to 10 for immunophenotyping (10 µg/mL final concentration). Cells were labeled at 4 °C for 20 min, washed twice with 1.0 mL of 1% BSA/PBS, pelleted, and resuspended in 100 µL of 1% BSA/PBS. In addition to the triple labeled cell populations, conditions of single antibody labeled MGCs and Fluorescence Minus One controls (samples labelled with 2 of 3 antibodies) were prepared to establish compensation values for correction of fluorescence signal crossover in samples labeled with multiple fluorophores, and for setting fluorescence signal thresholds for detection of the positive marker populations. The remaining unlabeled cell population (50 µL) was labeled with propidium iodide (#556463, BD Pharmingen™) to establish gating areas for the singlet, healthy cell population. Following labeling, cells were immediately analyzed in the live state by flow cytometry.

For identification of cells undergoing DNA replication during treatment, a Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit was used (#C10632, Thermo Fisher). After cells were analyzed in the live state for A2B5/CD11bc/GLAST or O4/CD11bc/GLAST labelling, the remaining triple antibody labelled samples were fixed, permeabilized and labeled with Alexa Fluor 488, according to the manufacturer's instructions.

Flow cytometry was performed using an Accuri C6 flow cytometer set to a medium flow rate and set volume (50 µL) of cells to facilitate comparison of total cell counts across treatment conditions and markers. Raw data files were extracted to FCS Express (De Novo Software, Glendale, CA) for analysis. For live cell flow analysis, an initial gate on the healthy (PI-negative), singlet (linear peak area to peak width) was applied and used to calculate the total cell count within the sample. Microglia were identified based on the intensity of the CD11bc signal and the astrocyte population was identified based on above threshold GLAST intensity. To aid in identifying the A2B5 or O4 positive population, the CD11bc(+) and GLAST(+) populations were gated out to reveal the oligo-lineage cell population. As with the CD11bc(-) and GLAST(+) population, control conditions without antibody labeling allowed for identification of threshold setting for the A2B5(+) or O4(+) population. For EdU(+) cells, the analysis cell gate was determined by PI(+) nuclei along a linear peak area to peak width plot and based on the intensity of the Click-It-488 fluorophore.

Duplicate treatments for each treatment condition and a minimum of three independent experiments were performed for all reported results. Average cell counts from each experiment, treatment and marker were used to calculate overall condition means and standard deviation. A one-way ANOVA with a Dunnett's post-hoc statistical analysis for comparison to vehicle control conditions was performed for each indicated cell marker (GraphPad Software, La Jolla, CA).

Total ASM Activity Assay

MGCs were plated on 100 mm petri dishes at a density of 10×10^5 cells/cm^2 and cultured for 13 days. 10 µg/mL of either rHIgM22 or control IgM were then added to the

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cells in serum containing culture medium, after a complete medium change. Alternatively, rHIgM22 or Human IgM treatment was performed after washing cells twice with DMEM/F12 in modified DMEM/F12 media supplemented with N2 supplement (1:100), 2 mM GlutaMax, penicillin/streptomycin and 0.1% BSA. ASM activity was assessed on cell lysates after different times of incubation with rHIgM22 or isotype control IgM. [^{3}H]SM (12 pmol) was mixed with 500 pmol of non-labelled SM, suspended in 0.2% Triton X-100 in $CHCl_3$:CH_3OH 2:1 (v/v) and dried under N_2 flux. 25 µL of 250 mM sodium acetate pH 5.1 were added for each sample. MGCs were collected in 0.2% Triton X-100 in H_2O. 25 µg protein of cell lysates were added to 25 µL of reaction substrate and samples were incubated for 2 h at 37 °C. The reaction was stopped by the addition of 200 µL of $CHCl_3$:CH_3OH 2:1 (v/v) followed by centrifugation at 16,100×*g* for 20 min. The amount of SM hydrolyzed was determined through autoradiography after thin layer chromatography separation of the substrate, [^{3}H]SM, and the reaction product, [^{3}H]ceramide. Negative controls were performed using heat-inactivated cell lysates (100 °C for 3 min).

[3H]Sphingosine Metabolism and Evaluation of Cellular and Extracellular S1P

MGCs were plated on 60 mm dishes at a density of 10×10^5 cells/cm^2 and cultured for 13 days, whereas astrocytes were plated on 35 mm dishes at a density of 4.5×10^4 cells/cm^2 and cultured for a week before proceeding with the experiment. At the time of the experiment, the medium was gently removed and cells were incubated for 24 h in medium supplemented with 1% FBS in the presence or absence of 10 µg/mL control IgM or rHIgM22. At the end of the treatments, the cells were pulsed with 20 nM D-erythro-[3-^{3}H]sphingosine ([^{3}H]Sph, 0.4 µCi/mL), for 45 min, in the presence of medium only, or medium containing control IgM or rHIgM22 in the presence or absence of AG1296 [24–26]. Subsequently, cells were harvested, total lipids were extracted at 4 °C with chloroform/methanol, and partitioned by adding 0.1 M NH_4OH, as previously reported [25, 27]. After centrifugation, the upper alkaline aqueous phase, containing intracellular S1P, was evaporated under nitrogen stream and associated radioactivity was determined by liquid scintillation counting. The methanolized organic phase and the aqueous phase were analyzed by HPTLC using chloroform/methanol/water (55:20:3 by vol) as solvent system. The [^{3}H]-labeled sphingolipids were recognized and identified as previously described.

Extracellular S1P was extracted from pulse medium and purified as described elsewhere [24, 25, 28]. Briefly, a two-step partitioning was performed, first in alkaline conditions followed by a back extraction of the aqueous phase obtained in acidic conditions. The acidic organic phase obtained, containing S1P, was evaporated under nitrogen stream; the aqueous phase, containing tritiated water produced from [^{3}H] S1P degradation, was purified by fractional distillation and counted for radioactivity [27].

