Chapter 4

Myelin elicits different responses in regionally-distinct oligodendrocyte progenitor cells and in microglia and macrophages *in vitro*

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(submitted for publication)

Abstract

Multiple sclerosis (MS) is a chronic disease of the central nervous system hallmarked by the formation of demyelinated lesions. For remyelination to occur, remyelination-inhibiting myelin debris has to be removed by microglia and infiltrated peripheral macrophages after which oligodendrocyte progenitor cells (OPCs) differentiate into mature myelinating oligodendrocytes. Remyelination is faster and more extensive in grey matter (GM) lesions than in white matter (WM) lesions. Here, we compared the effect of rat and MS GM and WM myelin on OPC proliferation and differentiation, either directly or indirectly by altering the activation of bone marrow-derived macrophages (BMDMs) and microglia. We reveal that although the composition of rat and MS myelin differ between GM and WM, GM and WM myelin induced a similar effect regarding OPC behavior and microglia and BMDM activation. Remarkably, rat myelin coatings suppressed wmOPC proliferation more potently than gmOPC proliferation, whereas MS myelin coatings only inhibited gmOPC differentiation. In addition, exposure to rat myelin differently altered the activation status of pre-polarized alternatively activated BMDMs and microglia, and slightly increased the mRNA expression of the pro-OPC differentiation factors galectin-3 in BMDMs and LIF in microglia. This myelin-induced phenotype shift had hardly an effect on OPC differentiation, indicating that the detrimental effect of myelin is *in vitro* primarily directly on OPCs, rather than indirect via secreted factors from BMDMs and microglia. Hence, our findings indicate that cells native to the demyelinated area are likely more important determinants of remyelination efficiency than regional differences in myelin composition.

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS). Accumulation of clinical disability in MS patients reflects an increasing burden of axonal injury and loss, caused by repeated episodes of inflammatory demyelination and failure of reparative processes, such as remyelination[4]. Remyelination is a natural regenerative response to demyelination, and is characterized by the mobilization of oligodendrocyte progenitor cells (OPCs) and the generation of mature oligodendrocytes (OLGs) that enwrap their myelin sheaths around denuded axons[3]. Notably, OPCs are present in approximately 70% of MS lesions that reside in the white matter (WM)[15,17,18]. In fact, remyelination is more profound in grey matter (GM) MS lesions than in WM MS lesions[26,27]. Similar findings are observed in an experimental model in which demyelinated axons in the cortex (GM area) are remyelinated more efficiently than demyelinated axons in the corpus callosum (WM area)[24,25]. This suggests a more supportive micro-environment towards remyelination in GM than WM, intrinsic differences in remyelination capacities of OPCs in GM and WM, or a combination of both.

A well-established immediate inhibitory factor for remyelination following demyelination is myelin debris[51]. Myelin debris-associated proteins, including Eprhin3B, but not lipids, directly prevent OPC differentiation by modulating Fyn, RhoA and PKC-mediated signaling[427,428]. Given that myelin is less abundant in GM than in WM, quantitative differences in myelin debris load in lesions can account for differences in remyelination in GM and WM. Alternatively, regional heterogeneity in myelin composition can contribute to regional differences in remyelination efficiency. While a comprehensive comparative analysis of the composition of GM and WM myelin is still lacking, homogenates from human CNS WM are relatively enriched in lipids, while CNS GM homogenates are more abundant in proteins[195,196]. Furthermore, myelin components including fatty acids ethanolamine, lecithin and serine glycerophosphatides are more abundant in GM homogenates, while cholesterol, cerebroside and sulfatide are more abundant in WM homogenates[195,196]. Moreover, the concentration of myelin proteins and the enzymatic activity of 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) differ more than is accountable to the regional difference in myelin content[197]. In MS, during periods of disease activity,

sphingolipids, including galactosylceramide (GalCer) and sterols are reduced and the phospholipid content is increased in GM and WM homogenates, which at disease inactivity is normalized in GM, but not WM[429]. This indicates that additional MS-induced regional heterogeneity in myelin composition may contribute to differences in the formation and resolution of GM and WM MS lesions.

Ample evidence indicates that peripheral-derived macrophages and resident microglia control remyelination. For instance, macrophages and microglia avidly clear myelin debris by phagocytosis[430–432], thereby relieving the lesion microenvironment of inhibitory myelin debris. In addition, macrophages and microglia are reported to release pro-regenerative factors that promote remyelination[52] (reviewed in [49]). We and others further demonstrated that ingestion of myelin by phagocytes reshapes the phenotype of macrophages and microglia to one that is typically associated with CNS repair and is accompanied by reduced expression of inflammatory mediators[53,54] (reviewed in [433]). Activation of lipid signaling pathways, such as liver-X-receptors and peroxisome proliferator-activated receptors, by myelin-derived lipids underlies the less-inflammatory, reparative phenotype of myelin-containing macrophages[55,56]. However, prolonged exposure to myelin promoted the induction of an inflammatory, repair-suppressive phenotype. This disease-promoting phenotype shift relied on elevated stearoyl-CoA desaturase-1 activity, the rate-limiting enzyme in the formation of monounsaturated fatty acids from saturated fatty acids[434]. These findings stress the role of lipid metabolism in directing the pro-regenerative phenotype of macrophages and microglia in MS lesions, and the importance of efficient and timely myelin debris clearance.

Given the essential role of myelin debris in controlling OPC maturation and macrophage and microglia function, we postulated that differences in protein and lipid composition between GM and WM myelin may contribute to regional differences in remyelination efficiency. Therefore, we investigated whether OPC proliferation and differentiation are distinctly modulated by rat and MS GM and WM myelin *in vitro*, either in a direct or in an indirect manner by differentially interfering with bone marrow-derived macrophage (BMDM) and microglia polarization and/or modifying their pro-regenerative properties. Our findings revealed that GM and

WM myelin impair OPC proliferation and/or differentiation and affect BMDMs and microglia to a similar extent. However, rat and MS myelin elicit distinct responses in gmOPCs and wmOPCs, as well as in BMDMs and microglia, which may contribute more to regional differences in remyelination efficiency than regional differences in myelin composition.

Results

Rat and MS GM and WM myelin differ in proportions of lipids and proteins

Although studies on CNS GM and WM homogenates indicate that myelin has a different lipid and protein composition[195,196], direct evidence on purified GM and WM myelin is still lacking. Therefore, we first compared the proportion of the myelin-typical lipids GM1, GalCer and sulfatide, and of myelin-specific proteins CNP, myelin basic protein (MBP) and proteolipid protein (PLP) in myelin that was isolated from pooled cortex or corpus callosum of adult male rats. Dot blot analysis revealed that the proportion of GalCer to sulfatide was higher in rat GM myelin than in WM myelin (Fig. 1a, 1.26 *vs* 0.72 respectively). The proportion of ganglioside GM1 to sulfatide was also higher in rat GM myelin (Fig. 1a, 1.74 *vs* 1.09 respectively). Western blot analysis demonstrated also differences in the proportions of myelin-specific proteins. More specifically, both the MBP to PLP and CNP to PLP ratio were lower in GM rat myelin than in WM myelin (Fig. 1b, MBP/PLP 0.31 *vs* 0.67; CNP/PLP 0.10 *vs* 0.59, respectively), indicating that PLP was relatively more abundant in rat GM myelin. We next performed similar comparative analysis between GM and WM myelin obtained from a MS patient. Notably, the proteome and lipidome of rodent and human myelin are 80% similar[435]. Similar to rat myelin, the proportion of GalCer to sulfatide was higher in MS GM myelin than WM myelin (Fig. 1c, 1.29 *vs* 0.90, respectively). Contradictory to rat myelin, the proportion of GM1 to sulfatide was lower in MS GM myelin than in MS WM myelin (Fig. 1c, 1.35 and 3.36 respectively). A lipidomic approach was taken to further define the lipidome of MS GM and WM myelin. Within different phospholipid classes, only minor differences between MS GM and WM myelin were observed (Fig. S1). In general, phosphatidylcholine (PC) and phosphatidylserine (PS) species were more prominently present in MS GM myelin than in MS WM myelin (Fig. S1a,b), while sphingomyelin (SM) was more abundant in MS WM myelin than in MS GM myelin (Fig. S1c). Also, contrary to rat myelin, the proportions of MBP and CNP to PLP were both higher in MS GM myelin than WM myelin (Fig. 1d, MBP/PLP 1.52 *vs* 0.69; CNP/PLP 0.55 *vs* 0.14, respectively). Hence, although in a different manner, both rat and MS GM and WM myelin differ in their proportions of highly abundant myelin lipids and proteins. As OPCs directly encounter myelin debris in demyelinated areas, we next assessed whether rat GM

