On the cause of multiple sclerosis

Bijlard, Marjolein

Chapter 4

Sulfatide-mediated control of ECM-dependent oligodendrocyte maturation

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ABSTRACT

In the central nervous system, the extracellular matrix (ECM) compound laminin-2, present on developing axons, is essential in regulating oligodendrocyte maturation. For example, laminin-2 is involved in mediating interactions between integrins and growth factors, initially localizing in separate membrane microdomains. The galactosphingolipid sulfatide is an important constituent of these microdomains, and may serve as a receptor for laminin-2. Here we investigated, whether sulfatide interferes with ECM-integrin interactions and, in this manner, modulates oligodendrocyte maturation. Our data reveal that disruption of laminin-2-sulfatide interactions impeded oligodendrocyte differentiation and myelin-like membrane formation. On laminin-2, but not on (re)myelination-inhibiting fibronectin, sulfatide laterally associated with integrin α6 in membrane microdomains. Sulfatide was partly excluded from membrane microdomains on fibronectin, thereby likely precluding laminin-2-mediated myelination. Anti-sulfatide antibodies disrupted integrin α6-PDGFαR interactions on laminin-2, and induced demyelination in myelinated spheroid cultures, but intriguingly stimulated myelin-like membrane formation on fibronectin. Taken together, these findings highlight the importance of laminin-sulfatide interactions in the formation of functional membrane microdomains essential for myelination. Thus, laminin-sulfatide interactions might control the asynchronous localized differentiation of oligodendrocytes, thereby allowing myelination to be triggered by axonal demand. Given the accumulation of fibronectin in multiple sclerosis lesions, the findings also provide a molecular rationale for the potential of anti-sulfatide antibodies to trigger quiescent endogenous oligodendrocyte progenitor cells in axon remyelination.

INTRODUCTION

Myelination of axons by oligodendrocytes (OLGs) is a carefully regulated process, involving the integration of both temporal and spatial signals that are provided by neighbouring neuronal target cells. Thus, in the developing central nervous system (CNS), localized axonal laminin-2, an extracellular matrix (ECM) protein, is instrumental in regulating OLG maturation, promoting survival, differentiation and myelination [19,63,99,216,223,248–250]. The interaction of laminin-2 with transmembrane cell adhesion molecules such as integrin α6β1 or α-dystroglycan appears essential in these events [223,251]. One of the underlying mechanisms is the laminin-2-mediated facilitation of integrin-growth factor interactions, originating from merging of initially separated, integrin and growth factor-containing membrane microdomains, also defined as 'lipid rafts' [63,64,113]. Besides amplification of growth factor signalling, the axon-directed mobilization of membrane microdomains is also imperative in paronode formation [59,60], fyn-mediated process extension [61,65] and protein trafficking towards the myelin membrane [9,26], highlighting the importance of the proper formation and functioning of microdomains in OLG myelination.

The galactosphingolipids, galactosylceramide (GalC) and its sulphated derivative sulfatide, are important constituents of oligodendroglial microdomains [61]. These myelin-enriched lipids are expressed prior to the onset of myelination [222], i.e., just before 'activation' of axonal contact signals such as laminin-2, suggesting that these typically raft-associated lipids might be involved in establishing early interactions between myelin membranes and axons. Indeed, in addition to its ability to bind to dystroglycan and integrin, laminin-2 also displays binding affinity towards sulfatide [94–98]. Interestingly, both GalC and sulfatide as such are able to initiate signalling pathways [252–254], while sulfatide has been postulated to negatively regulate OLG differentiation [82,83], although this galactolipid is necessary for paranode assembly and myelin maintenance [73–76].

ECM cues have attracted widespread attention in many diseases of the CNS, including multiple sclerosis (MS). During CNS development, the expression of ECM molecules is tightly regulated. Consequently, following CNS myelin injury, the ECM undergoes extensive remodeling, which leads to alterations in ECM expression profiles [255–258]. For example, sulfated proteoglycans and fibronectin are readily and transiently expressed following demyelination, while laminins accumulate upon remyelination. A careful and timely regulation of expression of distinct ECM molecules, such as (re)myelination-inhibiting fibronectin and (re)myelination-promoting laminin-2 is thus crucial in maintaining the proper physiological environment for development of oligodendrocyte progenitor cells (OPCs) into mature, myelinating cells. Evidently, a misbalance in expression of these ECM entities and ensuing defects in ECM-mediated signaling might thus contribute to an impaired differentiation of OPCs into mature OLGs. Indeed, fibronectin aggregates, present in MS lesions, reduce differentiation of OPCs in demyelinated lesions, and thereby impede remyelination [259].

The current study was undertaken to investigate the role of molecular players in ECM-dependent OLG maturation and the underlying mechanisms, in particular the role of sulfatide. We provide evidence that stimulation of laminin-2-facilitated differentiation of OPCs, and hence myelination, is dependent on sulfatide. Moreover, interactions between PDGFαR and integrin α6 in membrane microdomains, as promoted by laminin-2, were dependent on sulfatide. Interestingly, fibronectin precluded the proper integration of sulfatide in these microdomains, thereby likely frustrating the ability of laminin-2 to facilitate myelin-like membrane formation. Intriguingly, addition of anti-sulfatide antibodies triggered myelin-like membrane formation in OLGs that were cultured on fibronectin, thus overcoming its inhibitory effect. Hence, these findings may explain the quiescence of OPCs in MS lesions, and provide a challenging option for the potential application of anti-sulfatide antibodies in promoting axon remyelination in a fibronectin, i.e., remyelination inhibitory, microenvironment.

MATERIALS AND METHODS

Cell culture

Primary oligodendrocytes: Primary OLGs were isolated from 1-3 day old Wistar rats as described previously [213,260]. Isolated OPCs were plated on 8 well chamber slides (Nunc, Naperville, IL, 15,000 cells/well) or 10 cm tissue culture dishes (Nunc, 1 x 10^6 cells/dish), pre-coated with either poly-l-lysine (PLL, 5 μg/ml, Sigma, St. Louis, MO), fibronectin (10 μg/ml, Sigma), or laminin-2 (10 μg/ml, Sigma), for immunocytochemical studies and biochemical analysis, respectively. To block lipid binding sites on the different substrates, pre-coated dishes were incubated with either soluble sulfatide (40 μg/ml, Sigma) or GalC (40 μg/ml, Sigma) for 2 hrs at 37°C prior to plating. After plating, cells were synchronized by culturing in SATO [213] supplemented with the growth factors FGF-2 (10 ng/ml, Peprotech, London, UK) and PDGF-AA (10 ng/ml, Peprotech). After 2 days, differentiation was initiated by growth factor withdrawal and switching to SATO supplemented with 0.5% FCS (Bodinco, Alkmaar, The Netherlands). Antibodies (O4, O1 and R-mAb) and inhibitors of galactolipid biosynthesis [30 μM fumonisin B1 (Sigma) and 30 μM sodium chlorate (Fluka BioChemica, Buchs, Switzerland)] were added 3 days after initiating differentiation, i.e., at the onset of myelin-like membrane formation. Cells were analyzed 7 days after initiating differentiation.

OLN-93 cells: The rat-derived oligodendroglia derived cell line, OLN-93 (a kind gift of Dr. Christiane Richter-Landsberg, University of Oldenburg, Germany) were cultured as described [236]. Cells were plated on pre-coated PLL (5 μg/ml), laminin-1 (10 μg/ml), laminin-2 (10 μg/ml) or fibronectin (10 μg/ml) bacterial 96 well plates (Greiner Bion-one, Alphen aan de Rijn, the Netherlands) for the adhesion assays (50,000 cells/well), on 8 well permanox chamber slides (Nunc) for immunocytochemistry (5,000 cells/well) and on 10 cm tissue culture dishes for immunoprecipitation analysis (500,000 cells/well). Cells were grown in DMEM supplemented with 10% FCS for 3 days, unless otherwise indicated.

Hybridoma culture: The hybridoma cell lines O4, O1 and R-mAb producing monoclonal antibodies against sulfatide, GalC, and both GalC and sulfatide, respectively were a kind gift of Guus Wolswijk [217,261]. The hybridoma cell lines were cultured in heat-inactivated 10% FCS in DMEM. Upon antibody production, cells were grown without medium change for 2 weeks in the absence of FCS. Antibodies were concentrated from the hybridoma culture supernatants by ammonium sulfate (Sigma) precipitation, and dialysed against the appropriate buffer. For the antibody perturbation experiments, the antibodies were used at 10 μg/ml, as determined by a Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Spheroid cultures: Whole brain spheroid cultures were prepared as described previously [262]. After 4 weeks the spheroids were plated in six-well plates at approximately 1 plate per flask. The following day, the medium was removed from the spheroids and replaced by 2 ml new medium with or without addition of 0.135 μM lysolecithin to induce demyelination.

Lysolecithin was 3 times completely replenished. After 7 days of exposure to lysolecithin, the medium was replaced by 2 ml of fresh lysolecithin-free medium. Antibodies (O1 and O4, 1:10) were added to the cultures once at day 8 after induction of demyelination or to untreated spheroids.