The fractions containing cellular and extracellular S1P were submitted to HPTLC separation on silica gel plates using *n*-butanol/acetic acid/water (3:1:1, v/v/v) as solvent system. Standard [^{3}H]S1P was chromatographed on the same plate and used as internal standard. At the end of the chromatography, HPTLC plates were dried and submitted to digital autoradiography (^{T}Racer Beta-Imager, Biospace, Paris, FR) and S1P and other radioactive sphingolipids were quantified by M3Vison software (Biospace, Paris, FR).

Immunoblotting Analysis

Cells were lysed with lysis buffer (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/mL aprotinin).

In order to evaluate PDGFαR, SK1, pSK1, and SK2 expression, cell proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked with 3% BSA in TBS with 0.05%-Tween20, incubated overnight with anti-SK1 (1:1000), anti-pSK1 (1:500), anti-SK2 (1:3000), anti-PDGFαR (1:1000), and anti-GAPDH (1:5000) primary antibodies and finally with a goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:2000) using GAPDH as loading control.

In order to evaluate SGPL1 and SPP1 expression, cell proteins were resolved by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. Membranes were then blocked with 5% Milk in TBS with 0.05%-Tween20, incubated overnight with anti-SGPL1 (1:500), anti-SPP1 (1:500), and anti-GAPDH (1:5000) primary antibodies and finally with a goat anti-rabbit horseradish peroxidase-linked secondary antibody (1:2000) using GAPDH as loading control.

In all cases bound antibodies were visualized by ECL (LiteABlot Plus and LiteABlot Turbo Chemiluminescent Substrate). For quantitative measurements, membranes were acquired by UVITEC Cambridge technology (Eppendorf). Image analysis was performed using NINEAlliance software.

Steady-State Labeling of MGC [1-^{3}H]sphingosine and Lipid Analysis

MGCs were plated on 100 mm petri dishes at a density of 10×10^5 cells/cm^2 and cultured for 13 days. Cell sphingolipids were steady-state metabolically labeled by 2 h pulse/48 h

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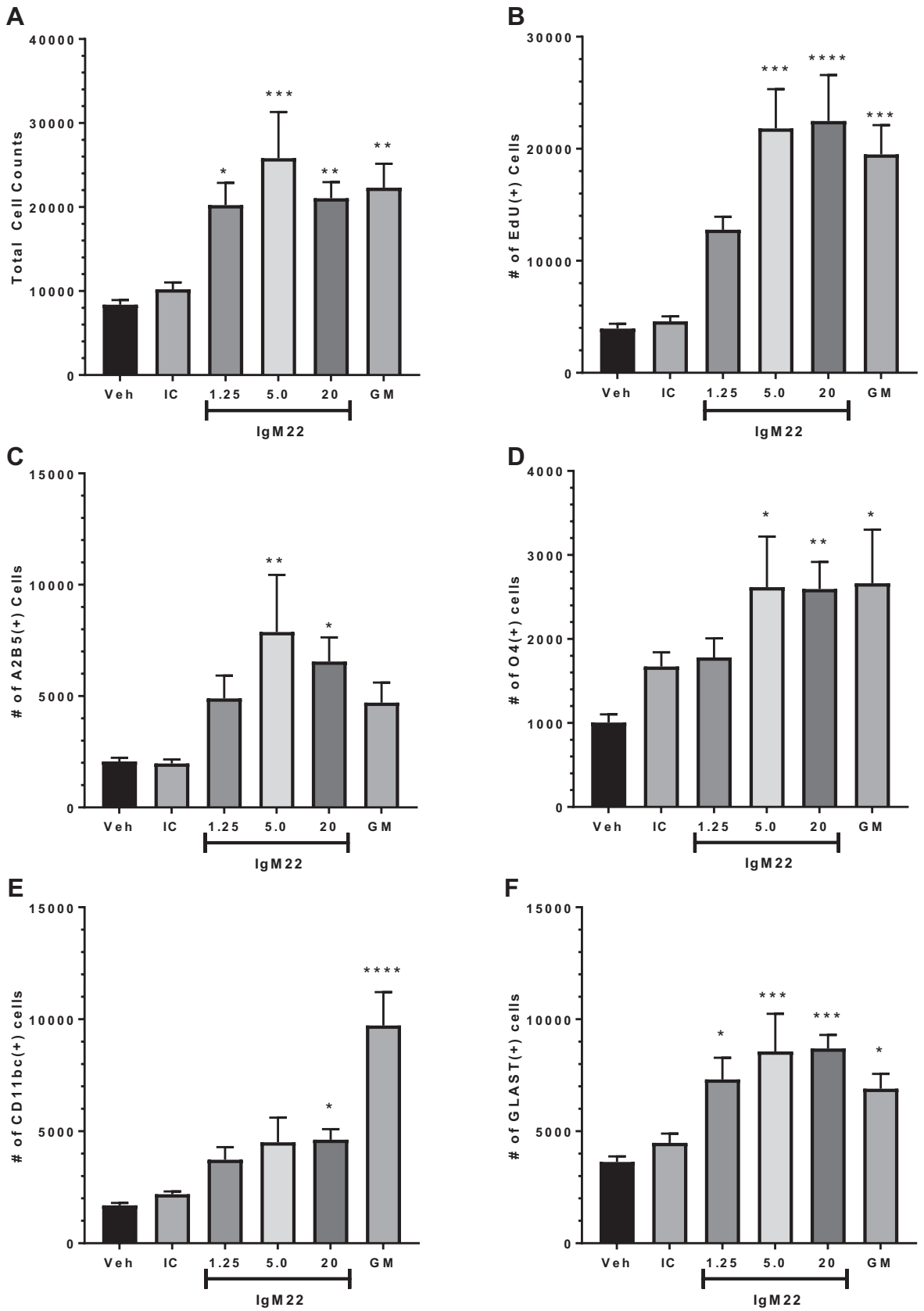

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◂**Fig. 1** MGCs proliferative response to 48 h rHIgM22 or control treatments. Immunophenotyping by flow cytometry of mixed glial cell cultures following 48 h of treatment with SFM + vehicle (Veh), SFM + isotype control (IC), SFM + IgM22 at 1.25, 5.0 or 20 µg/mL, or growth media (GM). Recovered cells were labeled with fluorochrome labeled primary antibodies against the OPC marker A2B5, the immature OL marker O4, the microglial marker CD11bc, the astrocyte marker GLAST or were processed for the presence of EdU incorporation following an 18 h pulse using Click-it detection. Cell counts for equal volume of isolated cells was performed for **a** total, **b** EdU(+), **c** A2B5(+), **d** O4(+), **e** CD11bc(+) or **f** GLAST(+) cells. One-way ANOVA with a Dunnett's post-hoc analysis was performed for each marker versus the vehicle control condition. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$. *Error bars* = ± SEM

chase with 3×10^{-8} M [1-^{3}H]sphingosine as described previously.