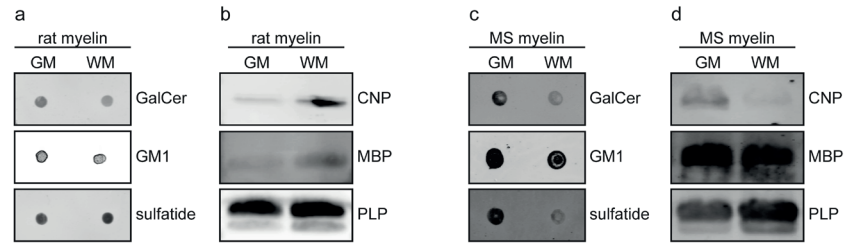

Fig. 1. Rat and MS GM and WM myelin differ in their proportions of lipids and proteins. Myelin was isolated from pooled cortex (grey matter; GM) or corpus callosum (white matter; WM) from 10 adult male rats or from postmortem GM and WM brain tissue of a multiple sclerosis (MS) patient. GM and WM myelin were subjected to dot blot analysis for the quantification of myelin-typical lipids galactosylceramide (GalCer), GM1, and sulfatide (**a**,**c**), and to Western blot analysis for the quantification of myelin-specific proteins CNP, MBP and PLP (**b**,**d**). Total protein used was 5 µg (GM1), 10 µg (CNP, PLP, MBP) or 25 µg (GalCer, sulfatide) (**a-d**). Note that the ratio of GalCer and GM1 to sulfatide is lower in rat WM myelin compared to GM myelin (**a**, GalCer/sulfatide 0.77 *vs* 1.26, GM1/sulfatide 1.74 *vs* 1.09), while the ratio of CNP and MBP to PLP is much lower in GM myelin compared to WM myelin (**b**, CNP/PLP 0.10 *vs* 0.59, MBP/PLP 0.32 *vs* 0.67). The ratio of GalCer to sulfatide is also lower in MS WM myelin compared to MS GM myelin (**c**, GalCer/sulfatide 0.87 *vs* 1.29) while, in contrast to rat myelin, the ratio of CNP and MBP to PLP are higher in GM myelin compared to WM myelin (**d**, CNP/PLP 0.55 *vs* 0.14, MBP/PLP 1.52 *vs* 0.69).

and WM myelin differently affect OPC proliferation and differentiation, which are important parameters for successful OPC-based remyelination[3].

Rat WM myelin coatings impair wmOPC proliferation more than rat GM myelin coatings impair gmOPC proliferation

Previous findings demonstrated that wmOPCs distinctly respond to environmental cues than gmOPCs[44,144,148] (reviewed in [436]). Therefore, we examined both the response of wmOPCs and gmOPCs to rat GM and WM myelin. For this purpose, gmOPCs and wmOPCs were cultured on an inert PLL-coating or on an additional coating of rat GM or WM myelin. Upon 48-hours exposure to the mitogens PDGF-AA and FGF-2, the percentage of A2B5-positive cells expressing the proliferation marker Ki67 was significantly reduced when gmOPCs and wmOPCs were grown on either a coating of rat GM myelin or rat WM myelin compared to gmOPCs and wmOPCs that were cultured on PLL (Fig. 2a,b, gmOPCs + GM myelin $p=0.022$; wmOPCs + GM myelin $p<0.001$; gmOPCs + WM myelin $p<0.001$; wmOPCs + WM myelin $p<0.001$). However,

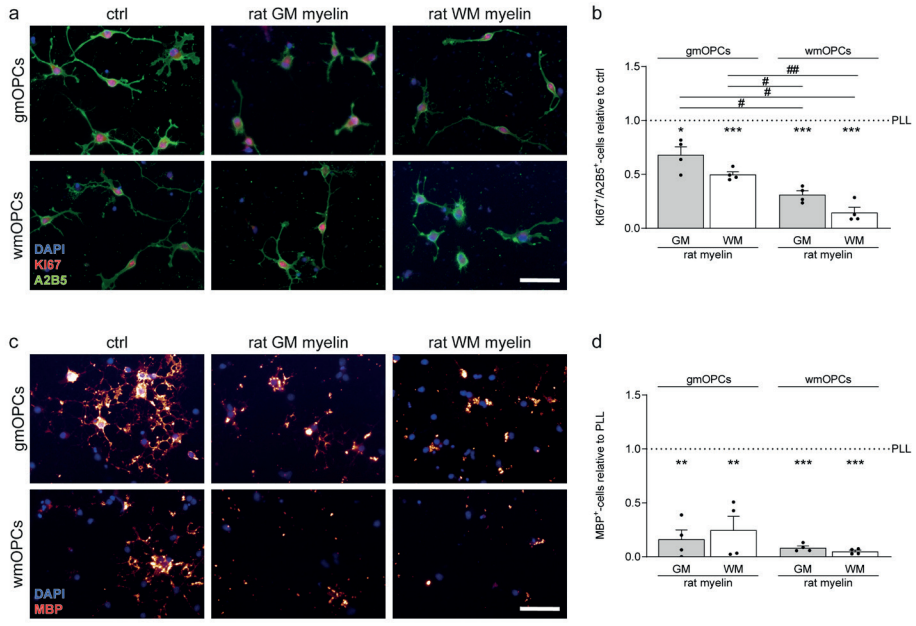

Fig. 2. Rat WM myelin coatings impair wmOPCs more than rat GM myelin coatings impair gmOPCs. Oligodendrocyte progenitor cells (OPCs) isolated from the cortex (gmOPCs) and non-cortex (wmOPCs) of neonatal rat forebrains were cultured on inert poly-L-lysine (PLL) or a rat GM or WM myelin coating (40µg/cm^2). Rat myelin was isolated from pooled cortex (grey matter; GM, grey bars) and corpus callosum (white matter; WM, white bars) of 10 adult male rat brains. OPCs were cultured for 48 hours in the presence of PDGF-AA and FGF-2 (**a**-**d**), followed by differentiation for six days (**c**,**d**). (**a**) Proliferation is determined by immunocytochemistry for the proliferation marker Ki67 (red). OPCs are stained by the OPC marker A2B5 (green). Nuclei are visualized with DAPI (blue). Representative images of four independent experiments are shown. (**b**) Quantitative analysis of the percentage of Ki67-positive of total A2B5-positive cells. (**c**) Differentiation was determined by immunocytochemistry for the differentiation marker MBP (red). Nuclei are visualized with DAPI (blue). Representative images of four independent experiments are shown. (**d**) Quantitative analysis of the percentage of MBP-positive cells of DAPI-stained cells. Bars represent relative means to PLL-coated control, which was set to 1 in each independent experiment (horizontal line). Error bars show standard error of the mean (SEM). The percentage Ki67-positive cells of A2B5-positive cells in the PLL-coated control was 65.2±4.7% for gmOPCs and 53.0±4.1% for wmOPCs (n=4, at least 150 cells analyzed per independent experiment). The percentage MBP-positive cells in the PLL-coated control was 29.3±5.0% for gmOPCs and 35.8±6.1% for wmOPCs (n=4, at least 150 cells analyzed per independent experiment). Statistical analyses were performed using a one-sample t-test (*p<0.05, **p<0.01, ***p<0.001) to test for differences between GM and WM treatment of gmOPCs or wmOPCs and their respective PLL-coated control, and a one-way ANOVA with Tukey post-test was used to compare between all treatment groups (# p< 0.05). Scale bar is 50 µm.

both a coating of GM myelin and WM myelin reduced wmOPC proliferation more prominently than gmOPCs (Fig. 2a,b gmOPCs + GM myelin vs wmOPCs + GM myelin p=0.037; gmOPCs + GM myelin vs wmOPCs + WM myelin p=0.012; gmOPCs + WM myelin vs wmOPCs + GM myelin p=0.021; gmOPCs + WM myelin vs wmOPCs + WM myelin p=0.003). Rat GM and WM myelin coatings both markedly decreased the percentage of gmOPCs and wmOPCs positive for the differentiation marker MBP compared to cells that were cultured on PLL (Fig. 2c,d, gmOPCs GM myelin p=0.002; gmOPCs WM myelin p=<0.001; wmOPCs GM myelin p=0.001; wmOPCs WM myelin p=<0.001). No significant differences between WM and GM myelin or gmOPCs and wmOPCs were found. Given that gmOPCs do not encounter WM myelin and wmOPCs do not encounter GM myelin *in vivo*, these findings indicate that while both rat GM and WM myelin coatings impaired OPC proliferation and differentiation, wmOPC proliferation was more affected by a rat WM myelin coating than gmOPC proliferation by a rat GM myelin coating. A previous study demonstrated that human CNS myelin coatings, similar to rat CNS myelin coatings inhibit rat OPC differentiation[437]. Therefore, we next addressed whether MS GM and WM myelin distinctly modulate OPC proliferation and differentiation.

MS myelin coatings impair gmOPC, but not wmOPC differentiation

In contrast to rat myelin coatings, coatings of MS GM and WM myelin hardly affected the percentage of Ki67-positive of A2B5-positive gmOPCs and wmOPCs (Fig. 3a,b), indicating that MS myelin hardly affected OPC proliferation. A coating of MS GM myelin significantly reduced the percentage of MBP-positive gmOLGs, but not wmOLGs, by approx. 50% (Fig. 3c,d, p<0.001). MS WM myelin coatings reproducibly, but not significantly, reduced gmOPC, but not wmOPC differentiation (Fig. 3c,d, p=0.065). Notably, MS myelin affected OPC differentiation less potently than rat myelin (Fig. 2c,d). Hence, MS GM and WM myelin provoked similar responses to OPCs, which were distinct from responses elicited by rat GM and WM myelin coatings. While no effect of MS myelin coatings on OPC proliferation was observed, MS myelin coatings reduced gmOPC, but not wmOPC differentiation. Upon demyelinating injury, peripheral-derived macrophages and resident microglia clear remyelination-inhibiting myelin debris, which alters their activation[53–56,438–444] (reviewed in [433]). Therefore, in addition to a direct effect on OPC behavior, rat and MS GM and WM

