

University of Groningen

On the cause of multiple sclerosis

Bijlard, Marjolein

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:
2016

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Bijlard, M. (2016). *On the cause of multiple sclerosis: Molecular mechanisms regulating myelin biogenesis*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

**TNF α induces actin cytoskeletal rearrangements in
mature oligodendrocytes and reallocates MBP;
consequences for (re)myelination**

Marjolein Bijlard*, Hande Ozgen*, Jenny C. de Jonge, Dick Hoekstra and Wia Baron

Department of Cell Biology, University of Groningen, University Medical Center Groningen,
Groningen, The Netherlands

*equal contribution

Manuscript in preparation

ABSTRACT

Multiple sclerosis (MS) is a demyelinating disease, characterized by inflammation, demyelination, failure of remyelination, and axonal loss. Pro-inflammatory cytokines are elevated in MS and contribute to MS pathology. Here, we examined the effect of the pro-inflammatory cytokine TNF α on myelin membrane integrity of oligodendrocytes, grown in primary and mixed myelinated cultures. Interestingly, treatment of myelinated spinal cord-derived cultures caused a reduction of the length of the MBP-positive internodal segments. Exposure of mature oligodendrocytes to TNF α did not affect protein and mRNA levels of the structural myelin protein MBP; neither did the cytokine interfere with cell survival and oligodendrocyte differentiation. However, in TNF α -treated oligodendrocytes, MBP protein was localized mainly in the cell body and primary processes, rather than in the myelin sheets. Upon removal of TNF α the preferential localization of MBP in the sheets was restored. Remarkably, while the myelin typical galactolipid galactocylceramide redistributed to TX-100-soluble membranes upon TNF α treatment, the altered localization of MBP is not reflected by detergent (in)solubility. However, TNF α treatment perturbed the organization of the actin cytoskeleton, which was accompanied by a redistribution of MBP from actin-dependent to actin-independent membrane microdomains. The ensuing decompaction appears to interfere with the barrier function of MBP, given that treatment with TNF α causes a similar redistribution of other myelin proteins, including PLP and CNP. Hence, our findings revealed that transient exposure to TNF α alters the internodal length of myelin by altering the actin cytoskeleton and the localization of myelin proteins and lipids, thereby likely allowing for myelin remodeling, while persistent exposure to TNF α might eventually interfere with myelin compaction.

INTRODUCTION

In the central nervous system (CNS) oligodendrocytes (OLGs) form myelin sheaths that are necessary for correct impulse conduction and support of neurons. Damage to OLGs and myelin has deleterious consequences, as reflected by the chronic neurodegenerative disease multiple sclerosis (MS), which is characterized by inflammation, demyelination, failure of remyelination and axonal loss. The pro-inflammatory cytokine TNF α appears to be one of the key players in MS pathology. This is illustrated by elevated TNF α levels in MS lesions^{21,22,279}, and in patient-derived T-cells^{280,281}, and TNF α levels in the cerebrospinal fluid (CSF) correlate with the degree of disability^{282,283}. Moreover, in MS patients blood levels of TNF α are upregulated before and during exacerbations^{281,284}, while patients with relapsing-remitting MS show a concomitant transient increase of TNF α levels²⁸⁵. In anti-cytokine therapy, a diminished production of TNF α reduces the incidence and causes a delay in the onset of EAE²⁸⁶, without affecting, however, the disease severity once EAE has been established²⁸⁷. Unfortunately, clinical trials with monoclonal anti-TNF antibodies were unsuccessful, and even resulted in exacerbated inflammation^{288,289}, indicating that TNF α has both adverse and beneficiary effects in MS.