After 24 h of chase, 10 µg/mL of either rHIgM22 or Human IgM (Sigma) were then added to the cells. After another 24 h, cells were collected, centrifuged and lysed in ice cold water. Following lyophilization, lipids were extracted with chloroform/methanol/water, 2:1:0.1, by volume, subjected to a two-phase partitioning, and radioactive lipids were separated by monodimensional HPTLC and quantitatively analyzed by digital autoradiography [29].

Other Experimental Procedures

The protein content was determined by the Bio-Rad DC assay kit using BSA as the reference standard. Radioactivity associated with cells, with medium, and with lipid extracts was determined by liquid scintillation counting.

Statistical Analysis

Experiments were run in triplicate, unless otherwise stated. Data are expressed as mean value ± SD and were analyzed by unpaired Student's t-test. *p* Values are indicated in the legend of the figures.

Results

Effects of rHIgM22 Exposure on the Proliferation of MGCs

The effects of rHIgM22 on growth rates and composition of mixed glial cultures was evaluated by flow cytometry using antibodies known to identify each of the major cellular components of the culture. CD11bc reactivity was used to identify the microglial population, astrocyte-specific glutamate transporter (GLAST) reactivity was used to identify the astrocyte population, immature oligodendrocytes (OLs) were identified by O4 immunoreactivity and oligodendrocyte precursor cells (OPCs) were detected by A2B5 antibody reactivity. In addition to immunophenotyping the cultures, total cell counts and EdU incorporation was used to assess the proliferative state of the culture.

Mixed glial cultures were treated on DIV 5 by replacing the culture medium with serum free medium (SFM) containing rHIgM22 at 1.25, 5.0 or 20 µg/mL, SFM plus the PBS vehicle, SFM with a human poly-clonal isotype control IgM at 20 µg/mL, or with growth medium containing 10% FBS. Cultures were incubated for 48 additional hours after which the cell populations were subjected to antibody labeling or detection of EdU incorporation as described in the "Material and Methods" section. For EdU experiments, the cells were treated with 10 µM of EdU for 18 h prior to the cell harvest at 48 h post-treatment.

Treatment with rHIgM22 promoted a significant increase in the total cell number and EdU incorporation compared to the serum free, vehicle control conditions and did so in a dose dependent manner (Fig. 1a, b; Table 1). Furthermore, rHIgM22 maintained culture growth rates and EdU incorporation similar to the serum containing growth medium (GM) condition. In contrast, an isotype control IgM had no impact on cell proliferation over the course of 48 h as there was no difference in cell or EdU number when compared to the vehicle control condition.

Immunophenotyping of the cultures following treatment demonstrated a pleotropic effect of rHIgM22 on the proliferative rate of all the cells in the culture (Fig. 1c–f; Table 1). However, the effect of rHIgM22 on the astrocyte (GLAST(+)) and OPC (A2B5(+)) cell population was noticeably more pronounced than the effect on CD11bc(+) microglia proliferation (Fig. 1e).

Watzlawik and collaborators [20] showed that rHIgM22 effect on OPC proliferation in mixed glial cultures was mediated by PDGFαR. Thus, we assessed the effect of treatment with 10 µg/mL rHIgM22 (an antibody concentration within the effective range for the stimulation of astrocyte proliferation in MGCs) on the PDGFαR protein levels. As shown in Fig. 2, rHIgM22 treatment was able to induce a time-dependent increase in PDGFαR levels, as measured by western blotting (the maximal effect was a fivefold increase after 48 h). Treatment with isotype control IgM under the same experimental conditions had no effect on PDGFαR levels.

Effect of rHIgM22 Exposure on ASM Activity and Ceramide Levels in MGCs

Recent evidence highlights the importance of increased ceramide levels in the induction of apoptosis, astrocyte activation and neuronal damage in MS [30, 31]. In particular, ceramide generated from sphingomyelin via the enzyme acid sphingomyelinase (ASM) is a key mediator for the detrimental events observed in mouse models of MS. On the other hand, genetic deficiency or pharmacological inhibition of

Table 1 MGCs proliferative response to 48 h rHIgM22 or control treatments

	CD11(+) (%)	GLAST(+) (%)	A2B5(+) (%)	O4(+) (%)	EdU(+) (%)	Total cells (%)
Vehicle	100.0	100.0	100.0	100.0	100.0	100.0
Isotype control	129.2	123.6	95.6	166.4	116.5	122.1
1.25 µg/mL rHIgM22	219.7	201.8	237.4	177.3	324.2	241.8
5.0 µg/mL rHIgM22	265.7	236.2	381.8	260.4	553.4	308.6
20 µg/mL rHIgM22	272.9	239.9	317.7	258.2	570.3	251.5
Growth media	573.6	190.3	228.2	265.1	494.8	266.6

Data plotted in Fig. 1 have been calculated as percentage respect to vehicle-treated cells (representing 100%) for each treatment for the different cell populations

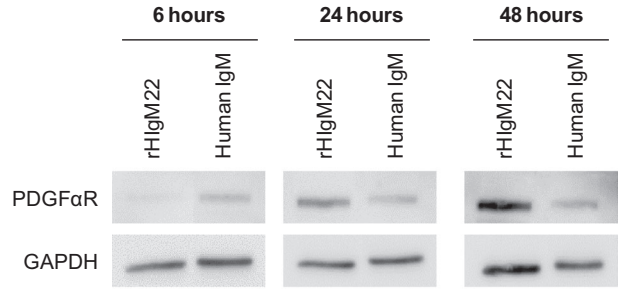

Fig. 2 Effect of rHIgM22 or control treatments on the expression of PDGFαR in MGCs. MGCs at the 13th day of culture were treated with a single dose (10 µg/mL) of rHIgM22 or of a non-immunogenic human IgM (as the negative control) for different times (6, 24 or 48 h). After treatment, cells were harvested, lysed and the same amount of protein (50 µg) for each sample was separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed by western blotting using specific anti-PDGFαR and anti-GAPDH antibodies