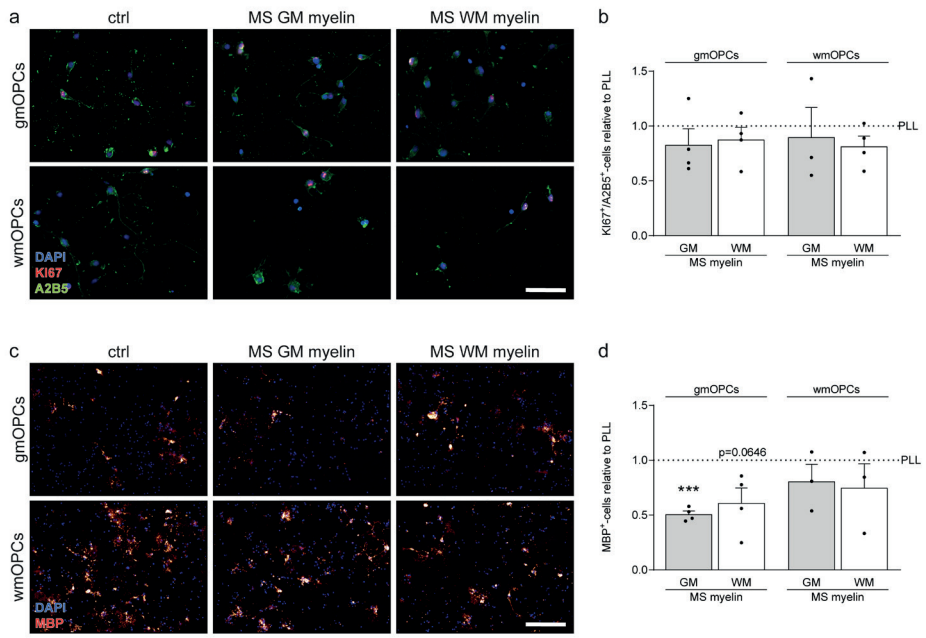

Fig. 3. MS myelin coatings impair gmOPC, but not wmOPC differentiation**.** Oligodendrocyte progenitor cells (OPCs) isolated from the cortex (gmOPCs) and non-cortex (wmOPCs) of neonatal rat forebrains were cultured on poly-L-lysine (PLL) or a MS myelin coating (40µg/cm^2). MS myelin was isolated from grey matter (GM, grey bars) and white matter (WM, white bars) human postmortem brain tissue of a MS patient. OPCs were cultured for 48 hours in the presence of PDGF-AA and FGF-2 (**a-d**), followed by differentiation for six days (**c**,**d**). (**a**) Proliferation is determined by immunocytochemistry for the proliferation marker Ki67 (red). OPCs are stained by the OPC marker A2B5 (green). Nuclei are visualized with DAPI (blue). Representative images of three to four independent experiments are shown. (**b**) Quantitative analysis of the percentage of Ki67-positive of total A2B5-positive cells. (**c**) Differentiation was determined by immunocytochemistry for the differentiation marker MBP (red). Nuclei are visualized with DAPI (blue). Representative images of three to four independent experiments are shown. (**d**) Quantitative analysis of the percentage of MBP-positive cells of DAPI-stained cells. Bars represent relative means to PLL-coated control, which was set to 1 in each independent experiment (horizontal line). Error bars show standard error of the mean (SEM). The percentage Ki67-positive cells of A2B5-positive cells in the PLL-coated control was 42.5±9.8% for gmOPCs and 41.3±19.0 % for wmOPCs (n=3-4, at least 150 cells analyzed per independent experiment). The percentage MBP-positive cells in the PLL-coated was 21.9±3.1% for gmOPCs and 32.2±18.2% for wmOPCs (n=3-4, at least 150 cells analyzed per independent experiment). Statistical analyses were performed using a one-sample t-test (*p<0.05, **p<0.01, ***p<0.001) to test for differences between GM and WM treatment of gmOPCs and wmOPCs and their respective PLL-coated control, and a one-way ANOVA with Tukey post-test was used to compare between all treatment groups (not significant). Scale bar is 50 µm (**a**) or 200 µm (**c**).

myelin may indirectly affect OPC differentiation by modulating the phenotype of infiltrating macrophages and/or resident microglia, which was examined next.

Exposure to rat and MS myelin interferes with pre-polarized M2-like bone marrow-derived macrophage and microglia activation states

Previous findings indicate that myelin-internalization markedly impacts the inflammatory and reparative features of macrophages and microglia[54–56,439,440,442]. To examine whether GM and WM myelin differently modulate their phenotype when polarizing, rat BMDMs and microglia were pre-polarized towards (IFNγ+LPS-activated) M1-like and (IL4-activated) M2-like cells or not pre-polarized (M0) for 24 hours before the cells were additionally exposed to myelin (Fig. 4a). Previously, we have demonstrated that at these conditions both microglia and BMDMs polarize to the M1-like or M2-like phenotype at the mRNA and protein level, 24 and 48 hours after induction, respectively[328,445]. Alternatively activated M2-like BMDMs and microglia still had increased mRNA expression levels of the M2 profile marker *Arg1* 24 hours after a 48-hour exposure to IL4 (Fig. 4b,c, BMDMs p=0.013; microglia p=0.038). The mRNA levels of M1 profile markers *Ccl5* and *Nos2* were, except for one outlier, hardly altered in classically activated M1-like BMDMs 24 hours after a 48-hour exposure to IFNγ+LPS (Fig. 4b). Classically activated microglia had reproducibly, but not significantly, higher transcript levels of the M1 profile markers *Ccl5* and *Nos2* 24 hours after the 48-hours induction.

When exposing pre-polarized BMDMs for 24 hours to rat GM and WM myelin (Fig. 4a), transcript levels of *Nos2* were reduced in M0 and pre-polarized M2-like BMDMs 24 hours after myelin withdrawal, but not in pre-polarized M1-like BMDMs compared to BMDMs that were not exposed to myelin (Fig. 4d, M0 + GM myelin p=0.031; M1+ GM myelin p=0.503; M2 + GM myelin p=0.002; Fig. 4e, M0 + WM myelin p=0.007; M1 + WM myelin p=0.506; M2 + WM myelin p=0.001). Reduced *Nos2* expression was not observed in pre-polarized BMDMs that were exposed to MS GM and WM myelin (Fig. 4f,g), nor after uptake of rat or MS GM and WM myelin by pre-polarized microglia (Fig. 4h,i). In fact, *Nos2* transcripts were significantly increased when pre-polarized M2-like microglia were exposed to rat GM myelin (Fig. 4h, p=0.048) and reproducibly increased in rat WM myelin-treated pre-polarized M2-like microglia (Fig. 4i). No changes in the expression of *Ccl5* were observed when pre-polarized microglia were treated with rat GM and WM myelin. In contrast, *Ccl5* transcripts were significantly increased when pre-polarized M2-like BMDMs were exposed to MS GM myelin (Fig.

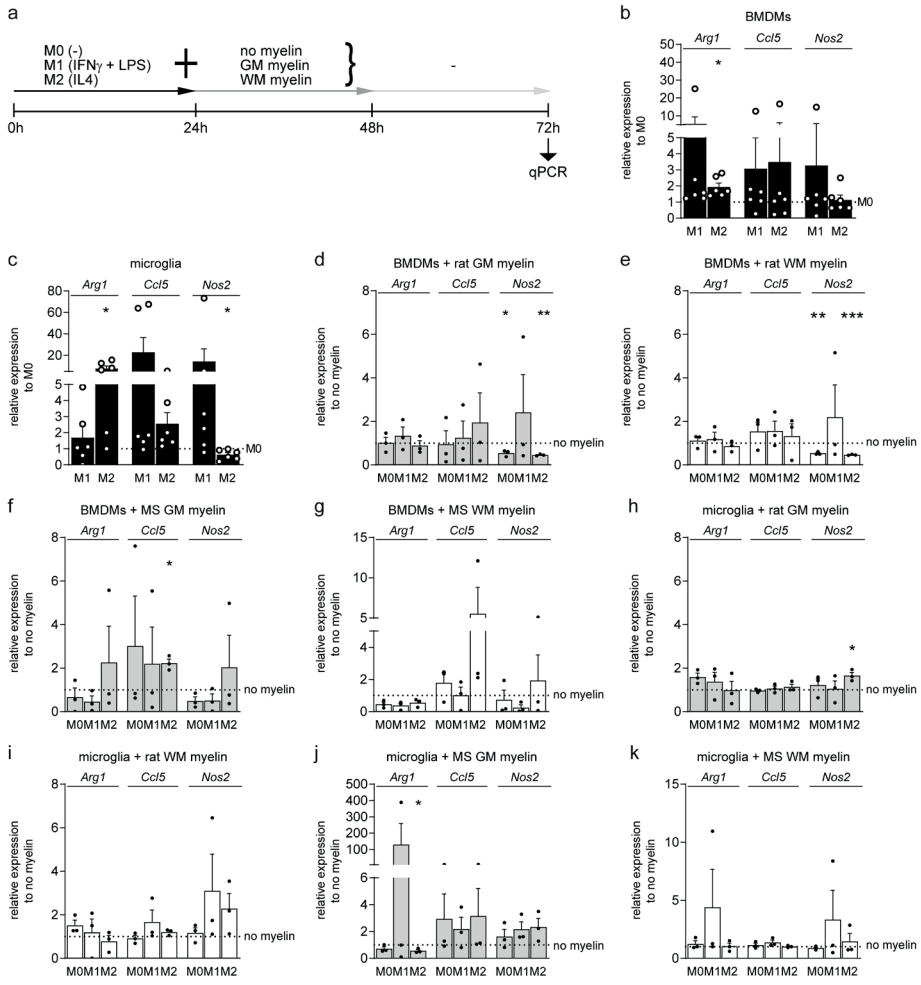

Fig. 4. Exposure to rat or MS myelin interferes with pre-polarized M2-like bone marrow-derived macrophage and microglia activation states. Bone marrow-derived macrophages (BMDMs) and resident microglia isolated from neonate rats were either kept naïve (M0), pre-polarized to pro-inflammatory activated cells by exposure to interferon-gamma (IFNγ) and lipopolysaccharide (LPS) (M1-like), or pre-polarized to alternatively activated cells by exposure to interleukin (IL)4 (M2-like). After 24 hours BMDMs and microglia were additionally treated with 10 µg/ml grey matter (GM) or white matter (WM) myelin debris for 24 hours. Cells were cultured for another 24 hours before analysis. Rat myelin was isolated from cortex (GM, grey bars) and corpus callosum (WM, white bars), and MS myelin from GM (grey bars) and WM (white bars) postmortem brain tissue of a MS patient. Cells were subjected to RT-qPCR analysis of pro-inflammatory activation profile markers *Ccl5* and *Nos2*, and alternative activation profile marker *Arg1*. (**a**) Schematic representation of the experimental set-up. (**b**,**c**) mRNA expression levels of activation profile markers in BMDMs (**b**) and microglia (**c**) relative to naïve conditions (M0) (n=6). (**d**-**k**) mRNA expression levels of activation profile markers in (pre-polarized) BMDMs (**d**-**g**) and