Constructs

The cDNAs encoding ceramide sulfatide transferase (CST) and ceramide galactosyltransferase (CGT) were kind gifts of Drs. Matthias Eckhardt (University of Bonn, Germany) and Brian Popko (University of Chicago, Chicago, IL), respectively. For cloning, the *cst* and *cgt* genes were inserted into the *EcoRI* site of into the retroviral vector pLXIN (Clontech Biosciences, Mountain View, CA). Recombinant plasmids were grown in TOP10 cells (Invitrogen, Paisly, UK), and plasmids with the cDNA insert in the correct orientation with respect to transcription were identified by restriction analysis. The orientation and the integrity of the obtained pLXIN constructs were confirmed by DNA sequencing. The production of retroviral particles and the subsequent infection and selection of OLN-93 cells were performed according to [263]. OLN-93 cells were first infected with *cgt* and subsequently selected, which was followed by a second infection with *cst* (OLN-GS). An OLN-MOCK cell line was obtained by retroviral infection with vector only (pLXIN). The transduction efficiency was nearly 100%.

Analysis of cellular glycosphingolipids

OLN-93 cells were washed three times with phosphate buffered saline (PBS), harvested by scraping in PBS, centrifuged at 10,000 rpm at room temperature (RT), followed by lipid extraction of the cell pellet according to Ref. 264. Lipids were separated on TLC plates using $C_3H_6O_2/CH_3CH(OH)CH_3/CHCl_3/CH_3OH$/25% KCl (25:25:25:10:9, v/v/v/v/v) as the running solvent. To visualize the glycosphingolipids, the plates were dried, and sprayed with 10% H_2SO_4 and 5% CH_3OH and heated to 120°C.

Lateral crosslinking

Cells were incubated on ice with 4% bovine serum albumin (BSA) for 10 min, and loaded with anti-sulfatide O4 antibody (1:5) for 30 min. Cells were washed twice with PBS, and incubated with rabbit-anti-mouse IgM (1:50, Jackson ImmunoResearch, Westgrove, PA) at 37°C. After 30 min, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde (PFA, Merck, Darmstadt, Germany) in PBS for 20 min on ice, and post-fixed with ice-cold methanol for 10 min at RT to prevent artifacts. To visualize cross-linked sulfatide patches, the cells were subsequently incubated with TRITC-conjugated goat-anti-rabbit (1:50, Jackson ImmunoResearch) for 30 min at RT. The samples were analysed with a Leica SP8 AOBS CLSM confocal microscope (Leica Microsystems, Heidelberg, Germany).

Immunocytochemistry

Antibody staining of the cell surface lipids GalC and sulfatide, with O1, O4 and R-mAb were performed on live cells at 4°C. After blocking non-specific binding with 4% BSA in PBS, cells were incubated with primary antibody for 30 min, washed three times and incubated for 25 min with appropriate FITC- or TRITC-conjugated antibodies (Jackson ImmunoResearch). The cells were fixed with 4% PFA in PBS for 20 min at RT, after which the nuclei were stained with DAPI (1 μg/ml). For staining of intracellular antigens, cells were gently fixed, first with 2% PFA for 15 min at RT, followed by 4% PFA for 15 min at RT. Fixed cells were permeabilized with ice cold methanol for 10 min, and subsequently blocked with 4% BSA in PBS for at least 30 min at RT. The cells were incubated for 1-2 hrs with primary antibody (MBP, Serotec, Oxford, UK, 1:250) at RT. The cells were washed with PBS and incubated with TRITC-conjugated secondary antibodies supplemented with DAPI for 25 min at RT. The samples were analyzed with an immunofluorescence microscope (Olympus AX70), equipped with analySIS software. OLGs were characterized by morphology, i.e., cells with a typical astrocytic morphology were excluded, and in each experiment at least 500 cells were scored as either MBP-positive or MBP-negative. In addition, positive cells bearing MBP-positive membranous structures spread between the cellular processes were identified as myelin-like membrane-forming, irrespective of the extent of membrane formation.

Immunohistochemistry

The spheroids were harvested at 0, 8 and 14 days after the induction of demyelination. After washing with ice-cold PBS, spheroids were prepared for immunological staining. Following an incubation in 4% PFA during 1.5 hrs, the spheroids were maintained in 16% sucrose overnight at 4°C, and subsequently quickly frozen in Tissue-Tek® (Sakura Finetek, Zoeterwoude, the Netherlands) for cryostat sectioning (6 μm sections). Cryostat sections were air-dried for 20 min and incubated with normal mouse serum for 1 hr, after which the sections were incubated overnight at 4°C with the primary antibodies [SMI99 (Covance, Princeton, NY, 1:500), anti-laminin 1+2 (Abcam, Cambridge, UK, 1:300), anti-fibronectin (Millipore, Chemicon, Bedford, MA, 1:50), and R-mAb (1:10)]. After washing, sections were incubated with the appropriate Alexa-conjugated secondary antibodies (Invitrogen, 1:500) for 1 hr. The sections were counterstained with Hoechst (Invitrogen, 1:5,000) and embedded in mounting medium. Omission of the primary antibody was included as a negative control. Pictures were taken on a Leica DM6000 (Leica LAS AF software, Leica Microsystems, Bensheim, Germany) and processed using Adobe Photoshop 6.0. Immunoreactivity in unprocessed pictures was quantified by the image analysis program AnalySIS software (Soft Imaging System GmbH, Münster, Germany). The threshold was set such that the total surface of each spheroid was included in the analysis. The mean immunointensity per spheroid was automatically measured.

Adhesion assay

Adhesion assays were performed as described [265], with some minor modifications. Non-tissue culture 96-wells plates (Greiner) were coated with laminin-1 or -2 for at least 3 hrs at 37°C, after which the wells were washed with PBS and blocked for 30 min with 1% heat-inactivated BSA at 37°C. After washing, 50,000 cells (OLN-MOCK or OLN-GS) per 50 µl per well were allowed to adhere for 30 min at 37°C. For blocking experiments, OLN-GS cells were pre-incubated with O4 (1:5), or anti-integrin β1 (BD Pharmingen, 1:200) antibodies for 30 min at 37°C. The cells were washed two times with PBS, and adhered cells were fixed for 15 min with ice-cold methanol. Cells were stained with 50 µl 0.2% crystal violet (in 2% ethanol, Sigma), washed with water after which the stain was solubilised in 50 µl 1% SDS. Adhesion was quantified by measuring the absorbance at 570 nm after 30 min. To estimate 100% adhesion, a similar amount of OLN-93 cells (in triplicate) as plated in the wells was centrifuged, fixed, stained, washed, resuspended in 50 µl 1% SDS and transferred to the 96 well plate, to measure the absorbance (A) as for the adhered cells. The 0% adhesion value was established by measuring the absorbance of culture media, collected from cell-free wells, incubated at identical conditions. The % adhesion of the cells was calculated as $(A_{sample}-A_{0\%})/(A_{100\%}-A_{0\%})$ x 100% with triple determinations.

Membrane microdomain isolation

Cells were washed with ice-cold PBS, scraped on ice in 375 µl TNE-lysis buffer [50 mM Tris-HCl, pH 7,5, 150 mM NaCl, 5 mM EDTA, 1% TX-100 and protease cocktail inhibitors (Complete Mini, Roche Diagnostic Corp., Mannheim, Germany)], and homogenized by passage through a 25 gauge needle. Lysates were incubated for 30 min at 4°C and protein content was determined. Membrane microdomains were subsequently isolated using density gradient centrifugation. Equal amounts of protein in 40% Optiprep (Lucron Bioproducts, Milsbeek, the Netherlands, 750 µl) were overlaid with 2.25 ml 30% and 2.25 ml 10% Optiprep (in TNE). Gradients were centrifuged overnight at 40,000 rpm (SW55 Beckman, 4°C). Membrane microdomains are visibly floating between the 10% and 30% OptiPrep layer. A 750 µl fraction was taken from this area and used for immunoprecipitation (250 µl), followed by Western blot analysis using non-reducing SDS sample buffer, and dotblot analysis. For total gradient analysis, fractions of 750 µl were collected from the top (fraction 1) to the bottom (fraction 7) of the gradient.

Immunoprecipitation

Cells were washed and scraped in PBS, and lysed on ice for 30 min in TNE-lysis buffer. For immunoprecipitation proteins and lipids of the membrane microdomain fractions, surface proteins were first biotinylated as described [112]. The protein content was determined by a Bio-Rad DC Protein Assay, using BSA as a standard. Equal amounts of protein (25 µg for primary OLGs, 50 µg for OLN-GS) or an equal volume (75 µl of a pooled fraction 3-4) of

membrane microdomains were incubated with 75 µl protein G-Sepharose-beads (Amersham, BioSciences, Buckinghamshire, UK) in TNE-lysis buffer with the appropriate antibody (anti-integrin α6, Millipore, Chemicon, 1:100; anti-integrin αv, 1:100, Chemicon; O4, 1:20 or O1, 1:20) overnight head over head at 4°C. Where O4 or O1 were used, protein G-Sepharose was first pre-incubated for 2 hrs at 4°C with the linker antibody rabbit-anti mouse IgM. Protein G-Sepharose beads were washed four times with IP-wash buffer (TNE-lysis buffer supplemented with 0.2% SDS) and once with PBS. Washed Protein G-Sepharose beads were resuspended in 2% SDS if proteins are immunoprecipitated, after which lipid analysis using dotblots were performed (see below). In the case where lipids are immunoprecipitated, the non-immunoprecipitate (supernatant) and washed protein G-Sepharose beads (immunoprecipitate) were subjected to another immunoprecipitation procedure (re-IP, [112]) using anti-integrin α6 antibodies. Washed Protein G-Sepharose beads were resuspended in non-reducing SDS sample buffer, and after 5 min at 95°C proteins were separated by SDS-PAGE followed by Western blotting, and detection of (surface) integrin α6 using SA-HRP (GE Healthcare, 1:2000) and ECL detection (see below). Immunoprecitation of PDGFαR followed by Western blot detection of integrin α6 was performed as described previously [63].