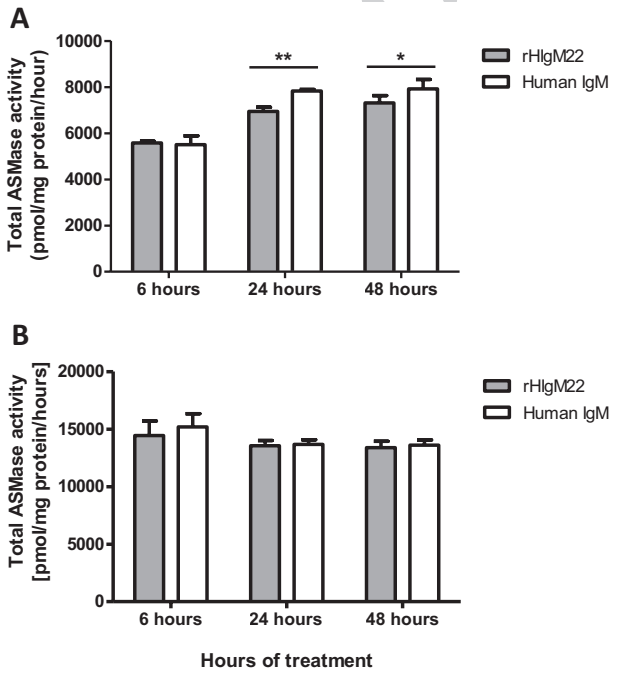

Fig. 3 Effect of rHIgM22 or control treatments on the in vitro activity of ASM in MGCs. MGCs at the 13th day of culture were treated with a single dose (10 µg/mL) of rHIgM22 or of a non-immunogenic human IgM (as the negative control) for different times (6, 24 or 48 h) in serum containing growth medium (**a**) or in the absence of serum (**b**). After treatments, cells were harvested and lysed in 0.2% Triton X-100. Total ASM was assessed in vitro by determining the hydrolysis of SM as mixed micelles as described under "Materials and Methods". The amount of hydrolyzed SM was determined through autoradiography after HPTLC separation of the substrate, [^{3}H]SM, and the reaction product, [^{3}H]ceramide. Data are expressed as mean ± SD of six experiments, *$p < 0.05$; **$p < 0.001$

ASM effectively protects against demyelination, detrimental neuroinflammatory response and development of symptoms, suggesting that the ASM/ceramide axis is central in the onset and progression of the disease [30, 31]. Thus, we determined the effect of rHIgM22 or isotype control IgM on the total activity of ASM in MGCs by cell-free assay using mixed micelles of SM and Triton X-100 as the substrate. Antibody treatment was performed either in serum containing medium or in the absence of serum, as described in "Materials and Methods". No SM hydrolysis was detected in negative controls performed by incubating heat-inactivated samples under the same conditions. After 6 h of incubation, in vitro ASM activity was identical in rHIgM22 and control cells, and identical to the enzyme activity measured at time 0. Treatment with either rHIgM22 or control IgM for 24 and 48 h in the presence of serum determined a significant increase in the ASM activity with respect to the starting incubation time of 6 h (Fig. 3a). However, the increase of ASM activity after 6 h was higher for control-treated cells (+41% and +44% at 24 and 48 h, respectively) than for rHIgM22-treated cells (+25% and +31% at 24 and 48 h, respectively). Thus, in the presence of serum, the enzyme activity was slightly but significantly lower in rHIgM22-treated than in control cells. On the other hand, when antibody treatment was performed in the absence of serum (the same experimental condition used to assess the effect of rHIgM22 on MGCs proliferation), ASM activity was

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constant along time of treatment and not significantly different in rHIgM22-treated versus control IgM-treated cells (Fig. 3b). In addition, we measured the levels of radioactivity incorporated into ceramide after steady state labeling with tritiated sphingosine in the presence of serum (Fig. 4a), or after pulse labeling with radioactive sphingosine in the absence of serum (Fig. 4b). In both cases, radioactive ceramide levels were not significantly different in rHIgM22-treated versus control IgM-treated cells, suggesting that rHIgM22 treatment had no effects neither on ceramide levels nor on its turnover.