microglia (**h**-**k**) exposed to rat GM myelin (**d**,**h**, n=3), rat WM myelin (**e**,**l**, n=3), MS GM myelin (**f**,**j**, n=3) or MS WM myelin (**g**,**k**, n=3), relative to their untreated (pre-polarized) controls ('no-myelin'). Bars represent relative means to the indicated control, which was set to 1 in each independent experiment (horizontal line). Error bars show standard error of the mean (SEM). Normality was tested using a Shapiro-Wilk normality test if applicable. Statistical analyses were performed using a one-sample t-test (*p<0.05, **p<0.01, ***p<0.001) to test for differences between treatments and their respective control.

4f, p=0.023) and the same reproducible trend was observed upon exposure to MS WM myelin (Fig. 4g). While rat GM and WM myelin exposure did not alter *Arg1* mRNA levels in pre-polarized BMDMs (Fig. 4d,e) and microglia (Fig. 4h,i), the mRNA levels of the M2 profile marker were significantly lower in pre-polarized M2-like microglia that were exposed to MS GM myelin (Fig. 4j, p=0.042). An opposite differential response to MS GM and WM myelin was observed for BMDMs. Exposure to WM MS myelin (Fig. 4g), but not GM MS myelin (Fig. 4f), reproducibly, but not significantly reduced *Arg1* mRNA levels by approx. 50% in M0, M1- and M2-like BMDMs (Fig. 4g, M0 p=0.056; M1 p=0.054; M2 p=0.090) and Nos2 mRNA levels in pre-polarized M1-like cells (Fig. 4g, M1 p=0.051). Hence, exposure to either rat GM and WM myelin induced alterations in mRNA levels of M1 profile markers of pre-polarized M2-like cells, which are distinct between BMDMs and microglia. While exposure to rat myelin mainly altered M1-profile markers in M0 and pre-polarized M2-like cells, exposure to MS WM myelin reduced the mRNA levels of the M2-profile marker *Arg1* and M1 profile marker *Nos2* in pre-polarized M1-like BMDMs. As macrophage and microglia activation states distinctly modulate OPC differentiation[52], we examined next whether myelin may interfere with mRNA expression levels of BMDM- and/or microglia-derived trophic factors that positively modulate OPC differentiation.

Exposure to rat myelin, but not MS myelin, increases mRNA expression of galectin-3 in bone marrow-derived macrophages

Macrophages and microglia secrete several factors that enhance or accelerate OPC differentiation, including insulin-like growth factor 1 (IGF1)[446–448], galectin-3[449–451] and members of the IL6 family, IL6[452–454], ciliary neurotrophic factor (CNTF)[455,456], leukemia inhibitory factor (LIF)[457,458] and oncostatin M (OSM)[459–461]. Therefore, we determined whether GM and WM myelin affect the mRNA expression of these pro-OPC differentiation factors. Our findings show that 24 hours after a 48-hour treatment with IFNγ+LPS to induce polarization towards M1-like BMDMs, only *Cntf*

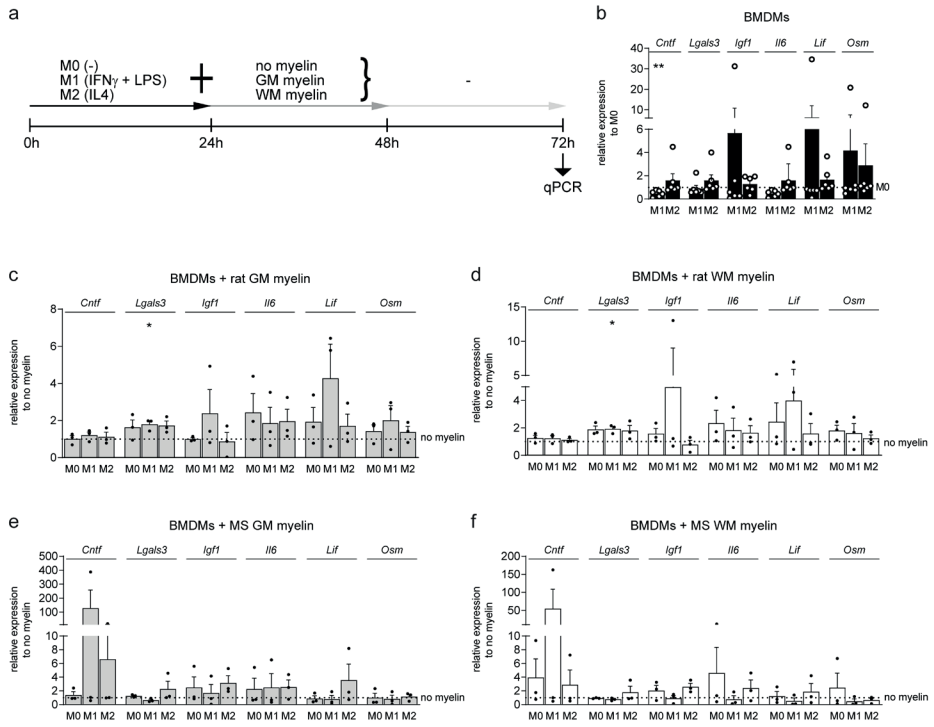

Fig. 5. Exposure to rat, but not MS myelin, increases mRNA expression levels of galectin-3 in bone marrow-derived macrophages. Bone marrow-derived macrophages (BMDMs) isolated from neonate rats were either kept naïve (M0), pre-polarized to pro-inflammatory activated cells to interferon-gamma (IFNγ) and lipopolysaccharide (LPS) (M1-like), or pre-polarized to alternatively activated cells interleukin (IL)4 (M2-like). After 24 hours BMDMs were additionally treated with 10 µg/ml grey matter (GM) or white matter (WM) myelin for 24 hours. Cells were cultured for another 24 hours before analysis. Rat myelin was isolated from cortex (GM, grey bars) and corpus callosum (WM, white bars), and MS myelin from GM (grey bars) and WM (white bars) postmortem brain tissue of a MS patient. Cells were subjected to RT-qPCR analysis of *Cntf*, *Lgals3*, *Igf1*, *Il6*, *Lif* and *Osm,* which are pro-OPC differentiation factors (**a**) Schematic representation of the experimental set-up. (**b**) mRNA expression levels of pro-OPC differentiation factors relative to naïve conditions (M0, n=6). (**c**-**f**) mRNA expression levels of pro-OPC differentiation factors when exposed to rat GM myelin (**c**, n=3), rat WM myelin (**d**, n=3), MS GM myelin (**e**, n=3) and MS WM myelin (**f**, n=3), relative to their untreated (pre-polarized) control ('no-myelin'). Bars represent relative means to the indicated control, which was set to 1 in each independent experiment (horizontal line). Error bars show standard error of the mean (SEM). Normality was tested using a Shapiro-Wilk normality test if applicable. Statistical analyses were performed using a one-sample t-test (*p<0.05, **p<0.01) to test for differences between treatments and their respective control.