Dotblot

The cells were washed three times with PBS and harvested by scraping into 1 ml PBS. Cells were centrifuged at 10,000 rpm at RT and pellets were lysed in TNE-lysis buffer. Equal amounts of protein in equal volumes (max. 10 µl) or equal volumes of Optiprep gradient fractions were applied onto nitrocellulose membranes. When dried, membranes were incubated for 1 hr at RT in blocking solution (5% nonfat dry milk in PBS), followed by an overnight incubation with the indicated primary antibodies (ammonium sulfated precipitated, O4 1:20, O1, 1:20). After washing with PBS containing 0.1% Tween 20 (PBS-T), the membranes were incubated for 2 hrs with HRP-conjugated anti-IgM antibodies in 1% nonfat dry milk in PBS-T. For detection of GM1, membranes were incubated with HRP-conjugated CTB (Sigma, 1:1000) for 1 hr at RT. Signals were detected by enhanced chemilluminescence (ECL; Amersham Pharmacia Biotech), scanned and quantified with Scion Image software (Scion Corp., Frederick, MD).

SDS-PAGE and Western blot analysis

Samples were loaded onto 10% SDS-polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane by semi-dry blotting (BioRad, Hercules, CA). The membranes were blocked with 5% nonfat dry milk in PBS either for 1 hr at RT or overnight at 4°C in blocking solution. After washing with PBS-T, membranes were incubated with appropriate primary antibodies in PBS-T supplemented with 0.5% non-fat dry milk (3 hrs at RT or overnight at 4°C). The membranes were washed 3 times with PBS-T and incubated for 1-2 hrs with the appropriate HRP-conjugated antibodies or SA-HRP (GE Healthcare, 1:2000) in PBS-T with 0.5% milk. Signals were detected by enhanced chemilluminescence (ECL; GE Healthcare).

Statistics

Data are expressed as mean + standard deviation (SD) and were obtained from at least three independent experiments. Statistical analysis was performed using the one sample t-test when compared relative to control, which was set to 100% in each independent experiment. When absolute values of more than two means were compared, statistical significance was calculated by one way ANOVA followed by the Newman-Keuls posttest. In all cases a p value of $p< 0.05$ was considered significant (* $p<0.05$, ** $p< 0.01$, *** $p<0.001$).

RESULTS

Sulfatide is not essential for laminin mediated oligodendrocyte adhesion

Axonal laminin-2 triggers OLGs to initiate myelin membrane biogenesis, whereas in absence of such signal, OLGs go into apoptosis [99,250]. Since laminin-2 displays binding sites for sulfatide [94,96], which is enriched on the surface of (maturing) OLGs [222], we first assessed whether laminin-mediated OLG adhesion depends on sulfatide. Since primary OLGs only acquire sulfatide expression during development, we made use of the OLG-derived cell-line OLN-93 [236]. OLN-93 cells express relatively low levels of GalC and sulfatide. To increase these levels we retrovirally overexpressed ceramide galactosyltransferase (CGT) and ceramide sulfatide transferase (CST), which mediate GalC and sulfatide biosynthesis, respectively, from ceramide. The galactolipid-overexpressing OLN-93 cells are referred to as OLN-GS cells. As shown in Figure 1A, compared to control mock-transduced cells (OLN-MOCK, i.e., vector-only), OLN-GS cells significantly overexpress GalC and sulfatide. A double band for GalC is visible, likely representing non-hydroxylated (upper band) and hydroxylated (lower band) GalC. Importantly, both GalC and sulfatide, as visualised with O1 and O4 antibody respectively, are largely expressed on the OLN-GS cell surface, while being non-detectable at the surface of OLN-MOCK cells (Fig. 1B). Following treatment of the cells with antibodies, cellular adhesion to laminin was determined by an adhesion assay. As laminin-1 is expressed by astrocytes upon injury [266], and also harbors a sulfatide binding site [95,98], this substrate was also included. Pretreatment of the cells with the anti-sulfatide IgM antibody, O4, slightly reduced laminin-2-mediated adhesion, whereas laminin-1-mediated adhesion was not affected (Fig. 1C). However, next to sulfatide, integrin $\alpha 6\beta 1$ and α-dystroglycan are the only additionally known laminin-receptors expressed in OLGs [251]. So far, integrin $\alpha 6\beta 1$ has been detected in OLN-93 cells [19], whereas the presence of α-dystroglycan remains to be determined. Interestingly, as shown in Figure 1, a preincubation of the cells with the function-perturbing anti-integrin $\beta 1$ antibody reduced adhesion of OLN-GS to laminin-1 and -2 by 50% and 30%, respectively. Adhesion of the anti-integrin $\beta 1$ antibody-treated cells to laminin-2 was further reduced when the cells had been co-treated with the anti-sulfatide antibody O4 (Fig. 1C). However, the $\beta 1$ integrin receptor seems to play the most dominant role, as co-treatment with the sulfatide antibody further diminished laminin-2 mediated adhesion from 30 to 50% only, whereas no additional effect on laminin-1 mediated adhesion was detected. Taken together, these data suggest that sulfatide only modestly contributes to the facilitation of the interaction between (early) myelinating OLGs and laminin-2-expressing axons. Given the ability of both sulfatide and laminin-2 to modulate the ubiquity of membrane microdomains [61,63,113], and the importance of these domains in myelination [9,26], we next examined whether sulfatide-laminin 2 interactions are relevant in propagating downstream signals, promoting differentiation and subsequent myelination.

Sulfatide is essential for laminin-2 dependent differentiation of oligodendrocytes

To assess the relevance of laminin-sulfatide interactions in laminin-2-dependent OLG maturation, the laminin-2-coated culture dishes were preincubated with sulfatide prior to plating OPCs. In this way, potential sulfatide-laminin interactions were blocked and binding of oligodendroglial sulfatide to laminin-2 prevented. As shown in Figure 2A and B, relative to control, i.e., OPCs grown on laminin-2-coated substrates without sulfatide treatment, prior blocking of laminin-2-sulfatide interactions significantly decreased the number of MBP-positive cells, suggesting that OLG maturation, as reflected by MBP expression, was substantially suppressed at these conditions. Of note, the sulfatide-block did not affect adherence of OPCs to the laminin-2 substrate, as OPCs do not express sulfatide on their surface yet, and likely attach to laminin-2 via surface-expressed integrin $\alpha 6\beta 1$ (cf. Fig. 1) and/or α-dystroglycan [251]. Morphologically, the cells appear less mature and display a less complex morphology, although they do express CNP, the first myelin-specific protein expressed during development (data not shown). In contrast, when the laminin-2-coated dishes were similarly pre-incubated with GalC, the precursor of sulfatide, which is also enriched at the surface of maturing OLGs [222], the OPCs displayed an MBP expression profile, which was virtually indistinguishable from control (Fig. 2B). It should be noted that myelin-like membrane

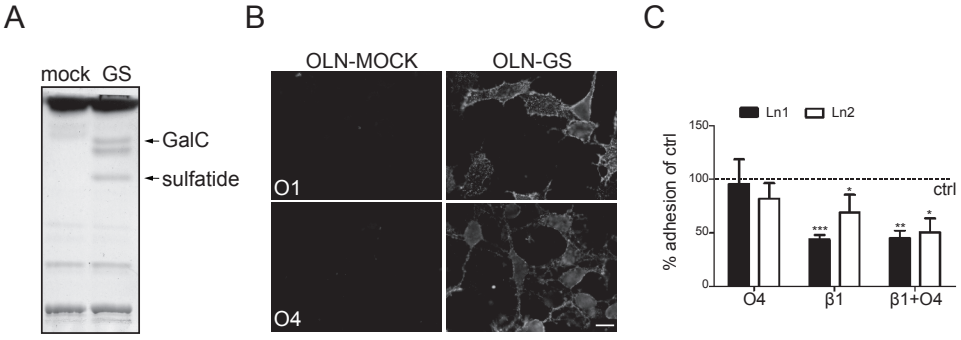


Figure 1. Sulfatide is not essential for adhesion to laminin-2

OLN-93 cells that express GalC and sulfatide on their cell surface (OLN-GS) were retrovirally generated as described in Materials and Methods. **A)** TLC analysis of cellular glycosphingolipids in OLN-MOCK (vector only) and OLN-GS cells. Note that OLN-GS cells express GalC and sulfatide, whereas OLN-MOCK do not express these galactosphingolipids at detectable levels. **B)** Cell surface staining of GalC and sulfatide in OLN-MOCK and OLN-GS cells, as visualized with O1 and O4 antibody, respectively. Scale bar is 20 µm. **C)** OLN-GS cells were pre-incubated for 30 min at 37°C with the indicated antibodies and plated on laminin-1 (Ln1) or laminin-2 (Ln2)-coated 96 wells plates. Cells were allowed to adhere for 1 hr, after which the relative % of adherent cells as compared to control (vehicle)-treated cells on the same substrate was determined using a colorimetric assay. Each bar represents the mean + SD of 3-5 independent experiments. The horizontal line represent control cells on the same substrate, which was set to 100% in each independent experiment. The absolute % adherence for control on laminin-1 was 21.5±10.5% and on laminin-2 33.9±9.9%. Statistical differences with control cells cultured on the same substrate, as assessed with a one sample t-test are indicated (* $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$).

formation of the few OLGs that do mature in MBP-positive cells, when cultured on sulfatide-pretreated laminin-2 substrate, is not perturbed (Fig. 2C). Pre-incubation of ´inert´ PLL-coated culture dishes with sulfatide neither interfered with the number of MBP-positive cells (Fig. 2A, B) nor with the percentage of MBP-positive myelin-like membranes (Fig. 2A, C). Interestingly, whereas differentiation was not affected when cells were cultured on fibronectin-coated dishes pre-incubated with either sulfatide or GalC, (Fig. 2A, B), pre-incubation with sulfatide, but not GalC, increased myelin-like membrane formation on this (re)myelination inhibiting ECM substrate (Fig. 2A, C) that is expressed upon demyelination [255,258,259].