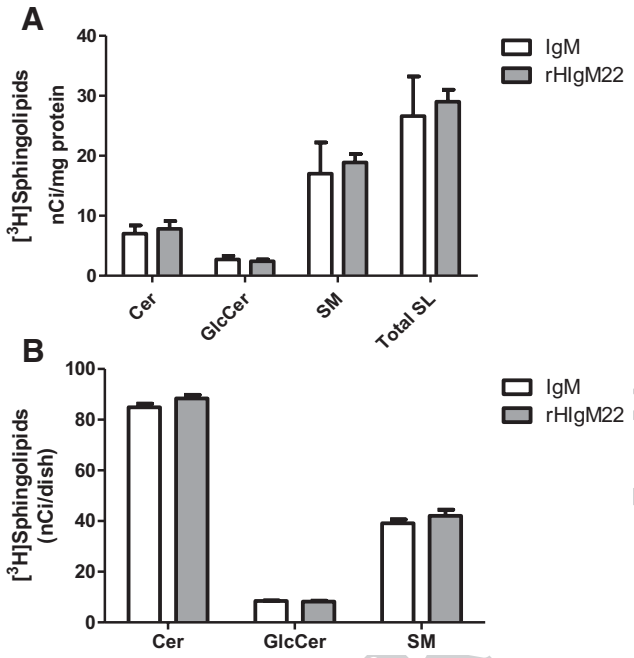

Fig. 4 Effect of rHIgM22 or control treatments on radioactive sphingolipid levels after steady-state or pulse labeling with [^{3}H]Sphingosine. **a** Lipid pattern of MGCs treated with 10 μg/mL of either rHIgM22 or Human IgM for 24 h. Cell sphingolipids were steady-state metabolically labeled by 2 h pulse/48 h chase with 3×10^{-8} M [1-^{3}H]sphingosine. Cell lipids were extracted with chloroform/methanol/water, 2:1:0.1, by volume, subjected to a two-phase partitioning. Organic phases were separated by monodimensional HPTLC using chloroform/methanol/water, 110:40:6, by volume, as a solvent system and quantitatively analyzed by digital autoradiography. **b** radioactivity incorporated into ceramide, glucosylceramide and sphingomyelin after short pulse labeling with radioactive sphingosine. MGCs at the 13th day of culture were treated with a single dose (10 μg/mL) of rHIgM22 or of a non-immunogenic human IgM for 24 h. IgM (white bars) and rHIgM22 treated cells (gray bars) were pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/mL) for 45 min. At the end of pulse, cells were harvested and submitted to lipid extraction and partitioning as described in Materials and Methods. The methanolized organic phase was analyzed by HPTLC and digital autoradiography of HPTLC using chloroform/methanol/water, 55:20:3 by volume. Radioactivity incorporated in ceramide (Cer), glucosylceramide (GlcCer) and sphingomyelin (SM) is shown. All values are the mean ± SD of at least three independent experiments

S1P in MGC and Astrocytes

An increasing number of studies demonstrates that the sphingolipid mediator S1P, usually exerting biological effects opposite to those of ceramide (in particular acting as anti-apoptotic stimulus and positive regulator of cell proliferation), is a relevant player in MS [13, 14]. For this reason, we evaluated whether rHIgM22 effects could be related to an altered production and metabolism of S1P. To this purpose, MGCs, treated with or without 10 μg/mL isotype control IgM or rHIgM22 for 24 h were submitted to pulse experiments with tritiated sphingosine ([^{3}H]Sph). After pulse, lipids associated with cells and culture media were extracted and [^{3}H]S1P levels were determined as described in "Materials and Methods". After short time (45 min) pulse, we found comparable levels of incorporated radioactivity in untreated, control IgM- and rHIgM22-treated cells (608,861.5, 613,279.5, and 639,040.2 dpm/dish respectively). Both control IgM and rHIgM22 induced a significant increase of the radioactivity associated with intracellular S1P (+155% and +228% respectively) compared to untreated cells (Fig. 5a). Similarly, as shown in Fig. 5b, treatment with both control IgM and rHIgM22 was able to increase the amount of the labeled [^{3}H]S1P associated with the extracellular milieu (+32% and +70% respectively). Of relevance, the amount of extracellular [^{3}H]S1P was significantly higher (by about 24%) in the rHIgM22-treated cells compared to control IgM-treated cells.

Since we showed that rHIgM22 treatment was able to upregulate the expression of PDGFαR at the protein level (Fig. 2), we investigated whether PDGFαR activation might be relevant for the increased production and release of S1P in MGCs. To this aim, we measured the production and release of S1P in MGCs upon treatment with rHIgM22 or control IgM in the absence or in the presence of the selective inhibitor of PDGFαR activation, AG1296. As shown in Fig. 6, treatment with PDGFαR inhibitor AG1296 had no effect on the intracellular [^{3}H]S1P levels neither in rHIgM22 treated nor in control IgM treated cells (Fig. 6a). On the other hand, AG1296 treatment strongly inhibited the rHIgM22-induced release of [^{3}H]S1P to the extracellular milieu (−42% vs. rHIgM22-treated cells), while it had no effects on extracellular [^{3}H]S1P levels in control-treated cells (Fig. 6b).

In the attempt to identify the metabolic source of the increased S1P in the MGCs treated with or without IgM and rHIgM22, we evaluated whether treated cells could be characterized by different activity and/or expression of the key enzymes involved in S1P synthesis and catabolism. We found that SK1 and SK2 showed a superimposable expression in control IgM-treated and rHIgM22-treated cells (Fig. 7). Since SK1 can be activated through ERK1/2-mediated phosphorylation on Ser^{225} [32], we assessed whether the increase in S1P production and release in treated cells could be correlated with

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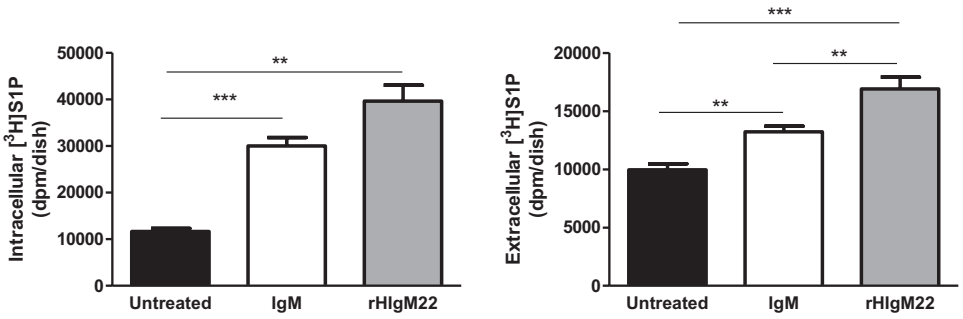

Fig. 5 Effect of rHIgM22 or control treatments on the production and release of S1P in MGCs. MGCs at the 13th day of culture were treated with a single dose (10 μg/mL) of rHIgM22 or of a non-immunogenic human IgM (as the negative control) for 24 h. Untreated (black bars), IgM (white bars) and rHIgM22 treated cells (gray bars) were pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/mL) for 45 min. At the end of pulse, S1P from cells and media was extracted and analyzed as described in "Materials and Methods". Radioactivity incorporated in intracellular (**a**), and in extracellular S1P (**b**) is shown. $^{**}p<0.005$, $^{***}p<0.001$ (t-test). All values are the mean ± SD of at least three independent experiments

SK1 phosphorylation level. To this purpose, we performed immunoblotting analysis by using a phospho-specific antibody recognizing SK1 (Fig. 7). SK1 phosphorylation was not affected by control IgM or rHIgM22 treatment. All together, these results suggest that the increased production and release of S1P in MGCs does not seem to be mediated by regulation of the biosynthetic enzymes, sphingosine kinase 1 and 2. S1P levels can be reduced by dephosphorylation due to the activity of a specific phosphatase (SPP1), or by cleavage by the S1P lyase (SGPL1). SPP1 protein level were unchanged along time upon control IgM or rHIgM22 treatment, while SGPL1 levels were reduced by ~40% in rHIgM22-treated cells versus control treated cells at 24 h, suggesting that a reduced expression of this enzyme could be at least in part responsible for the increased S1P release induced by rHIgM22 in MGCs (Fig. 7).