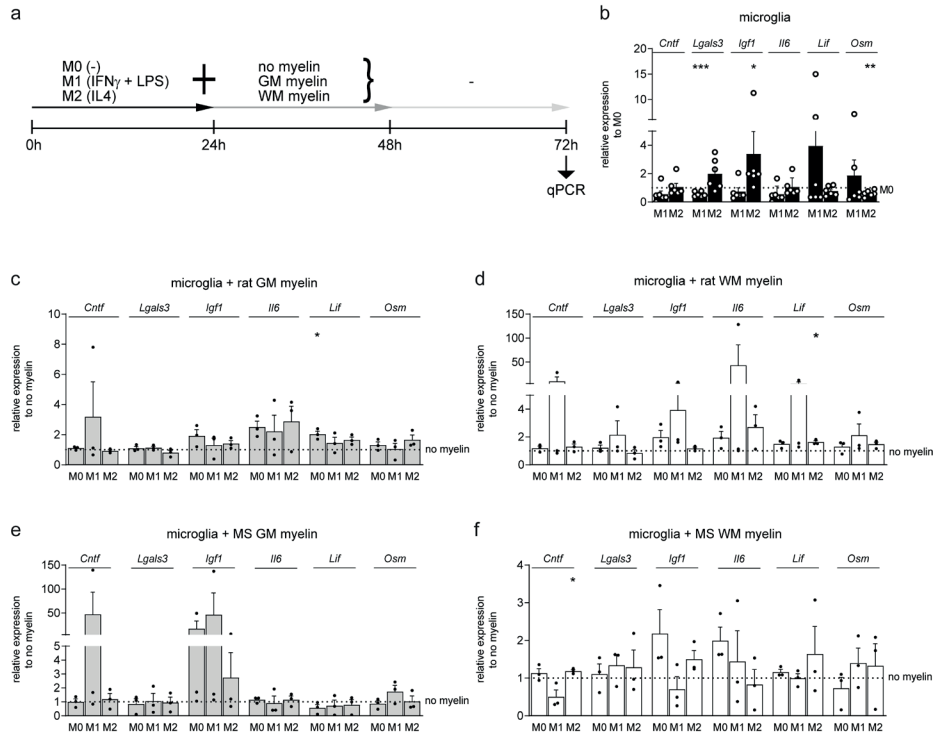

Fig. 6. Exposure to rat, but not MS myelin increases mRNA expression levels of LIF in microglia. Microglia from neonate rats were either kept naïve (M0), pre-polarized to pro-inflammatory activated cells by exposure to interferon-gamma (IFNγ) and lipopolysaccharide (LPS) (M1-like), or pre-polarized to alternatively activated cells by exposure to interleukin (IL)4 (M2-like). After 24 hours microglia were additionally treated with 10 µg/ml grey matter (GM) or white matter (WM) myelin debris for 24 hours. Cells were cultured for another 24 hours before analysis. Rat myelin was isolated from cortex (GM, grey bars) and corpus callosum (WM, white bars), and MS myelin from GM (grey bars) and WM (white bars) postmortem brain tissue of a MS patient. Cells were subjected to RT-qPCR analysis of *Cntf*, *Lgals3*, *Igf1*, *Il6*, *Lif* and *Osm,* which are pro-OPC differentiation factors. (**a**) Schematic representation of the experimental set-up. (**b**) mRNA expression levels of pro-OPC differentiation factors relative to naïve conditions (M0, n=6). (**c**-**f**) mRNA expression levels of pro-OPC differentiation factors when exposed to rat GM myelin (**c**, n=3), rat WM myelin (**d**, n=3), MS GM myelin (**e**, n=3) and MS WM myelin (**f**, n=3), relative to their untreated (pre-polarized) control ('no-myelin'). Bars represent relative means to the indicated control, which was set to 1 in each independent experiment (horizontal line). Error bars show standard error of the mean (SEM). Normality was tested using a Shapiro-Wilk normality test if applicable. Statistical analyses were performed using a one-sample t-test (*p<0.05, **p<0.01, ***p<0.001) to test for differences between treatments and their respective control if data could not be tested for normality or were normally distributed, when normality failed a Wilcoxon signed rank was used (**b**, *Igf1*, p<0.05).

mRNA expression levels were lower compared to untreated Mo BMDMs (Fig. 5b, p=0.005), while in alternatively IL4-activated M2-like BMDMs *Cntf* transcripts were unaltered (Fig. 5b). When pre-polarized BMDMs were transiently exposed to rat GM and WM myelin for 24 hours (Fig. 5a), *Lgals3* transcripts were more abundantly expressed 24 hours after myelin withdrawal (Fig. 5c,d). This effect is reproducible in Mo and pre-polarized alternatively activated M2-like BMDMs, and significant for pre-polarized classically activated M1-like BMDMs (Fig. 5c,d, Mo + GM myelin p=0.250; M1 + GM myelin p=0.048; M2 + GM myelin p=0.097; Mo + WM myelin p=0.0734; M1 + WM myelin 1p=0.035; M2 + WM myelin p=0.170). Exposure to MS GM and WM myelin did not have an effect on the mRNA expression of the indicated pro-OPC differentiation factors (Fig. 5e,f). Hence, exposure of BMDMs to rat, but not MS, GM and WM myelin slightly alters mRNA expression levels of galectin-3.

Exposure to rat myelin, but not MS myelin, increases mRNA expression levels of LIF in microglia

Next, the effect of rat and MS myelin from GM and WM on the mRNA levels of pro-OPC differentiation factors in (pre)-polarized microglia was assessed. Classically activated M1-like microglia have decreased *Lgals3* mRNA expression levels (Fig. 6b, p=0.002) 24 hours after a 48-hour IFNγ+LPS exposure, while *Igf1* transcripts were increased and *Osm* transcripts decreased in alternatively M2-like microglia compared to untreated Mo microglia (Fig. 6b, *Igf1* p=0.031; *Lgals3* p=0.007). When transiently exposing microglia for 24 hours to rat GM and WM myelin, expression of *Lif* was significantly increased in rat GM myelin-exposed Mo and rat WM myelin-exposed pre-polarized M2-like microglia 24 hours after myelin withdrawal compared to the respective (pre-polarized) microglia that were not exposed to myelin (Fig. 6c, Mo + GM myelin p=0.035; Fig. 6d, M2 + WM myelin p=0.024). Rat WM myelin-exposed Mo and rat GM myelin-exposed pre-polarized M2-like microglia had a similar trend (Fig. 6d Mo + WM myelin, p=0.089; Fig. 6c M2 + GM myelin p=0.073), indicating that the effect is likely not a GM or WM myelin dependent effect. *Igf1* transcripts were reproducibly, but not significantly higher upon treatment of pre-polarized M1-like microglia with MS GM myelin, but not MS WM myelin (Fig. 6e,f). In contrast, exposure to rat WM myelin, but not rat GM myelin, substantially increased *Igf1* transcripts in pre-polarized M1-like microglia (Fig. 6c,d). Therefore, exposure to

myelin induced subtle changes in gene expression of pro-OPC differentiation factors, which appeared to depend on the pro-OPC differentiation factor, cell type, their activation state, myelin species and on MS myelin from different regions.

Exposure to rat or MS myelin does not alter bone marrow-derived macrophage and microglia modulation of OPC differentiation by secreted factors *in vitro*

As macrophages and microglia orchestrate remyelination via secreted factors[49,462,463], it was assessed whether secreted factors from myelin-exposed classically or alternatively activated BMDMs and microglia distinctly affect differentiating OPCs. To this end, gmOPCs and wmOPCs were differentiated for 6 days in the presence of BMDM- or microglia-conditioned medium obtained from the same cells as used for the analysis of the mRNA expression levels (Fig. 7a). Treatment with conditioned medium obtained from Mo, M1-like and M2-like BMDMs increased the percentage of MBP-positive gmOLGs, but not wmOLGs (Fig. 7b, Mo p=0.024; M1 p=0.045; M2 p=0.061). For gmOPC differentiation the same trend was observed for Mo microglia-conditioned medium (Fig. 7c, gmOPC Mo p=0.094), indicating a distinct response of gmOPCs and wmOPCs to microglia and BMDM secreted factors. Secreted factors from Mo and pre-polarized M1- and M2-like BMDMs that were exposed to rat or MS GM and WM myelin hardly affected gmOPC and wmOPC differentiation compared to secreted factors from their respective (pre-polarized) control that was not exposed to myelin (Fig. 7d,e). Similarly, the percentage of MBP-positive gmOLGs was not altered upon exposure of gmOPCs to conditioned medium of (pre-polarized) microglia that were exposed to rat or MS GM and WM myelin (Fig. 7f,g). In contrast, secreted factors from pre-polarized M2-like microglia exposed to rat GM and WM myelin reproducibly, but not significantly, decreased wmOPC, but not gmOPC differentiation (Fig. 7e, GM myelin p=0.052; WM myelin p=0.073). Hence, secreted factors of GM and WM myelin-exposed pre-polarized BMDMs and microglia hardly altered OPC differentiation compared to non-myelin exposed BMDMs and microglia.