Cytokines regulate a wide variety of cellular functions in developing and mature brain. TNF α , for example, is highly expressed in the embryonic brain²⁹⁰. However, mouse studies revealed that TNF α signalling is not essential for brain development or behaviour^{291,292}. Rather, it is upon CNS injury that cytokines seem to play a more prominent role. Infiltrating cells of the immune system, including lymphocytes and monocytes, but also residential glial cells, produce numerous cytokines in response to a changing environment, e.g. in demyelinating lesions, and they may shape cellular reactions, in either a (transient) inhibitory or beneficiary manner, that influence repair capacity. TNF α has been claimed to be essential for correct remyelination by inducing proliferation of oligodendrocyte progenitor cells (OPCs), as observed in a toxin-induced demyelination model²⁹³. However, in other studies it has been shown that TNF α hampers both OPC differentiation to mature myelin producing OLGs and OLG survival^{294–298}. For conveying its activity, TNF α can bind to two related receptors, 55 kDa TNFR1 and 75 kDa TNFR2, and is therefore capable of many and opposing cellular responses^{299,300}. Cells of the OLG lineage express both TNF receptors; TNFR2 is particularly expressed during early development³⁰¹, and contributes to remyelination²⁹³. By contrast, TNFR1 expression is not developmentally regulated and has been suggested to mediate primarily demyelination²⁸⁸.

Upon CNS injury, TNF α levels will be upregulated and, in addition to OPCs, also existing 'healthy' myelin membranes will become exposed to the cytokine. Therefore, we examined the influence of TNF α on mature OLGs, focussing on the dynamics of myelin basic protein (MBP), a major myelin protein, located at the cytoplasmic surface of the myelin membranes. MBP is a basic, membrane-associated adhesive structural protein, and imperative for myelination^{204–206}. Its adhesive properties organize the close apposition and compaction of the inner membrane leaflets, where it may also act as an 'molecular sieve' for myelin

components³². MBP isoforms that lack exon-II are transported to the myelin sheath in their mRNA form^{12,13,226}, which is thought to circumvent premature adhesion of membranes^{9,204,206}, whereas exon-II containing MBP isoforms remain in the cell body, where they localize to the nucleus and cytoplasm^{206,227,302}. Here we report that TNF α induces a reorganization of the actin cytoskeleton, along with a lateral redistribution of MBP from actin-dependent to actin-independent membrane microdomains. In addition we observed a reallocation of MBP towards the cell body and primary processes, which may lead to shortening of existing myelin segments. The *in vivo* significance of these data may relate to a beneficiary effect of TNF α upon remyelination, allowing existing myelin segments to shorten in order to provide space for newly formed myelin segments and paranodes for ‘remodeling’.

MATERIALS AND METHODS

Cell culture

Primary oligodendrocytes: Primary OLGs and astrocyte cultures were generated by a shake-off procedure as described previously²²⁰. Enriched OPCs were resuspended in SATO medium containing 10 ng/mL PDGF-AA (Peprotech, Rocky Hill, NJ) and 10 ng/mL FGF-2 (Peprotech). For immunocytochemical studies, OPCs were plated on poly-L-lysine (PLL, 5 µg/ml, Sigma, St. Louis, MO)-coated 13-mm glass coverslips (VWR, Amsterdam, The Netherlands) in a 24-well plate at 30,000 cells per well (500 µL), and for qPCR and Western blot analysis on PLL-coated 10-cm dishes (Nunc) at 1×10^6 cells per dish (6 mL). After 48 hrs, differentiation was induced by growth factor withdrawal, and cells were cultured in SATO supplemented with 0.5% fetal calf serum (FCS, Bodinco, Alkmaar, The Netherlands). 7 days after initiating differentiation the majority of the OPCs matured towards MBP-positive OLGs bearing myelin-like membranes (myelin sheets). These mature OLGs were exposed to 20 ng/ml recombinant rat TNF α (Peprotech) for 3 days, unless otherwise indicated. For the recovery experiments, the cells were allowed to recover in SATO+0.5% FCS for 24 hrs. For the TNFR1 blocking experiments, cells were treated with the antibody 1 hr prior to TNF α treatment. The TNFR1 blocking antibody (TNFR1, E20, 1:100, Santa Cruz, Huissen, Netherlands,³⁰³) was present throughout the experiment.