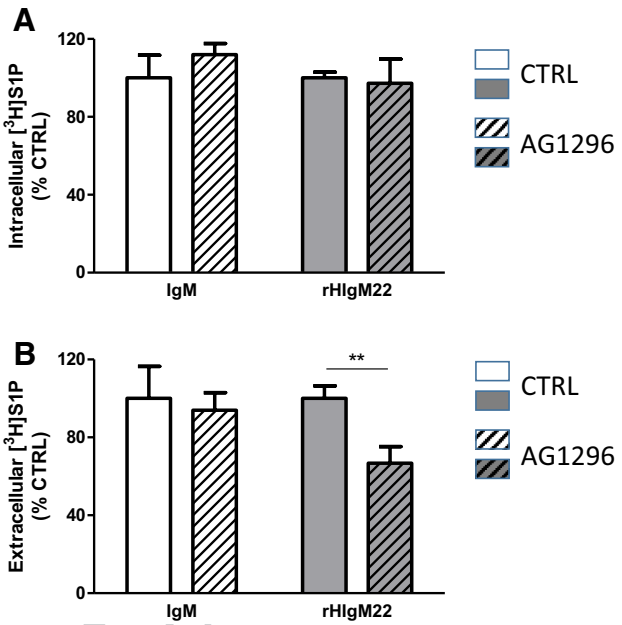

Fig. 6 Effect of AG1296 in rHIgM22 or control-treated cells on the production and release of S1P in MGCs. MGCs at the 13th day of culture were pretreated 30 min with AG1296 (20 μM) then treated with a single dose (10 μg/mL) of rHIgM22 or of a non-immunogenic human IgM (as the negative control) for 24 h. IgM (open white bars), rHIgM22 treated cells (open gray bars), IgM + AG1296 (hatched white bars) and rHIgM22 + AG1296 (hatched gray bars) were pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/mL) for 45 min. At the end of pulse, S1P from cells and media was extracted and analyzed as described in "Materials and Methods". Radioactivity incorporated in intracellular (**a**), and in extracellular S1P (**b**) is shown. $^{**}p<0.005$, $^{***}p<0.001$ (t-test). All values are the mean ± SD of at least three independent experiments

As described above, rHIgM22 treatment was able to increase significantly astrocyte proliferation in MGCs, while other populations were less or not affected. Fischer and collaborators demonstrated that both SK1 and $S1P_3$ are upregulated on reactive astrocytes in MS lesions, and in cultured astrocytes under pro-inflammatory conditions [12]. Thus, we next evaluated S1P production and release in the extracellular milieu in purified cultures of astrocytes, under the same experimental conditions used for MGCs. The levels of incorporated radioactivity after pulse were similar in untreated, control IgM- and rHIgM22-treated cells (278,543.5, 300,298.4 and 286,312.9 dpm/dish, respectively). As shown in Fig. 8, treatment with 10 μg/mL isotype control IgM or rHIgM22 for 24 h did not induce any significant change in the production and/or release of S1P in pure astrocyte cultures. This result suggest that other cell types present in MGCs should be responsible for the release of S1P.

Discussion

Naturally occurring antibodies or natural antibodies are immunoglobulins detectable in the serum of humans in the absence of a specific stimulation by a foreign antigen,

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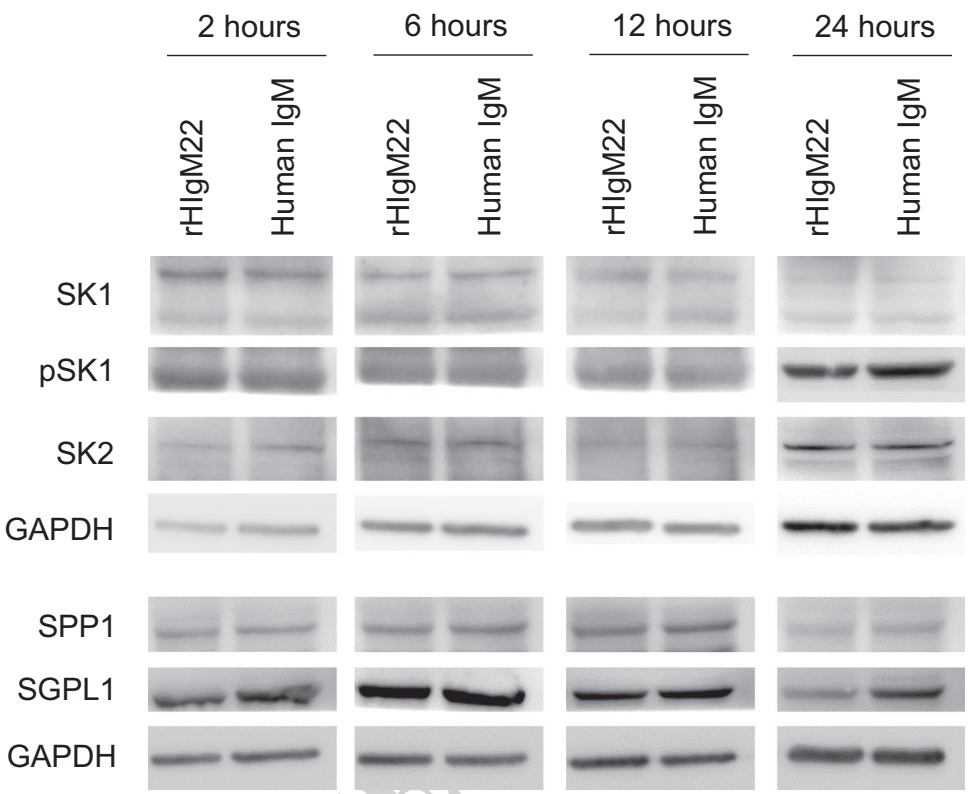