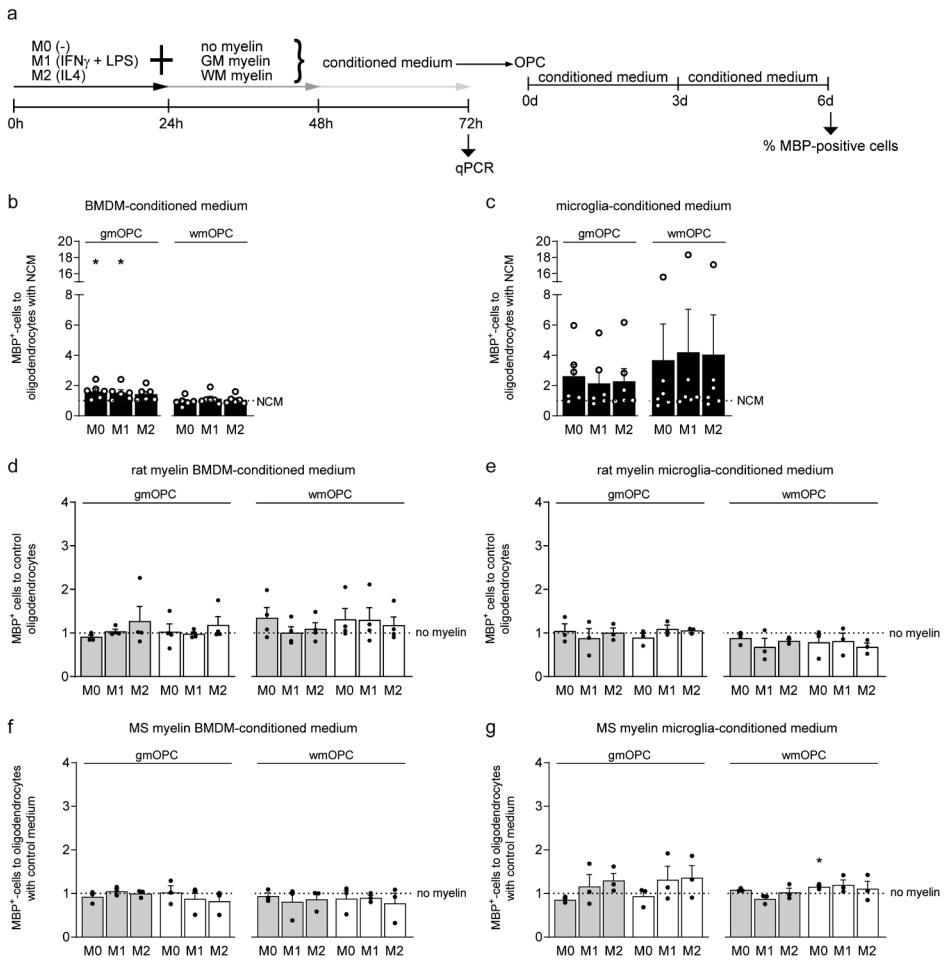

Fig. 7. Exposure to rat or MS myelin does not alter bone marrow-derived macrophage and modulation of OPC differentiation by secreted factors *in vitro*. Bone marrow-derived macrophages (BMDMs) and microglia isolated from new-born rats were either kept naïve (M0), pre-polarized to pro-inflammatory activated cells by exposure to interferon-gamma (IFNγ) and lipopolysaccharide (LPS) (M1-like), or pre-polarized to alternatively activated cells by exposure to interleukin (IL)4 (M2-like). After 24 hours, BMDMs and microglia were additionally treated with 10 µg/ml grey matter (GM) or white matter (WM) myelin debris for 24 hours. Cells were cultured for another 24 hours before analysis. Rat myelin was isolated from cortex (GM, grey bars) and corpus callosum (WM, white bars), and MS myelin from GM (grey bars) and WM (white bars) postmortem brain tissue of a MS patient. Oligodendrocyte progenitor cells (OPCs) isolated from the cortex (gmOPCs) and non-cortex (wmOPCs) of neonatal rat forebrains were cultured in the conditioned medium for six days, after which differentiation was determined by immunocytochemistry for the differentiation marker MBP (**a**) Schematic representation of the experimental set-up. (**b,c**) Differentiation of gmOPCs and wmOPCs cultured in BMDM- (**b**) and microglia(**c**)-conditioned medium relative to non-conditioned medium (NCM). (**d**-**g**) Differentiation of gmOPCs and wmOPCs cultured in BMDM- (**d,e**) and microglia- (**f,g**) conditioned medium obtained

from (pre-polarized) BMDMs exposed to rat GM (**d**,**f**, grey bars), rat WM myelin (**d**,**f**, white bars), MS GM myelin (**e**,**g**, grey bars) and MS WM myelin (**e**,**g**, white bars) relative to their respective (pre-polarized) control ('no-myelin'). Bars represent relative means to the indicated control, which was set to 1 in each independent experiment (horizontal line). Data was produced from three biological replicates of OPCs, to which three different batches of conditioned medium was added. Error bars show standard error of the mean (SEM). The percentage MBP-positive cells in the non-conditioned control was 52.9±10.0% for gmOLGs and 66.5±10.4% for wmOLGs. Normality was tested using a Shapiro-Wilk normality test if applicable. Statistical analyses were performed using a one-sample t-test (*p<0.05) to test for differences between treatments and their respective control.

Discussion

Previous studies have demonstrated that myelin obtained from total rat brain and human CNS tissue impairs OPC differentiation *in vitro*[427,428,437,471,472] and *in vivo*[51]. Here, we extended on these previous studies by taking into account regional differences between myelin and OPCs, and potential indirect effects on OPC behavior by myelin-exposed BMDMs and microglia. Our findings reveal that rat and MS myelin from GM and WM differ in their lipid and protein proportions. Despite these differences, rat WM and GM myelin coatings suppressed OPC proliferation and differentiation to a similar extent. Similarly, based on gene expression of activation profile markers and pro-OPC differentiation factors, only subtle differences between pre-polarized BMDMs and microglia that were exposed to rat GM and WM myelin were observed. Remarkably, gmOPCs and wmOPCs distinctly responded to rat myelin coatings, and from a pathological perspective indicate that rat WM myelin is more detrimental to wmOPC proliferation than rat GM myelin to gmOPC proliferation. In contrast, MS myelin coatings did not alter OPC proliferation, and inhibited gmOPC, but not wmOPC differentiation. Also, exposure to rat and MS myelin differentially modulated pre-polarized BMDMs and microglia, while gmOPCs and wmOPCs distinctly responded to secreted factors from BMDMs and microglia. Hence, both a distinct response of gmOPCs and wmOPCs and of macrophages and microglia to myelin may contribute more to regional differences in remyelination efficiency than regional differences in the composition of myelin per se.

Protein and lipid analysis of rat myelin revealed different proportions of prominent myelin constituents in rat GM and WM myelin. The ratios of MBP to PLP and CNP to PLP were lower in GM myelin compared to WM myelin, indicating that PLP was relatively more abundant in rat GM myelin. In contrast, relatively to GalCer and GM1, sulfatide was less abundant in GM myelin than WM myelin. As sulfatide and PLP have been implicated in modulating microglia responses[473–476], and as myelin phagocytosis drives the inflammatory and metabolic phenotype of macrophages and microglia[56,477], the observed regional differences in myelin composition may have a differential effect on BMDM and microglia polarization. However, our *in vitro* findings revealed that, independent of the region of origin, rat GM and WM myelin similarly interfered with M1-profile markers in pre-polarized M2-like cells.

Notably, mRNA levels of the M2 profile marker arginase-1 (*Arg1*) were not increased in (pre-polarized) BMDMs and microglia 24 hours after 24-hours myelin exposure, indicating that a disease-resolving macrophage phenotype, as observed in previous studies upon 24-hours myelin exposure[53–56,439,440], was not induced or resolved. Microglia display regional heterogeneity[30,478,479], and microglia from GM and WM may distinctly respond to myelin from GM and WM. However, injection of WM myelin induces a similar microglia phenotype in GM and WM[350], indicating that *in vivo* GM and WM microglia respond similarly to WM myelin. Exposure to myelin did not affect the activation state of pre-polarized M1-like BMDMs and microglia. This may relate to the fact that *in vitro*, classically activated, pro-inflammatory M1-like human BMDMs and microglia have less robust phagocytic activity than alternatively-activated M2-like BMDMs and microglia[432,480], which may result in the uptake of less or smaller myelin fragments and/or slower degradation of myelin.

Our data further demonstrated that pre-polarized M2-like BMDMs and microglia responded differently to rat myelin. More specifically, exposure to rat myelin decreased mRNA levels of the pro-inflammatory marker iNOS (*Nos2*) in naïve and pre-polarized M2-like BMDMs, while *Nos2* transcripts increased in myelin-exposed pre-polarized M2-like microglia. Yamasaki and coworkers revealed that monocyte-derived macrophages are mainly involved in active breakdown of myelin at the nodes of Ranvier, while microglia mainly phagocytose myelin debris[481]. Therefore, the increase in pro-inflammatory *Nos2* transcript levels upon myelin treatment by pre-polarized M2-microglia may be more physiological relevant for remyelination. In this regard it is relevant to mention that in mixed active/inactive WM MS lesions, microglia are preferentially present at the lesion edge[482], where also most iNOS-positive myeloid cells reside[483]. However, MS WM myelin did not alter *Nos2* transcript levels in BMDMs and microglia. In contrast, mRNA expression of *Ccl5*, another pro-inflammatory marker, increased in myelin-exposed pre-polarized M2-like BMDMs, which may add to the intermediate activation state of macrophages in WM MS lesions[52,484].

Both classically and alternatively activated macrophages and microglia are essential orchestrators of remyelination[52,485]. In most MS lesions, inactive OPCs[20,21] are present

and fail to differentiate[15,17,18]. Disrupted myelin debris clearance by macrophages and microglia in MS lesions may result in disturbances in their fine-tuned secretion of pro-OPC differentiation factors, thereby interfering with OPC differentiation. In our experimental *in vitro* design, *Lgals3* mRNA expression was increased in pre-polarized M1-like BMDMs upon rat GM and WM myelin exposure, whereas *Lif* transcripts were more abundantly present in rat GM and WM myelin-exposed M0 and pre-polarized M2-like microglia. This further corroborates that BMDMs and microglia differentially respond to myelin. The effect of myelin on pro-OPC differentiation factors in BMDMs and microglia at the gene expression level was however subtle, similar between rat GM and WM myelin, and not reflected in an increase in their OPC differentiation modulating capacities. Thus, while BMDM-conditioned medium increased gmOPC, but not wmOPC differentiation, secreted factors from rat GM or WM myelin-exposed BMDMs hardly altered gmOPC and wmOPC differentiation compared to secreted factors from BMDMs that were not exposed to myelin. Hence, secreted factors of myelin-exposed macrophages and microglia may not majorly contribute to the inhibitory effect of myelin on remyelination.