To obtain further support for the involvement and importance of sulfatide in laminin-2-dependent myelination, we next inhibited the *de novo* biosynthesis of sulfatide in OLGs at the onset of myelin membrane formation, i.e., at the developmental stage where laminin-2 signaling becomes important *in vivo*. Thus, 3 days after initiating differentiation, immature OLGs were exposed for 4 days to 30 μM fumonisin B1 (FB1), an inhibitor of ceramide synthase, thereby inhibiting the biosynthesis of GalC, sulfatide, and (complex) gangliosides. Alternatively, cells were treated similarly with 30 μM sodium chlorate, a competitive inhibitor of sulfation, thus inhibiting sulfatide biosynthesis, but not that of GalC and gangliosides. The effectiveness of these treatments was verified by dotblot analysis, demonstrating that FB1 inhibited GalC, sulfatide and ganglioside biosynthesis, while treatment with sodium chlorate effectively reduced the amount of sulfatide (Fig. 2D), without significantly affecting the pools of GalC and GM1. As a consequence of both treatments, the percentage of MBP-positive cells grown on a laminin-2 substrate was severely diminished as compared to control (Fig. 2E). In contrast, no significant changes in the number of MBP-positive cells were seen in galactolipid-depleted cells, grown on PLL, whereas differentiation, as reflected by MBP expression, was only slightly reduced for cells grown on fibronectin (Fig. 2E). Together, these data demonstrate a requirement of sulfatide in facilitating proper OLG differentiation on laminin-2, as reflected by frustration of the developmental expression of the myelin-specific protein MBP upon blocking sulfatide binding sites on laminin-2 or upon depletion of sulfatide, but not GalC or gangliosides. Since the sulfatide binding site on laminin-2 is adjacent to the integrin binding site [97], while sulfatide and integrin α6β1 both localize to membrane microdomains [63,64,113,267], it is then tempting to suggest that laminin-2 might induce a direct association between sulfatide and integrin α6β1, providing in this manner a cooperative mode of action relevant to laminin-2-controlled myelination.

Laminin-2 induces a lateral association of integrin α6β1 with sulfatide in membrane microdomains

To determine whether laminin-2 mediates a direct association between sulfatide and integrin α6β1, we performed co-immunoprecipitation assays. To this end, OPCs were cultured on either PLL, fibronectin or laminin-2 and allowed to differentiate. Subsequently total cell lysates were prepared, immunoprecipitated with anti-integrin α6 antibody, and examined for the presence of sulfatide, using dotblot analysis. As shown in Figure 3A, sulfatide was present

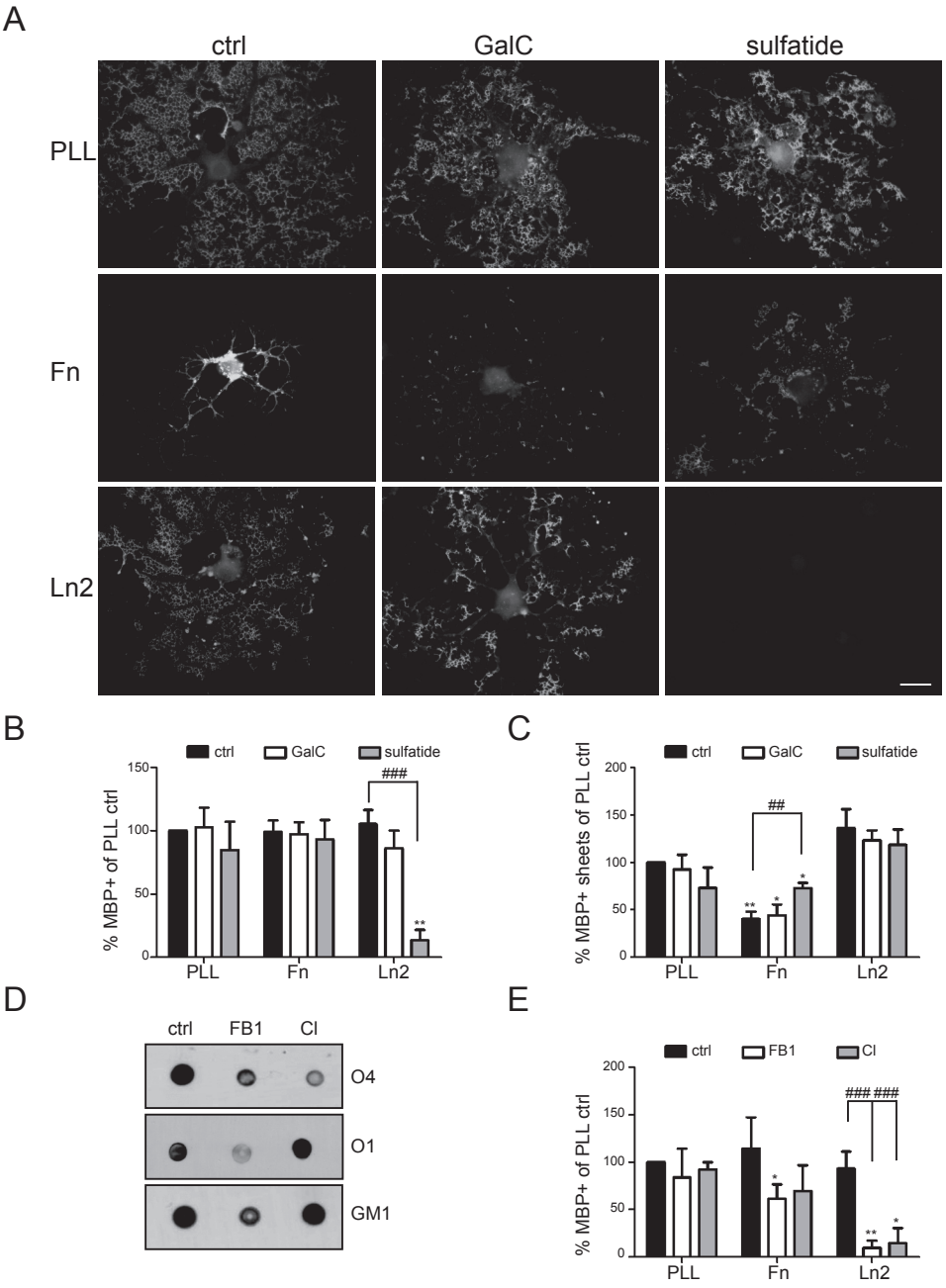
in association with integrin α6 in OLGs, cultured on a laminin-2 substrate, but not on PLL or fibronectin. A similar association was observed in OLN-GS cells. Furthermore, sulfatide did not co-immunoprecipitate with integrin αv, emphasizing the specificity of the sulfatide-integrin α6 interaction. Previously, we have shown that laminin-2, but not fibronectin, induces a redistribution of integrin α6β1, into GM1-positive membrane microdomains [63]. In addition, anti-sulfatide antibodies immunoprecipiated Fyn in OLGs [268], which associates with integrin α6β1 [269]. Therefore, we next determined whether integrin α6β1 associates with sulfatide in membrane microdomains, present at the OLG plasma membrane. To this end, membrane microdomains isolated by Optiprep density gradient centrifugation from surface biotinylated OLGs, were immunoprecipitated with anti-sulfatide antibodies. Subsequently, non-immunoprecipitates and immunoprecipitates were subjected to a re-immunoprecipitation with anti-integrin α6 antibodies. As shown in Figure 3B, immunoblot analysis using SA-HRP revealed that sulfatide associated with integrin α6 in membrane microdomains at the surface of cells cultured on laminin-2, to a lesser extent when the cells had been grown on PLL, but not on fibronectin. The association with sulfatide in membrane microdomains appears to be specific, as integrin α6 is only apparent in the non-immunoprecipitate fraction upon immunoprecipitation with anti-GalC antibodies (Fig. 3B). As integrin β4 is not expressed in OLGs, these findings reveal that laminin-2 induces a lateral association of integrin α6β1 with sulfatide in membrane microdomains.