Fig. 7 Effect of rHIgM22 or control treatments on the protein levels and phosphorylation of sphingosine kinases in MGCs. MGCs at the 13th day of culture were treated with a single dose (10 μg/mL) of rHIgM22 or of a non immunogenic human IgM (as the negative control) for 2, 6, 12 and 24 h. At the end of the treatment cells were lysed in 10 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/mL Aprotinin. The same amount of protein (40 μg) for each sample was separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed by western blotting using specific anti SK1, pSK1, SK2, SGLP1, SPP1 and anti GAPDH

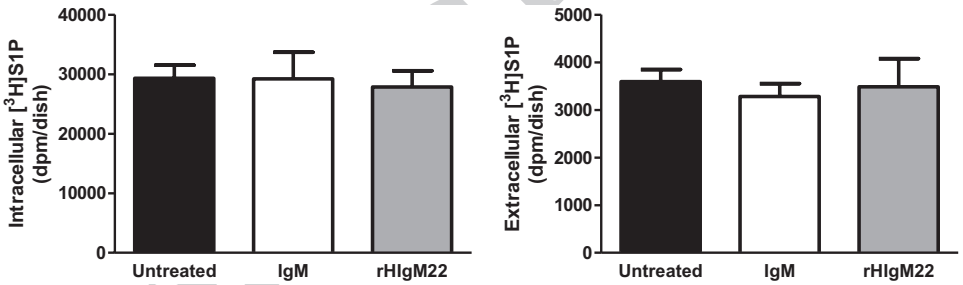

Fig. 8 Effect of rHIgM22 or control treatments on the production and release of S1P in pure astrocyte cultures. Cultured astrocytes were treated with a single dose (10 μg/mL) of rHIgM22 or of a non-immunogenic human IgM (as the negative control) for 24 h. Untreated (black bars), IgM (white bars) and rHIgM22 treated cells (gray bars) were pulsed with 20 nM [^{3}H]-Sph (0.4 μCi/mL) for 45 min. At the end of pulse, S1P from cells and media was extracted and analyzed as described in "Materials and Methods". Radioactivity incorporated in intracellular (**a**), and in extracellular S1P (**b**) is shown. $^{**}p<0.005$, $^{***}p<0.001$ (t-test). All values are the mean ± SD of at least three independent experiments

produced by B-cells in a T-cell-independent manner. Frequently, natural antibodies are multivalent and autoreactive. They are part of the innate immune system, however their physiological functions are multiple and still not fully understood. Nevertheless, it is clear that they are able to engage a variety of cell types in human tissues eliciting various biological responses [33]. rHIgM22 is the recombinant form of a human monoclonal IgM, sHIgM22, isolated from the serum of an individual affected by Waldenström macroglobulinemia, a condition characterized by elevated production of immunoglobulins. rHIgM22 was established after the serendipitous discovery by the group of Rodriguez that some natural antibodies, including sHIgM22, were able to induce remyelination in animal models of CNS demyelinating disease [34]. rHIgM22 binds to the surface of morphologically differentiated, MBP-positive rat and human OLs in culture [15], of O4-positive CG4 cells, to isolated myelin and to myelin in live mouse cerebellar slices [35]. The hypothesis that OLs represent the main cellular target of rHIgM22 was strengthened by the observation that binding was abolished in cerebellar slices from mice lacking cerebroside sulfotransferase, the enzyme responsible for the

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biosynthesis of sulfated glycolipids, the typical myelin lipids [36]. rHIgM22 treatment effectively promoted remyelination in both immune and non-immune mouse models of demyelination [15–19]. Magnetic resonance imaging showed that rHIgM22 reached demyelinated spinal cord lesions in Theiler's virus-infected mice, but it did not accumulate in CNS of control animals [37]. In humans, rHIgM22 was detected in the cerebrospinal fluid of treated MS patients in a phase I clinical trial. If we consider the substantial lack of treatments able to prevent the progression of demyelinating diseases, altogether these observations raise the hope that rHIgM22 could be effectively used to stimulate the endogenous mechanisms of myelin repair and represent a valuable option for the treatment of MS.

While it is clear that the ultimate effect of rHIgM22 is increased production of myelin by mature OLs, the mechanisms underlying this effect are still unclear and literature is in our opinion quite controversial. Only mature OLs, and not OLs at earlier stages of differentiation, neither astrocytes nor microglia, showed significant surface binding of rHIgM22 by immunofluorescence [38]. Early work from the Rodriguez group showed that sIgM22, similarly to other remyelination-promoting monoclonal antibodies, was able to induce Ca^{2+} transients in different glial populations [39] in rat MGCs. In particular, an early and rapid increase in intracellular calcium was observed in astrocytes (GFAP-positive cells), while a delayed and slower increase in Ca^{2+} concentration was observed in OPCs and OLs at different stages of differentiation, implying that surface reactivity of this antibody is not necessarily a predictor of its biological activity in a given cell type. The same group showed that rHIgM22 was able to stimulate proliferation of OPCs in MGCs, but not in purified OPC cultures [20], suggesting that antibody effect on OPC proliferation (and thus on remyelination) might be dependent on the recruitment of other cell types present in MGCs, i.e., astrocytes and or microglia. The proliferative effect of rHIgM22 in MGCs was mediated by the activation of PDGFαR, a well-known regulator of OPC proliferation and survival. Most glial cell-secreted PDGF is produced by astrocytes [40]. However, rHIgM22 treatment of MGCs was not able to significantly affect astrocytes and microglia proliferation, even if it slightly increased expression of GFAP and of the microglial marker CD68, suggesting some form of astrocyte/microglia activation upon rHIgM22 treatment. In this paper, we report a marked proliferative response of MGCs to rHIgM22 treatment under comparable experimental conditions (Fig. 1; Table 1). We observed a significant proliferative response, especially at higher antibody concentrations, in A2B5(+) and O4(+) OPCs, and, at a lesser extent, in CD11(+) cells. However, the most significant stimulation of proliferation at all antibody concentrations tested was observed for GLAST(+) astrocytes (Fig. 1). The effect of rHIgM22 on the astrocyte (GLAST(+)) and OPC (A2B(+)) cell population appeared to supersede the growth potential of serum containing media (GM). We surmise the increased numbers of O4-positive cells was a result of the proliferation of the OPCs population (A2B5(+)) which in a serum-free environment can lead to differentiation towards the O4-positive, OL fate. The results of these flow cytometry studies reveal the ability of rHIgM22 to maintain the growth potential of mixed glial cultures even in the absence of serum and suggests a more prominent function on astrocyte and oligodendrocyte precursor cells. Similar results have been obtained using imaging-based methods for the assessment of cell proliferation.