Regional differences in molecular composition of myelin did *in vitro* not elicit distinct responses in OPC behavior. Rat GM and WM myelin had a comparable detrimental effect on gmOPC and wmOPC proliferation and differentiation. While previous studies reported that myelin from the whole brain has no effect on OPC proliferation[428,471,472], we observed that OPC proliferation was reduced on a rat GM and WM myelin coating. This discrepancy may be explained by the use of a different combination of mitogens, assay methods and regional source of OPCs. Indeed, wmOPC proliferation was more severely affected by myelin coatings than gmOPC proliferation. The differential proliferative response of gmOPCs and wmOPCs to rat myelin may result in higher numbers of OPCs upon demyelination in GM areas, and contribute to the faster remyelination of axons in the cortex than axons in the corpus callosum[25]. Therefore, rat WM myelin is likely more detrimental to wmOPCs than rat GM myelin to gmOPCs. Remarkably, gmOPC, but not wmOPC differentiation was significantly reduced on a MS GM myelin coating. In addition, a coating of MS myelin hardly altered wmOPC and gmOPC proliferation. This indicates that MS myelin not only provoked different responses than rat myelin, but also that MS GM

myelin may be more detrimental for gmOPC differentiation than MS WM myelin for wmOPC differentiation. Hence, while a differential response of gmOPC and wmOPC proliferation to myelin may contribute to the more efficient remyelination in GM lesions in experimental models, the more detrimental effect of MS myelin on gmOPC differentiation hints to less efficient OPC differentiation in GM MS lesions.

Of particular interest for MS pathology is that MS GM and WM myelin elicit, although subtle, differences in activation state of pre-polarized M2-like BMDMs and microglia. More specifically, and in contrast to rat myelin, exposure to MS WM, but not GM myelin, reproducibly reduced mRNA levels of the M2 profile marker arginase-1 in pre-polarized M2-like BMDMs, while exposure to MS GM, but not WM myelin, decreased *Arg1* transcripts in pre-polarized M2-like microglia. We observed that in contrast to rat myelin, the ratio of GM1 to sulfatide is lower in MS GM myelin than in MS WM myelin, which may relate to a lower abundance of sulfatide in MS WM myelin. In fact, also the abundance of GalCer appeared lower in MS WM myelin, indicating a defective sphingolipid metabolism in MS. This in line with a comparative study on MS GM and WM homogenates[429]. In contrast to this study on homogenates, our lipidomic analysis demonstrates that SM levels are relatively more abundant in MS WM myelin compared to MS GM myelin. Another remarkable difference with rat myelin from different regions, is that the ratios of MBP to PLP and CNP to PLP were higher in MS GM myelin than in MS WM myelin. Although the difference in protein proportions between rat and MS myelin may be attributed to inter-species and disease differences, our preliminary data from GM and WM myelin from an Alzheimer's Disease (AD) and frontotemporal dementia (FTD) patient revealed that the MBP to PLP ratios in GM and WM myelin from AD and FTD were similar, indicating that PLP was relatively less abundant in MS GM myelin. Also Wheeler and coworkers reported less PLP in MS GM homogenates[429]. A comprehensive proteomic and lipidomic approach would be useful not only to reveal differences in myelin composition between MS GM and WM myelin, but also regional and pathological differences between MS myelin and myelin obtained from healthy subjects.

Taken together, our *in vitro* findings revealed that a regional difference in rat myelin composition may contribute only to a limited extent to the more efficient

remyelination in GM in experimental models. A distinct response of gmOPCs and wmOPCs to rat myelin and a differential effect of BMDMs and microglia upon rat myelin exposure, indicate that the local present cells in the demyelinated area are likely more important determinants for the myelin-induced inhibition of OPC proliferation and differentiation. Combined with less myelin debris[350] and less microglia being present in GM lesions[255], OPC differentiation in experimental models may proceed faster in GM than WM. Given the distinct effect of rat myelin and MS myelin, and a detrimental effect of MS myelin on gmOPC, but not wmOPC differentiation, our findings do not explain why remyelination in GM MS lesions is more robust than in WM MS lesions.

Materials and methods

Primary cell cultures

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Groningen (the Netherlands). All methods were carried out in accordance with guideline of the European Directive (2010/63/EU) on the protection of animals used for scientific purposes.

Mixed glial cultures

Mixed glial cultures were obtained from the cortex, a grey matter (GM) area, and mostly white matter (WM) parts of pooled neonatal (postnatal day 1-3) Wistar female and male rat brains (Envigo) as described[144]. WM includes WM tracts including corpus callosum, mixed WM/GM tracts including thalamus, hippocampus, and deep GM parts, including basal ganglia. Cells were cultured for 10-12 days in DMEM (Gibco, cat. no 41965) supplemented with 10% non-heat inactivated fetal bovine serum (FBS, Capricorn Scientific, cat. no. FBS-12A), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, cat. no. 15140), and 4 mM L-glutamine (Invitrogen, cat. no. 25030), followed by a shake-off procedure at 10-12 days in culture to obtain enriched microglia and OPCs.

Microglia

To detach microglia, flasks were shaken in an orbital shaker (Innova 4000, New Brunswick) at 150 rpm for 1 hour. Microglia of GM and WM mixed glial cultures were collected, mixed, and plated at a density of 2-3x10^6 cells per 10 cm Petri dish in MM medium [100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, cat. no. 15140), 4 mM L-glutamine (Invitrogen, cat. no. 25030), 1 mM pyruvate (Gibco, cat. no.11360-039), 10% (v/v) non-heat inactivated FBS in DMEM (Gibco, cat. no. 41965). Microglia (>95% Iba1-positive) were matured in the presence of 10 ng/ml macrophage colony stimulating factor (M-CSF, Peprotech, cat. no. 400-28) for 5-7 days.

Oligodendrocyte progenitor cells (OPCs)

OPCs from grey (gmOPCs) and white matter (wmOPCs) cultures were purified from the GM and WM mixed glia cultures after microglia were, by a 20-hour shake-off procedure at 220 rpm[144]. Shaken-off microglia and astrocytes were removed by differential adhesion on non-tissue culture dishes resulting in 95-97% Olig2-expressing OPCs, 1-3% GFAP-expressing astrocytes, <1% isolectin-B4-binding microglia and <1% TuJ1-expressing neurons in both GM and WM cultures. OPCs were cultured on poly-L-lysine (PLL)-coated (5 µg/ml) or on myelin-coated (see below) 13-mm glass coverslips at a density of 30.000 (gmOPCs) or 40.000 (wmOPCs) cells per coverslip and cultured in defined Sato medium[144]. To synchronize OPCs to the bipolar early OPC stage, cells were first exposed for 2 days to 10 ng/ml platelet-derived growth factor-AA (PDGF-AA; Peprotech, cat. no. 100-13) and 10 ng/ml human fibroblast growth factor-2 (FGF-2; Peprotech, cat. no. 100-18). Hereafter, OPCs were allowed to differentiate into mature oligodendrocytes in Sato medium supplemented with 0.5% FBS for 6 days. Microglia- or BMDM-conditioned medium was added at the onset of differentiation and refreshed at day 3 of differentiation.

Bone marrow derived macrophages (BMDMs)

Femora, tibia and humeri were dissected and pooled from postnatal day 1-3 female and male Wistar rats. Bone marrow cavities were flushed using MM medium to obtain bone marrow-derived monocytes. Cells were centrifuged for 10 min at 150 g and resuspended in MM medium containing 10 ng/ml M-CSF for maturation of monocytes into macrophages[464]. Cells were plated at a density of 2-3x10^6 in 10 cm Petri dishes and cultured for 5 days[465,466].

Myelin isolation and purification

Rat GM and WM myelin were obtained from 10 adult male Wistar rat pooled cortex and corpus callosum, respectively. Human GM and WM myelin-containing fractions from postmortem autopsy material from an MS patient (52 year old female, secondary progressive MS , disease duration 12 years, postmortem delay 6.75 hours) was kindly provided by Dr. Sandra Amor (Amsterdam UMC, the Netherlands). Written informed consents for brain autopsy and the use of clinical information and material for

research purposes were signed by donors. Myelin was purified by means of density-gradient centrifugation as described[467]. Briefly, brain tissue was homogenized in 0.32 M sucrose, 0.85 M sucrose was gently added and centrifuged at 75,000g for 30 min at 4°C. The myelin-containing interphase was taken and washed with water thrice by centrifuging at 75,000g for 15 min at 4°C. In the last wash, suspensions were centrifuged at 75,000g for 25 min at 4°C. Pellets were resuspended in 0.32 M sucrose and again 0.85 M sucrose was added gently. Suspensions were centrifuged at 75,000g for 30 min at 4°C. After taking the interphase and another wash with water (15 min centrifugation, 75,000g, 4°C), pellets were resuspended in water. Myelin was fragmented into debris by sonification for 30 min at 4°C. Protein concentrations were determined using a DC protein assay (Bio-Rad Laboratories, cat. no. 5000111) using bovine serum albumin (BSA) as a standard. Myelin was diluted to 10 mg/ml in water and stored at -20°C until further use.

Myelin exposure

OPCs were exposed to myelin by adherence. To this end, PLL-precoated coverslips were coated with myelin (40µg/cm^2) for 3 hours at room temperature (RT). Maturated microglia and BMDM were washed with PBS and gently scraped in 4% lidocaine (Sigma, cat. no. L-5647) in PBS. Cells were plated in a 12-well plate at a density of 0.5 *10^6 cells per well in MM medium. After one hour, cells were polarized into a classically activated phenotype using IFNγ (500 U/ml; Peprotech, cat. no. 400-20) and LPS (200 ng/ml; Sigma, cat. no. L4391), alternatively activated phenotype using IL4 (10 ng/ml; Bio-connect, cat. no. C61401), or left untreated. After 24 hours 10 µg/ml myelin debris was added for 24 hours. Then, myelin and cytokines were removed by rinsing with PBS and medium was replaced with Sato medium supplemented with 1% FBS. After 24 hours, the conditioned medium was filtered (0.45 µm filter; Whatman, cat. no. 10462100) and kept at -20°C until further processing. Cells were scraped in RNAprotect (Qiagen, cat. no. 76526) and kept at -20°C until further processing.