Sulfatide is essential for laminin-2 mediated co-clustering of integrin α6 and PDGFαR

Previously, we showed that binding to laminin-2 induces co-clustering of integrin α6β1 and PDGFαR in membrane microdomains, enabling subsequent activation of signaling pathways

Figure 2. Sulfatide is essential for oligodendrocyte differentiation on laminin-2

A-C) Oligodendrocyte progenitors were cultured on poly-L-lysine (PLL), fibronectin (Fn) or laminin-2 (Ln2) that were pre-coated with vehicle (ethanol, ctrl), GalC (40 μg/ml) or sulfatide (40 μg/ml) prior to plating. **A)** Representative images of MBP immunocytochemistry of oligodendrocytes (OLGs) cultured on PLL, fibronectin or laminin-2. Scale bar is 20 μm. The number of MBP-positive cells **(B)** and the number of MBP-positive cells bearing myelin-like membranes **(C)** were assessed 7 days after initiating differentiation. Each bar represents the mean + SD of 3 independent experiments. In each experiment, the data of vehicle-treated cells cultured on PLL was set at 100%. The percentage of MBP-positive cells or % myelin-like membranes in vehicle-treated cells cultured on PLL were 42.8±6.6% and 38.1±12.9 %, respectively. Statistical differences with vehicle-treated cells cultured on PLL, as assessed with an one sample t-test are indicated (* $p< 0.05$, ** $p< 0.01$), as well as statistical differences with the respective substrate control (## $p< 0.01$, ### $p< 0.001$). Note, the significant decrease in OLG differentiation on laminin-2 upon blocking of sulfatide binding sites prior to plating. **D, E)**. Immature OLGs (3 days after initiating differentiation) cultured on PLL, fibronectin, or laminin-2 were treated for 4 days with vehicle (DMSO), fumonisin B1 (FB1, 30 μM) or chlorate (Cl, 30 μM). **D)** Dotblot analysis of sulfatide, GalC and GM1 levels on PLL, as determined with anti-sulfatide (O4) antibodies, anti-GalC (O1) antibodies and CTB, respectively. **E)** Quantitative analysis of the number of MBP-positive cells (see B and C for more details). The percentage of MBP-positive cells in vehicle-treated cells cultured on PLL was 34.1±13.7%. Note that inhibition of *de novo* sulfatide biosynthesis in OLGs that were cultured on laminin-2, inhibited MBP expression, and therefore OLG differentiation.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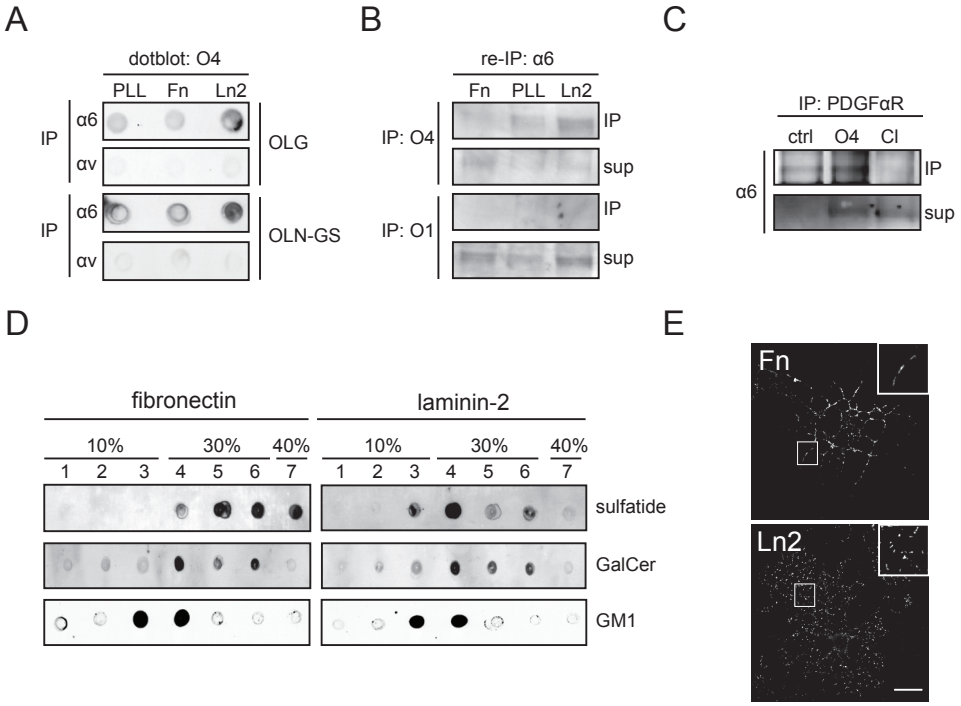

Figure 3. Sulfatide associates laterally with integrin α6β1 on laminin-2

A) Total cell lysates of primary immature oligodendrocytes (OLGs, 3 days after initiating differentiation) and OLN-GS cells cultured on poly-L-lysine (PLL), fibronectin (Fn) or laminin-2 (Ln2) were immunoprecipitated with anti-integrin α6 or anti-integrin αv antibodies and the immunoprecipitate was subjected to dotblot analysis using anti-sulfatide antibody O4. Note that sulfatide is only associated with integrin α6 in cells grown on laminin-2. **B)** Membrane microdomain fractions of surface biotinylated primary immature OLGs (3 days after initiating differentiation) cultured on PLL, fibronectin or laminin-2 were immunoprecipitated with anti-sulfatide (O4) or anti-GalC (O1) antibodies, after which the immunoprecipitate (IP) and non-immunoprecipitate (sup) were subjected to re-immunoprecipitation (re-IP) with anti-integrin α6 antibodies followed by Western blot analysis using SA-HRP. Note that sulfatide, but not GalC associates with integrin α6 in membrane microdomains at the surface of cells grown on laminin-2. **C)** Membrane microdomain fractions of mature OLGs cultured on laminin-2 that were untreated (ctrl), or 3 days after initiating differentiation treated with 30 μM sodium chlorate (Cl) or anti-sulfatide antibody (O4) were immunoprecipitated with anti-PDGFαR antibodies, after which the immunoprecipitate (IP) and non-immunoprecipitate (sup) were subjected to Western blot analysis using anti-integrin α6 antibodies. Note that both inhibition of sulfatide biosynthesis and treatment with anti-sulfatide antibodies disturbed the co-association of PDGFαR and integrin α6. **D)** Membrane microdomain and non-membrane microdomain fractions of primary OLGs cultured on fibronectin or laminin-2 were prepared using Optiprep step-density gradient centrifugation. The presence of sulfatide and GalC was analyzed by dotblotting using O4 and O1 antibodies, respectively. GM1 was used as a positive control for the presence of membrane microdomains. Representative experiment out of 2 independent experiments. Note that the relative level of sulfatide was significantly enriched in the membrane microdomain fraction on laminin-2 (fraction 3-4), whereas sulfatide was nearly absent in membrane microdomain fractions on fibronectin. **E)** Anti-sulfatide IgM antibodies (O4) were crosslinked with an anti-IgM antibody on the cell surface of mature OLGs at 37°C (7 days after initiating differentiation) cultured on fibronectin or laminin-2. Scale bar is 25 μm. Note that on laminin-2 smaller ´patches´ appeared as compared to cells grown on fibronectin.

required for OLG survival [63]. To determine whether sulfatide is essential for this co-clustering on laminin-2, we next examined whether PDGFαR and integrin α6 associate in membrane microdomains upon inhibition of sulfatide biosynthesis with sodium chlorate. Consistent with our previous results, in control cells grown on laminin-2, PDGFαR and integrin α6 co-precipitate while localizing in membrane microdomains, but not following treatment with sodium chlorate from immature OLGs onwards (Fig. 3C, IP). In fact, integrin α6, still present in membrane microdomains, appeared in the non-precipitated fraction (Fig. 3C, 'sup'). Of note, treatment with sodium chlorate seemingly did not perturb overall microdomain assembly as gradient analysis of control and treated cells revealed an identical distribution within the gradient of the membrane microdomain components, GM1 and caveolin (data not shown). Strikingly, upon treatment with the anti-sulfatide O4 antibody, which also interferes with laminin-2-sulfatide interactions, the integrin α6 present in membrane microdomains only partly associated with PDGFαR, whereas in the absence of the antibody hardly any, if at all, integrin α6 was present in the non-immunoprecipitate (Fig. 3C, 'sup', ctrl vs O4). Notably, upon treatment with the anti-sulfatide O4 antibody, the amount of membrane microdomain-associated integrin α6 increased. Given the importance of sulfatide in laminin-2 mediated myelin-like membrane formation, and the perturbing effect of fibronectin on this process, we next examined the lateral membrane distribution of sulfatide in OLGs, cultured on fibronectin or laminin-2.