Myelinated spinal cord cultures: Myelinated spinal cord cultures were generated from 15 days old Wistar embryo's (Harlan, the Netherlands), as described before, with minor modification³⁰⁴. Briefly, meninges were removed from isolated spinal cords, minced in 1 ml MEM, and subjected to enzymatic digestion by adding 100 µl of 2.5% trypsin solution (Sigma) and 80 µl of liberase (2.5 mg/ml; Roche, Diagnostics, Mannheim, Germany) for 20 minutes at 37°C. To stop digestion, 1 ml of SD solution [0.52 mg/ml trypsin soybean inhibitor (Sigma); 0.04 mg/ml bovine pancreas DNase (Roche); 3 mg/ml BSA] in L-15 medium] was added for 3 minutes. Next, cells were gently triturated and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in plating medium [PM; 50% DMEM (Gibco, Paisley, UK); 25% horse serum (Invitrogen); 25% HBSS (Gibco) and 2 mM glutamine], and cells were plated onto 13 mm coverslips with an astrocyte monolayer at a density of 150,000 cells/coverslip (500 µl/well). Notably, the astrocytes were derived from the remaining astrocyte monolayer after shaking off the OPCs. The monolayer was trypsinized and passaged once before cells were plated on PLL-coated (5 µg/ml) 13 mm coverslips (VWR) at a density of 60,000/coverslip in 10% FCS in DMEM (500 µl/well) for 3 days. The dissociated spinal cords were allowed to attach for 2 hrs, after which 500 µl of differentiation medium [(DM) DMEM supplemented with 1 mg/ml holotransferrin (Sigma), 20 mM putrescine (Sigma), 4 µM progesterone (Sigma), 6 µM selenium (Sigma), 10 ng/ml biotin (Sigma), 50 nM hydrocortisone (Sigma) and 10 µg/ml insulin (Sigma)] was added. Half of the volume of medium was replaced every second day with fresh DM. After 12 days *in vitro* (DIV), insulin was omitted. At 19 DIV, cultures were treated with or without 10 ng/ml TNF α . TNF α was added upon each medium change, i.e., every 2 days. The cultures were analysed at 26 DIV.

LDH and MTT assay

The LDH and MTT assay were performed as described previously²²⁰. Briefly, mature OLGs, i.e., 7 days after initiating differentiation, were exposed to the indicated TNF α concentrations for 3 days, after which the medium (LDH assay) and cells (MTT) were analyzed. To determine the cytotoxicity of TNF α , the release of LDH into the medium was measured using a commercial LDH assay kit (Roche, Indianapolis, IN) according to manufacturer's instructions. The effect on cell viability was determined with an MTT assay on the remaining cells. Cytotoxicity (LDH) and cell viability (MTT) are expressed as the percentage of control cells, which was set at 100%.

Isolation of Triton X-100-resistant membrane microdomains

Cells were subjected to 1% Triton X-100 (TX-100) extraction at 4°C, followed by 10-30-40% discontinuous OptiPrep density gradient centrifugation of equal protein amounts, as previously described⁶². Fractions were collected from top (1) to bottom (7), the membrane microdomains being present at fractions 3-4, after which equal volumes of each fraction were subjected to Western blot (60 μ l) and dotblot (10 μ l) analysis.

Isolation of detergent free lipid rafts

Detergent-free lipid rafts were isolated as described by Klappe et al.³⁰⁵. Primary cells were scraped in base buffer (20 mM Tris/HCl, pH 7.8, and 250 mM sucrose, 1 mM CaCl₂ and 1 mM MgCl₂) on ice and centrifuged at 250 g for 2 minutes. The pellet was suspended in 1 ml base buffer supplemented with a cocktail of protease (Complete Mini, Roche) and phosphatase (Calbiochem, Cocktail set II, La Jolla, CA) inhibitors. The suspension was homogenized by passaging 20 times through a 25-gauge needle and centrifuged at 1000 g for 10 minutes. This procedure was repeated, and the first and second post-nuclear supernatant (PNS) were combined. To equal protein amounts of the PNS (in a total volume of 2 ml), 2 ml of base buffer containing 50% OptiPrep (Axis-Shield PoC AS, Dundee, Scotland) was added. On top of this mixture, an 8 ml continuous gradient of 0–20% OptiPrep buffer was poured with the use of a gradient mixer. After centrifugation at 22,000 rpm for 90 min at 4°C (Beckman SW41 rotor), 9 fractions of 1.34 ml were collected (from top to bottom) and half of this fraction volume was subjected to TCA precipitation¹¹⁵ followed by Western blot and dotblot analysis.