This is apparently in contrast with the aforementioned report showing no apparent proliferation of astrocytes in response to rHIgM22 treatment. We have measured the proliferative response as total cell count and EdU labeling index by flow cytometry and imaging analysis with consistent result, while Watzlawik et al. analyzed the co-localization between the nuclear proliferation marker Ki-67 and cell type specific markers. This technical difference could explain the apparent discrepancy of the results. From this point of view, it might be worth to recall that GFAP staining requires cell permeabilization, while GLAST staining is performed on non permeabilized cells.

Interestingly, we add new pieces of evidence highlighting the importance of sphingolipid mediators in rHIgM22 signaling. Ceramide (Cer) and S1P are interconvertible bioactive sphingolipids, their levels are finely regulated, and they in turn differentially regulate cell growth and survival, modulating opposing signaling pathways. The balance between the levels of Cer and S1P, a concept known as "sphingolipid rheostat", and their regulatory effect on different pathways determines the fate of the cells [41–43]. In fact, elevation of cellular ceramide levels induces cell growth arrest and apoptosis [43], whereas S1P production is required for optimal cell proliferation induced by growth factors [44] and suppresses ceramide-mediated apoptosis [41]. Treatment of MGCs with rHIgM22 induced a slight but significant decrease in the in vitro activity of ASM (Fig. 3a), one of the major responsible factors for the stimulus-mediated production of Cer by SM hydrolysis. Previously it was shown that the Src family kinase Lyn (that was activated downstream to PDGFαR in MGCs treated with rHIgM22 [20]) associated with integrin receptors was able to suppress the activity of ASM thus preventing ceramide-induced apoptosis in mouse brain and cultured OLs [45]. Remarkably, the importance of ASM for the onset and progression of MS has been recently highlighted by the finding that genetic deficiency or pharmacological inhibition of ASM are protective against lesions in mouse models of MS [30, 31]. However, the inhibitory effect of rHIgM22 on ASM activity was absent in MGCs when antibody treatment was performed in the absence of serum (Fig. 3b). Moreover, steady-state and pulse labeling

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experiments with radioactive sphingosine showed no differences in the incorporation of radioactivity into Cer, in rHIgM22 treated versus control cells (Fig. 4).

On the other hand, rHIgM22 was able to induce a significant increase in the production of S1P and in its release in the culture medium in MGCs (Fig. 5). Remarkably, we demonstrated that the effect of rHIgM22 on the release of S1P from MGCs was strongly inhibited by the concomitant treatment with a selective inhibitor of PDGFαR, suggesting that antibody-mediated receptor activation is a requisite for S1P release. This, together with our finding, that rHIgM22 was able to upregulate PDGFαR protein levels (Fig. 2) and with previous data suggesting that rHIgM22 was able to activate PDGFαR [20], confirms the importance of PDGFαR as a mediator of rHIgM22 biological effects.

Sphingosine kinase 1 and S1P receptors are upregulated in reactive astrocytes in MS lesions. S1P by acting on $S1P_3$ receptors on astrocytes induced the secretion of the chemokine CXCL1, which in turn was able to recruit OPCs to the lesion area, with a possible positive impact on remyelination [6, 12]. Treatment with rHIgM22 on MGCs had no effects on the protein levels of SK1 and SK2, or on SK1 phosphorylation, the main known activation mechanism for SK1 (Fig. 7). Among catabolic enzymes responsible for the removal of S1P, treatment with rHIgM22 had no effects on the protein levels of the specific phosphatase SPP1, while it reduced the levels of the S1P lyase SGPL1. To our knowledge, this is the first report indicating the possible role of this enzyme in MS and/or mechanisms of myelin repair.

We tested the possibility that S1P production by rHIgM22 in MGCs could involve astrocytes, implying the possibility of a S1P-dependent autocrine loop controlling astrocyte proliferation in response to the antibody. However, rHIgM22 treatment under the same experimental condition was not able to affect the production or release of S1P in pure cultured astrocytes (Fig. 8). This finding is interesting but not particularly surprising considering that the main mode of action of extracellular S1P is paracrine rather than autocrine. In addition, rHIgM22 did not show significant binding to cultured astrocytes, and astrocytes express very low levels of sulfated antigens, that seem relevant for the binding of rHIgM22. Thus, another cellular population present in MGCs is likely involved in this event. S1P-producing cells in response to rHIgM22 remain to be identified. However, microglia seems to play very important roles in the lesion microenvironment. As for astrocytes, rHIgM22 did not show significant surface binding to microglia by immunofluorescence [38]. However, a recent paper showed that rHIgM22 can stimulate myelin phagocytosis by microglial cells, a crucial event in clearing the myelin debris that strongly inhibits OPCs maturation [9]. Thus, it is becoming evident that the myelin-repair effect of rHIgM22 requires the orchestration of the responses of multiple cellular populations in the lesion niche. The data presented here suggest that the balance between different sphingolipid mediators in the sphingolipid rheostat might play a significant role in this orchestration.

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