Fibronectin partly excludes sulfatide from membrane microdomains

To determine the presence of sulfatide in membrane microdomains of OLGs, we performed dotblots analysis. Interestingly, upon examination of membrane fractions by gradient analysis from OLGs cultured on laminin-2, sulfatide is primarily present in the raft-like membrane microdomains (Fig. 3D, fractions 3-4), whereas the lipid is primarily recovered in the non-raft fractions in OLGs grown on fibronectin (Fig. 3D, fractions 5-7). It should be noted that at these conditions the distribution of GalC and GM1 was very similar, both lipids localizing prominently in raft fractions (Fig. 3D, fractions 3-4), irrespective of the ECM on which the cells were grown. An apparent ECM-dependent difference in lateral distribution of sulfatide in the plasma membrane of OLGs was further demonstrated by live staining with anti-sulfatide antibodies. Thus, lateral cross-linking of anti-sulfatide antibodies on the surface of living mature OLGs at 37°C, as reflected by size, revealed a striking difference in the organisation of the sulfatide-containing domains. In cells cultured on fibronectin, anti-sulfatide antibodies were localized into relatively large patches upon crosslinking, whereas smaller patches were seen on cells cultured on laminin-2 (Fig. 3E). These data thus emphasize ECM-dependent differences in lateral membrane distribution of sulfatide, suggesting a more random and diffuse distribution in cells grown on fibronectin, as opposed to a more clustered distribution in cells cultured on laminin-2. Since ECM-integrin interactions in membrane microdomains is a relevant element of the machinery regulating OLG maturation, it is thus tempting to suggest a regulatory role of sulfatide in this process.

Fibronectin prevents laminin-2-mediated morphological differentiation of oligodendrocytes

Upon CNS myelin injury, as occurs in MS and as a result of toxin-induced lesions, OPCs face a mixed laminin-fibronectin ECM environment [255,257,258,270]. Since laminin-2 promotes, whereas fibronectin frustrates myelin-like membrane formation [19,223,248], it was thus of interest to examine which ECM dominates upon their simultaneous presence. Specifically, the reduced sulfatide levels in membrane microdomains in cells cultured on a fibronectin substrate (Fig. 3D), might imply that the simultaneous presence of both fibronectin and laminin-2 could be inhibitory to proper OLG maturation. Notably, a previous study showed that laminin-2 dominated over fibronectin in the presence of a myelination-allowing PLL substrate underneath, which might have influenced the outcome [223]. In addition, we have previously shown that whereas the number of MBP-positive cells is similar on fibronectin and laminin-2 (Fig. 2B, [248]), a clear difference in morphological differentiation of OPCs is apparent, when cultured on either substrate [248]. Therefore, we examined the influence of a pure mixed fibronectin-laminin-2 environment on the morphological differentiation of OPCs. Primary OPCs, cultured on fibronectin alone, laminin-2 alone or a mixed laminin-2/fibronectin substrate, were allowed to differentiate for 7 days. Morphological differentiation, as defined by the presence of secondary and tertiary processes, was examined by immunofluorescence staining with the OLG specific cell surface marker R-mAb (Fig. 4A), which detects the myelin-typical galactosphingolipids, GalC and sulfatide [217]. OLGs were scored either as cells with a more complex morphology (Fig. 4B (1)), typically observed when exposed to laminin-2, or as cells with a moderate morphology (Fig. 4B (2)), i.e., less secondary and

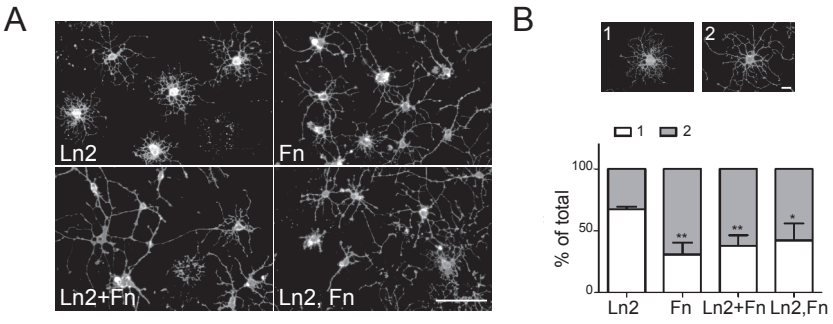

Figure 4. Fibronectin precludes laminin-2-directed signals

Morphological differentiation of primary rat oligodendrocytes (OLGs), cultured on laminin-2 (Ln2), fibronectin (Fn), a laminin-2/fibronectin mixture (Ln2+Fn) or fibronectin, layered on top of laminin-2 (Ln2, Fn). Cells were examined 7 days after initiating differentiation by immunostaining with R-mAb. **A)** Representative images of 4 independent experiments. Scale bar is 100 µm. **B)** Quantification of morphological differentiation on the different ECM substrates, as indicated above (A). The morphological appearance of at least 500 cells, in at least 4 independent experiments, were scored and identified as 'typical laminin-2' morphology (1) or as a 'typical fibronectin' morphology (2). Data are expressed as the mean + SD. Statistical differences with laminin-2 alone are indicated (one-way ANOVA followed by the Newman-Keuls posttest; * $p< 0.05$, ** $p< 0.01$). Note that when fibronectin is present, laminin-2 signals for promoting morphological differentiation are suppressed.

elongated primary processes, characteristic for OLGs cultured on fibronectin [248]. As shown in Figure 4A, morphological differentiation of OLGs is far more advanced when grown on laminin-2, as compared to fibronectin, consistent with previous findings [19,223,248]. On a mixed laminin-2/fibronectin (Ln2+Fn) substrate, the morphology of OLGs reflected more that observed for fibronectin, rather than the morphology seen on laminin-2. Similarly, when fibronectin was layered on top of a laminin-2 substrate (Ln2, Fn), a situation that could mimic that occurring in demyelinated lesions [255,257,258,270], the overall morphological appearance of the cells was similar to the morphology of fibronectin alone. Quantitative analysis confirmed the inhibitory effect of fibronectin over morphological differentiation-promoting laminin-2 signaling (Fig. 4B). Having thus determined that fibronectin suppresses laminin-2-mediated morphological differentiation *in vitro*, and given that sulfatide is partly excluded from membrane microdomains in cells grown on fibronectin (Fig. 3D), more direct evidence for a role of sulfatide in this process would be desirable. Therefore, we next examined whether anti-sulfatide antibodies could modulate ECM-mediated myelin-like membrane formation.

Anti-sulfatide antibodies prevent myelin-like membrane formation in cells cultured on laminin-2 and promote myelin-like membrane formation on fibronectin

A previous observed inhibition of OPCs by the anti-sulfatide IgM O4 antibody on the inert PLL substrate was dependent on the presence of serum components [83]. As serum components might mask pure antibody effects, we continuously exposed immature OLGs (3 days after initiating differentiation) to O4 antibody in the absence of serum components, until myelin-like membranes were formed (7 days after initiating differentiation). In case of cells grown on laminin-2, the presence of the O4 antibody caused an approximately three fold reduction in the number MBP-positive OLGs featuring myelin-like membranes, while the antibody did not affect the percentage of MBP-expressing cells (Fig. 5). Intriguingly, when anti-sulfatide O4 antibody was added to cells grown on fibronectin, an impressive increment in myelin-like membrane formation was observed (Fig. 5A, bottom row and 5C). In fact, in absolute numbers the percentage of MBP-positive myelin-like membranes on fibronectin in the presence of anti-sulfatide antibody is similar to the number obtained when cultured on laminin-2 (40.0±6.1% vs 46.5±16.5%). Moreover, the effect of the anti-sulfatide O4 antibody was specific since, relative to control, the anti-GalC IgM O1 antibody did not affect the number of MBP-positive myelin-like membranes of cells cultured on either ECM substrate (Fig. 5A, C). Importantly, the percentage of MBP-positive cells was not altered upon treatment with either antibody (Fig. 5B). Of interest, treatment with IgG R-mAb, which recognizes both GalC and sulfatide, only marginally interfered with myelin-like membrane formation (Fig. 5C), indicating that likely the IgM property of the anti-sulfatide O4 antibody is essential. Taken together, similar to preblocking ECM with sulfatide (Fig. 2), the anti-sulfatide O4 antibody interfered with myelin-like membrane formation on laminin-2 and fibronectin in a remarkably opposing manner. Given the accumulation of fibronectin (as aggregates) in MS lesions [257,259,270,271], these findings thus provide a molecular rationale for the potential of anti-sulfatide antibodies to trigger quiescent endogenous OPCs, potentially

capable of remyelinating axons in MS lesions. To examine the potential of anti-sulfatide antibodies *in vivo*, we next exploited the effect of anti-sulfatide antibodies in an *in vitro* model for (re)myelination, i.e., three-dimensional whole brain spheroid cultures.