Western and dot blot analysis

For Western blot analysis, 10 µg protein was mixed with SDS-reducing sample buffer, heated for 5 min at 95°C and subjected to SDS-PAGE (12.5% SDS-gel) and

Western blotting as described[385]. In brief, equal amounts of protein were loaded onto 12.5% SDS-polyacrylamide gels. Proteins were transferred onto PVDF membranes (Millipore, IPFL00010) after gel electrophoresis. For dot blot analysis 5 µg (GM1) or 25 µg (GalCer, sulfatide) protein were applied using 5 µl droplets onto a nitrocellulose membrane (BioRad, cat.no. 162-0112). After drying, the membrane was subjected to immunoblot analysis similar to that of the Western blots. Primary antibodies included anti-CNP (1:500; Sigma, cat. no. C5922), anti-MBP (1:250; Serotec, cat. no. MCA409S), anti-PLP (1:100; a kind gift of Dr. Vijay Kuchroo, Harvard Medical School, Boston, MA), anti-GalCer O1 (Sommer and Schachner, 1981) antibody (ammonium sulfatide precipitated, 1:3000) and anti-sulfatide O4 antibody (Sommer and Schachner, 1981) (ammonium sulfatide precipitated, 1:400), which were both a kind gift of Dr. Guus Wolswijk, NIN, Amsterdam, the Netherlands). Biotinylated-cholera toxin subunit B (1:1500; Sigma, cat. no. C8052) was used to visualize GM1. Appropriate secondary IRDye-conjugated antibodies (1:3000) and streptavidin (1:500) were applied for 1 hour at RT. Signals were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Quantification was performed with Scion Image.

Electrospray ionization mass spectrometry (ESI-MS/MS)

Lipids were analyzed using ESI-MS/MS as described[434,469]. In brief, ESI-MS/MS was used to analyze phospholipids on a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP system; SCIEX). To profile phospholipids, a precursor ion scan with varying collision energy (prec 184, 50 eV; nl 141, 35 eV; nl 87,–40 eV; prec 241,–55 eV) was used. Multiple reactions monitoring mode (average signal period 3 min) was used to quantify individual species. PC25:0 and PC43:6 (Avanti Polar Lipids) were added as a lipid standard calculated for the protein concentration of the original sample. A Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was used to measure protein concentration. Data correction was applied for carbon, hydrogen and nitrogen isotope effects[470]. Lipid species with signal intensity under five times the blank were omitted.

qPCR analysis

mRNA was extracted (Isolate II RNA Micro Kit; Bioline, cat. no. BIO-52075) according to manufacturer's instructions. Total RNA (100 ng) was reverse transcribed in the presence of dNTPs (Invitrogen, cat. no. 10297018) and oligo (dT)12–18 (Invitrogen, cat. no. 18418012) with M-MLV reverse transcriptase (Invitrogen, cat. no. 28025013). Real-time quantitative RT-PCR using iTaq Universal SYBR Green Supermix (Bio-Rad, cat. no. 172-5124) was performed to quantify gene-expression levels. Primer sequences are listed in table 1. Each measurement was performed in triplicate and amplification data was processed using the LinRegPCR method[391,392].

Immunocytochemistry

Proliferation assay

Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 20 min at RT. Nonspecific antibody binding was blocked using 4% BSA in PBS for 30 min, after which cells were incubated with A2B5 antibody (1:5 in BSA; kind gift of Dr. Thijs Lopez-Cardozo, Utrecht, the Netherlands) for 1 hour. Cells were washed thrice with PBS and incubated with FITC-conjugated goat-anti-mouse IgM antibodies (1:50; Jackson Immunoresearch, cat. no. 115-095-020) for 30 min at RT. Cells were then permeabilized by incubation with 0.1% Triton-X100 in 4% BSA in PBS for 30 min at RT, followed by incubation with anti-Ki67 (1 µg/ml; Abcam, cat. no. ab15580) in 4% BSA in PBS for 1 hour at RT. After three washes with PBS, TRITC-conjugated goat-anti-rabbit antibodies (1:50; Jackson Immunoresearch, cat. no. 111-025-003) and DAPI (nuclear stain, 1 µg/ml; Sigma-Aldrich, cat. no. 32670) were added and incubated for 30 min at RT. After washing three times with PBS, Dako mounting medium (Dako, cat. no. S3025) was used to mount coverslips.

Differentiation assay

Cells were fixed using 4% PFA in PBS and permeabilized using ice-cold methanol for 10 min at RT. Nonspecific antibody binding was blocked with 4% BSA in PBS for 30 min at RT. Cells were incubated with anti-MBP antibodies (1:250; Serotec) for 1 hour at RT. After rinsing thrice with PBS, TRITC-conjugated goat-anti-rat antibodies (1:50;

Jackson Immunoresearch, cat. no. 112-025-003) and DAPI were added and incubated for 30 min at RT. After washing thrice with PBS, Dako mounting medium was used to mount coverslips.

Microscopic analysis

A conventional immunofluorescence microscope (Leica DMI 6000B with Leica Application Suite Advanced Fluorescence software) with 40x objective was used for analysis. 150-250 cells were scored per condition for each experiment, quantifying Ki67-positive of total A2B5-positive cells (proliferation assay) and MBP-positive cells of DAPI-stained cells (differentiation assay).

Statistical analyses

Data are obtained from at least three independent cell culture experiments and expressed as means ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism 6.0. In experiments with a n>6 normality was tested where possibly using a Shapiro-Wilk normality test to assess the normal distribution of the data. Where testing for normality was not possible, or when normality passed, a one-sample t-test was used when relative levels were compared with control in case control-values were set to 1 in each independent experiment. When normality was not reached, a Wilcoxon-signed ranked test was used to compare relative levels to control-values which were set to 1 in each independent experiment. A one-way ANOVA with a Tukey post-test was used when comparing multiple treatment groups. P-values of <0.05, <0.01, and <0.001 were considered significant and indicated with *, **, *** or #, ##, ###, respectively.

Author contributions

D.H.L., J.F.J.B. and W.B. designed the project and obtained funding. W.B. supervised the study. D.H.L., J.F.J.B., L.O., J.V.S. and W.B. performed the experiments and acquired the data. D.H.L., J.F.J.B. and I.L.W. produced the figures and carried out the statistical analysis. D.H.L. wrote the draft manuscript text, J.F.J.B., J.J.A.H. and W.B. critically reviewed and revised the manuscript text. All authors have read and approved the manuscript.

Funding

This work was supported by a grant from the Dutch MS Research Foundation ('Stichting MS Research', D.H.L., J.F.J.B.). D.H.L. and I.L.W. are recipients of a personal PhD fellowship from the Graduate School of Medical Sciences, University of Groningen, the Netherlands. There was no input or approval of supporting agencies regarding study design, execution, analysis, interpretation, writing and decision to submit the article for publication.

Declaration of competing interests

The authors declare no competing interests.

Data availability

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

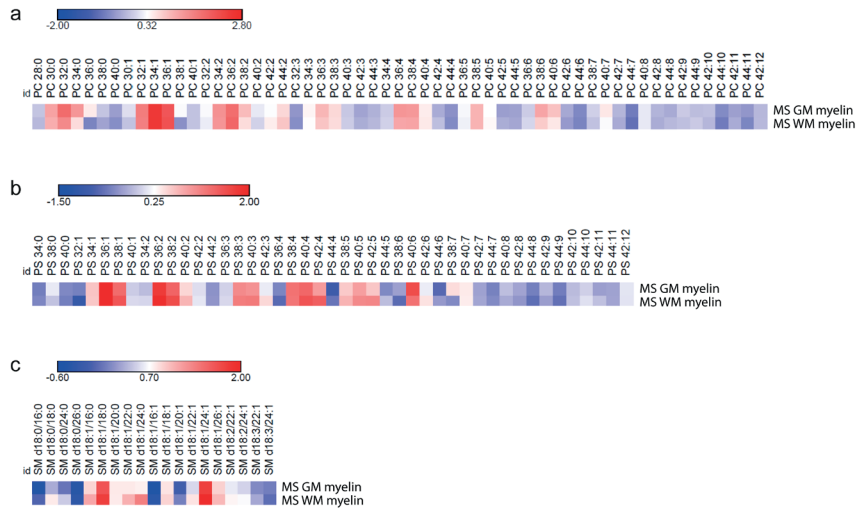

Figure S1. Lipidomic analysis of MS GM and WM myelin. MS myelin was isolated from postmortem grey matter (GM) and white matter (WM) brain tissue from a multiple sclerosis (MS) patient and subjected to ESI-MS/MS for analysis of phosphatidylcholine (PC, **a**), phosphatidylserine (PS, **b**) and sphingomyelin (SM, **c**) species. Visualized is the amount of lipid species (nanomolar) per mg total protein per sample on a log scale. Total amount of PC species was 802.7 and 756.3 (**a**), total amount of PS species was 745.4 and 529.2 (**b**) and total amount of SM species was 163.4 and 267.9 (**c**) nanomolar per milligram protein for MS GM and WM myelin respectively. Note that saturated PC and PS species are enriched in MS GM myelin (**a**,**b**) while SM is more abundant in MS WM myelin than in MS GM myelin (**c**).