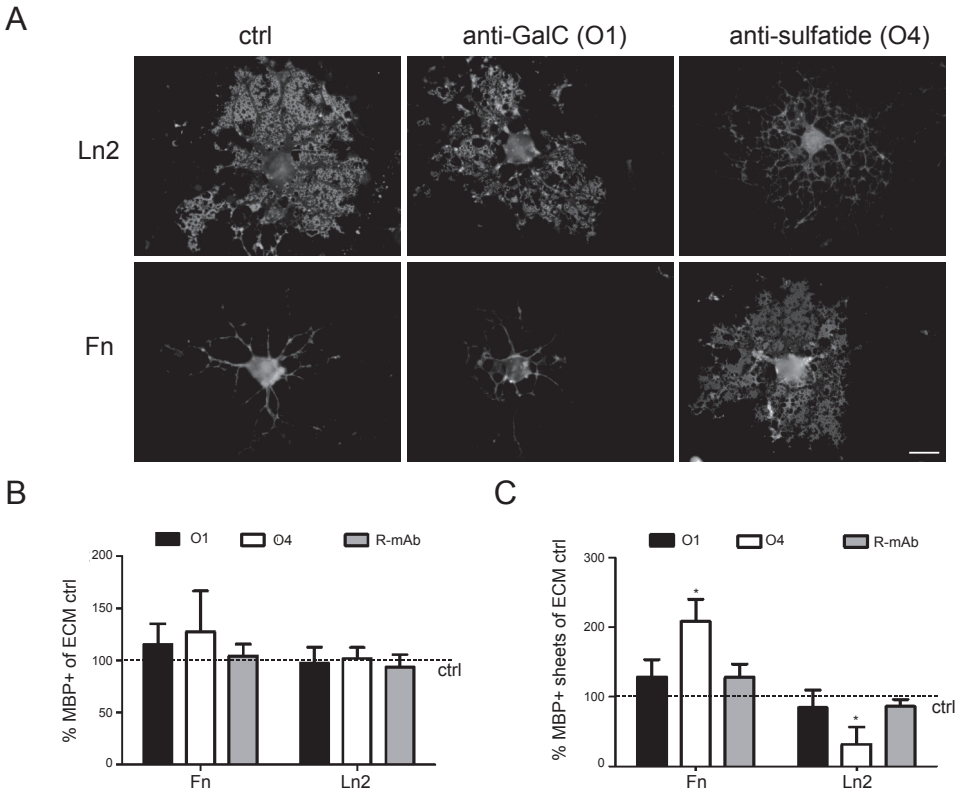

Figure 5. Anti-sulfatide antibodies modulate myelin-like formation both on laminin-2 and fibronectin

Immature oligodendrocytes (OLGs, 3 days after initiating differentiation) cultured on fibronectin (Fn) or laminin-2 (Ln2) were treated for 4 days with vehicle (PBS), anti-GalC (O1, IgM), anti-sulfatide (O4, IgM) or anti-GalC/sulfatide (R-mAb, IgG3) antibodies, and the expression of MBP examined as described in Materials and Methods. **A)** Representative images of MBP immunocytochemistry. Scale bar is 20 µm. **B, C)** Quantitative analysis of the number of MBP-positive cells (B) and the number of MBP-positive cells bearing myelin-like membranes (C). Each bar represents the mean + SD of 3-5 independent experiments. The horizontal line represent vehicle-treated cells, which was set to 100% in each experiment. The percentage of MBP-positive cells or % myelin-like membranes in vehicle-treated cells cultured on fibronectin were 42.4±17.3% and 19.2±6.8 %, and on laminin-2 49.5±9.2% and 46.5±16.5%, respectively. Statistical differences with vehicle-treated cells cultured on the respective substrate, as assessed with an one sample t-test are indicated (* $p < 0.05$). Note the perturbation of myelin-like membrane formation upon treatment with anti-sulfatide, but not anti-GalC antibodies, when cells were cultured on laminin-2, and the significant increase in myelin-like membrane formation upon addition of anti-sulfatide antibodies to OLGs grown on fibronectin.

Anti-sulfatide antibodies induce demyelination in myelinated spheroid cultures

Whole brain myelinating spheroid cultures represent an ideal *in vitro* model for monitoring (re) myelination [262]. Upon continuous rotation, single cell suspensions of whole embryonic rat brains re-aggregate into spheroids, in which myelination of axons occurs in a ´natural´ cellular and signaling environment. In addition, similarly as *in vivo*, lysolecithin-mediated demyelination can be induced in these spheroids, which is followed by partial remyelination [262]. To be able to interpret the effect of the anti-sulfatide O4 antibody on (re)myelination, we first investigated the deposition of laminin (1 and 2) and fibronectin at myelinating, demyelinating and remyelinating conditions, respectively. (Fig. 6A, B). Upon lysolecithin-induced demyelination, both the level of fibronectin and laminin increased relative to control (Fig. 6B, day 8). The level of laminin (1 and 2) was reduced at remyelination, whereas the level of fibronectin decreased only gradually (Fig. 6B, LPC lanes day 8 vs day 14). Notably, the fibronectin present in myelinated and remyelinated spheroid cultures resided predominantly at the edges of the spheroids, whereas upon lysolecithin-induced demyelination fibronectin particularly accumulated in the core of the spheroids (Fig. 6A, arrow). Importantly, qualitative and quantitative analysis of MBP expression confirmed demyelination upon exposure to lysolecithin, and subsequent occurrence of remyelination (Fig. 6D). As predicted from the data in OLG monocultures (Fig. 5), a single dose of anti-sulfatide O4, but not anti-GalC O1 antibody, at day 8, reduced the expression of MBP, to the same extent as lysolecithin in spheroid cultures (Fig. 6C, D). In contrast, addition of anti-sulfatide O4 antibody to demyelinated spheroids, i.e., in a fibronectin- and laminin-containing environment (Fig. 6A, B), hardly interfered with MBP expression as compared to control non-demyelinated cultures. However, the extent of MBP expression was significantly lower as compared to MBP expression in control remyelinating spheroids, indicating that the anti-sulfatide O4 antibody still might induce partly demyelination. Thus, given the effect of anti-sulfatide O4 antibodies in OLG monocultures (Fig. 5), we anticipate that in a demyelinated mixed fibronectin- and laminin-containing environment, the antibodies likely fulfill a dual role, i.e., they either facilitate remyelination or induce demyelination, which depends on the nature of the substrate that is encountered.

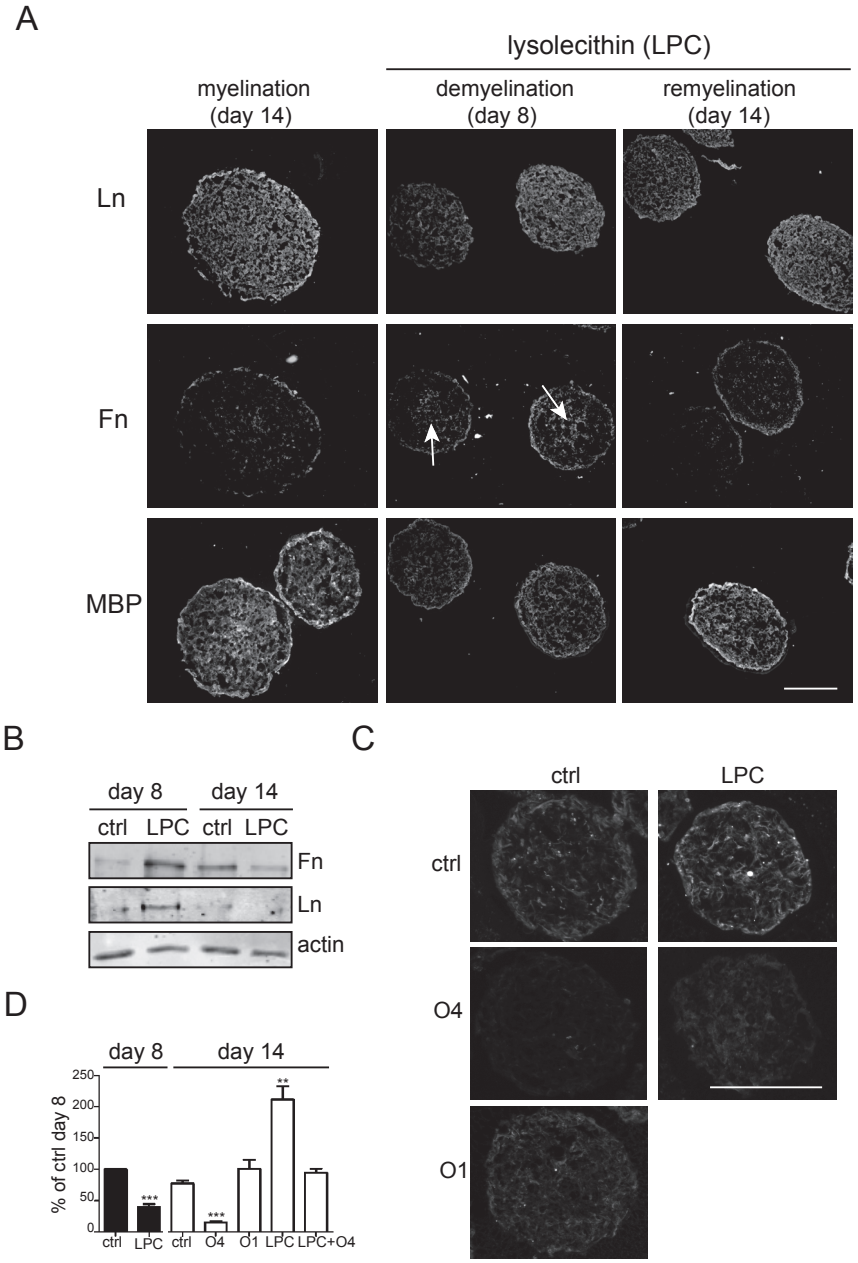
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Figure 6. Anti-sulfatide antibodies induce demyelination in myelinated spheroid cultures

A, B) 4 week old myelinated whole brain spheroid cultures (day 0) were left untreated or exposed to lysolecithin for 7 days, harvested at day 8 (demyelination) or at day 14 (remyelination), and analyzed for fibronectin (Fn) and laminin (Ln) expression by immunocytochemistry (A) and Western blot (B, 20 µg). Representative images out of 3 independent experiment are shown. Scale bar is 200 µm. Note the transient increased expression of fibronectin and laminin at demyelination. **C)** At day 8 (see A), myelinated and lysolecithin (LPC)-demyelinated spheroid cultures were cultured for another 6 days (day 14) in the absence (ctrl) or presence of a single dose of anti-sulfatide (O4) or anti-GalC (O1) antibodies added at day 8. MBP immunocytochemistry is performed as a marker for myelination, demyelination and remyelination. Representative images of MBP stainings are shown. Scale bar is 200 µm. **D)** Quantitative analysis of MBP expression of C. Each bar represents the mean + SD of 2-3 independent experiments. In each experiment, the data of control at day 8 was set at 100%. Statistical differences with control at day 8, as assessed with an one sample t-test are indicated (** $p< 0.01$, *** $p< 0.001$). Note that addition of anti-sulfatide (O4), but not anti-GalC (O1) antibodies at day 8 induced demyelination. In lysolecithin (LPC)-treated spheroid cultures the expression of MBP upon addition of anti-sulfatide antibodies (O4) was comparable to the level of control spheroids.

DISCUSSION

In the present work we have shown that sulfatide in conjunction with laminin-2, an ECM substrate that is present on developing axons, is necessary for OLG differentiation, thus governing OLG myelination. Our data support a mechanism in which laminin-2 engagement facilitates lateral interactions between integrin α6β1 and sulfatide in membrane microdomains, which likely provide a platform within the OLG plasma membrane that enables interactions between PDGFαR and integrin α6. This process is precluded by fibronectin, presumably because this ECM molecule prevents inclusion of sulfatide in raft-like membrane microdomains. Interestingly, in the presence of fibronectin, addition of anti-sulfatide IgM antibodies at the onset of myelin formation stimulated myelin-like membrane formation, whereas myelin-like membrane formation was precluded in the presence of laminin-2 at otherwise similar conditions. Thus, given the accumulation of remyelination-inhibiting aggregates of fibronectin in MS lesions [259], targeted delivery of anti-sulfatide antibodies, modified in a manner that they are not affecting laminin-2-OLG interactions at lesions sites, might be a promising starting point to induce/re-establish endogenous remyelination in MS.

Sulfatide has been proposed to act as a sensor and/or transmitter of environmental information and appears to function as a negative regulator of terminal OLG differentiation [83]. This hypothesis was based on experiments that did not take into account effects of sulfatide-ECM interactions. Here we show that the biosynthesis of sulfated glycosphingolipids in conjunction with laminin-2 was essential for the generation of MBP-positive OLGs. In addition, disruption of laminin-2-sulfatide interactions by anti-sulfatide antibodies or pre-blocking sulfatide-binding sites on laminin-2, impeded OLG myelin-like membrane formation, underscoring the importance of laminin-2-sulfatide interactions in myelination. In agreement with previous studies, sulfatide was not required for OLG differentiation on inert PLL [82,272]. Also, sulfatide was not essential for OLG maturation in the presence of fibronectin. Adhesion assays demonstrated that integrin α6β1 was a more important laminin receptor in adhesion as compared to sulfatide, and sulfatide does likely not prevent integrin binding [97,98]. Therefore, given that activation of integrin α6β1 initiates the translation of MBP [17], a role for sulfatide as a response modifier of integrin signaling is more likely.

We have previously shown that myelination-promoting integrin-growth factor interactions in OLGs originate from a laminin-2-mediated merging of initially separated, membrane signalling microdomains [63,64,112,113]. We hypothesized a role of sulfatide, being part of these domains, in this process. Indeed, as shown in the present study, the laminin-2 mediated interaction between microdomain localized PDGFαR and integrin α6 was precluded upon inhibition of sulfatide biosynthesis or treatment with anti-sulfatide O4 antibody. Moreover, laminin-2 induced a lateral raft-associated interaction of sulfatide with integrin α6β1 at the surface of OLGs. The distinct interactions between laminin-2 with either integrin α6β1 and sulfatide on the one hand, and sulfatide and integrin α6β1 on the other, lead us to propose that a laminin-integrin-sulfatide ternary complex might establish a signaling platform in

membrane microdomains that controls the timing of OLG maturation. Furthermore, since sulfatide expression precedes the establishment of axonal contact-dependent signals, its interaction with axonal laminin-2 might thus act as a focal point in OLG-axon adhesion that controls the asynchronous localized differentiation of OLGs, thereby allowing initiation of myelination on axonal demand. In support of a role of a sulfatide-laminin-2 interaction in the timing of MBP expression is the observation of an accelerated differentiation of OLGs in sulfatide -/- mice [82] and a delay in laminin-2 deficient mice [273]. A sulfatide-laminin-2 mediated initiation of myelination has also been reported in cultured Schwann cells [274]. However, an alternative sulfatide-independent route also exists as MBP expression and myelination proceed in sulfatide -/- mice [82]. Of interest, adult sulfatide-deficient mice maintain increased OLG numbers as a result of increased proliferation [275]. It is tempting to suggest that this increased proliferation is a result of disturbed PDGFαR-integrin α6β1 interactions in membrane microdomains, leaving PDGFαR available for proliferative signaling [64,112], as also observed for cells cultured on fibronectin [269].

Interestingly, whereas sulfatide localizes in membrane microdomains of cells grown on laminin-2, the lipid was hardly sequestered to such domains in cells cultured on the (re)-myelination-inhibiting ECM substrate fibronectin. Laminin-2 versus fibronectin-dependent differences in the lateral distribution of sulfatide is further reflected by evident differences in the size of crosslinked sulfatide-antibody patches. The specificity of the distinct ECM-dependent lateral membrane distribution of sulfatide was indicated by the fact that both GalC and the ganglioside GM1 were present in raft fractions of the cells, irrespective of the ECM substrates, which is also the case for PDGFαR, and integrin α6β1 [112,113]. It is thus intriguing why sulfatide, and not its immediate precursor GalC, is partly excluded from membrane microdomains in the presence of fibronectin, particularly since this ECM protein displays negligible sulfatide-binding activity [95]. However, GalC and sulfatide are distinctly localized, GalC being present in compact myelin and sulfatide in paranodal regions [27]. In addition, detergent extractions *in situ* revealed that GalC and sulfatide are present in different membrane microdomains in OLGs, i.e., GalC-positive microdomains are largely confined to myelin-like membranes, whereas sulfatide containing microdomains are restricted to cell body and primary processes (our unpublished observations) [27].

Of particular interest for MS pathology is our observation that inhibition of morphological OLG differentiation by fibronectin dominated over the promoting potential of laminin-2. These findings may suggest that fibronectin, as present in MS lesions, specifically prevents sulfatide segregation to membrane microdomains, thereby precluding proper laminin-2-sulfatide-integrin-growth factor interactions, and hampering proper axonal contact and paranode formation [73–76], where sulfatide largely localizes [27]. In this context, the application of anti-sulfatide antibodies to induce endogenous remyelination in MS lesions is of interest. Thus, the anti-sulfatide O4 antibody stimulated myelin-like membrane formation on fibronectin, which in the absence of anti-sulfatide antibodies prevents myelination. This beneficial effect likely relates to the pentameric IgM property of the antibody, as R-mAb, i.e., an anti-sulfatide/anti-GalC IgG3 antibody, hardly modulated myelin-like membrane formation on fibronectin.

Presumably, pentameric anti-sulfatide IgM antibodies may trigger a lateral redistribution of membrane microdomains by virtue of its multivalency [276], thereby facilitating lateral interactions that activate downstream signalling, required for OLG maturation. In support of this notion, our preliminary findings show that the membrane microdomain association of NF155, which becomes reduced in cells cultured on fibronectin [213], is promoted upon exposure to anti-sulfatide O4 antibodies. Moreover, addition of O4 to cells that were grown on fibronectin resulted in tyrosine phosphorylation of a protein of an apparent molecular weight of approximately 50-55 kDa, which is not the case upon O4 exposure of cells cultured on laminin-2. The significance of these preliminary findings, which are of clear potential interest, is currently investigated in our laboratory.

Our data also demonstrate that when added to immature OLGs, anti-sulfatide antibodies inhibit myelin-like membrane formation in cells cultured on laminin-2, implying an occurrence of the undesirable potential of the antibody to induce demyelination in healthy white matter. Indeed, our findings in myelinated spheroid cultures showed that a single dose of the anti-sulfatide O4 antibody, but not anti-GalC O1 antibody, significantly reduced MBP expression. Hence, the present work identified sulfatide as an important regulator of ECM-dependent OLG myelination, and also emphasizes the need for the development of anti-sulfatide antibodies, capable of inducing ECM-independent remyelination. Of interest, a natural human IgM antibody, rHIgM22, increases remyelination *in vivo. In vitro* and when cultured on fibronectin, this antibody promotes OPC survival, prolongs OPC proliferation in a PDGF-dependent manner [277], and inhibits differentiation, likely via a PDGFαR-integrin $\alpha v\beta 3$-Lyn signaling complex [278]. Given our present findings, it would be of interest to determine the effect of this antibody on laminin-2.

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