Western Blot and dotblot analysis

Cells were scraped in PBS, and lysed on ice for 30 min in TNE-lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% TX-100 and protease and phosphatase cocktail

inhibitors). For Western blot analysis, equal protein (lysates) or volume amounts (gradients) were mixed with SDS reducing sample buffer, heated for 5 min at 95°C and subjected to SDS-PAGE. Samples were loaded onto 15% SDS-polyacrylamide gels and subjected to Western blot analysis as described previously²²⁰. Primary antibodies used were polyclonal rat anti-MBP (1:2000, Millipore) and monoclonal mouse anti- β -actin (1:2000, Sigma). For dotblot analysis, equal volumes of the gradient fractions (10 μ l) were applied onto nitrocellulose membrane, and when dried subjected to similar immunoblot analysis as described above for the Western blots. Primary antibodies were anti-GalC antibody O1 (ammonium sulfate precipitated, 1:3000) and anti-sulfatide O4 antibody (ammonium sulfate precipitated, 1:400), which were both a kind gift of Dr. Guus Wolswijk²⁶¹. The signals were detected using appropriate IRDye®-conjugated secondary antibodies (Li-Cor Biosciences, Lincoln, NE) and the Odyssey Infrared Imaging System (Li-Cor Biosciences). The protein bands were quantified by imaging software ImageJ.

qPCR analysis

Total RNA from cells was isolated using the Invitrap Spin Cell RNA Mini Kit (Strattec molecular, Berlin, Germany). Total RNA (1 μ g) was reversed transcribed in the presence of oligo(dT)12-18 and dNTPs (Gibco) with superscript II reverse transcriptase (Roche) according to the manufacturer's instructions. qPCR amplifications were performed on copy DNA using primers specific for rat MBP with exon-II (forward, 5'-CACATGTACAAGGACTCACAC-3'; reverse 5'-GAAGAAGTGGACTACTGGGT-3'), rat MBP without exon-II (forward, 5'-ACTTGGCCACAGCAAGTACC-3'; reverse, 5'- TGTGTGAGTCCTTGCCAGAG-3') and the house-keeping genes HBMS (forward, 5'- CCGAGCCAAGCACCAGGAT-3; reverse, 5'-CTCCTTCCAGGTGCCTCAGA-3'), and HPRT1 (forward, 5'- GACTTGCTCGAGATGTCA-3'; reverse, 5'- ACCACCTGTGCTGTAG-3'). The results were analyzed with StepOne software and normalized to the house-keeping genes HBMS and HPRT1.

Immunocytochemical analysis

Monocultures: Cells were gently fixed with 2% paraformaldehyde (PFA) for 15 min at room temperature (RT), followed by incubation with 4% PFA for 15 min. Fixed cells were blocked and permeabilized, respectively with 4% bovine serum albumin (BSA) and 0.1% TX-100 for 30 min. The cells were incubated for 60 min at RT with polyclonal rat anti-MBP (1:100, Millipore, Temecula, CA), monoclonal anti-CNP (1:100, Sigma), monoclonal anti-PLP (4C2, 1:10, kind gift of Dr. Vijay Kuchroo (Harvard Medical School, Boston,²¹⁸), or monoclonal anti-mouse β -tubulin (1:500, Sigma) antibodies. After three times washing with phosphate buffered saline (PBS), cells were incubated with appropriate TRITC- or FITC-conjugated secondary antibodies (1:50, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted in 4% BSA in PBS for 30 min at RT, followed by three times PBS washing. During

secondary antibody incubation, DRAQ5 (1:500, Bistatus Limited, Leicestershire, UK) and TRITC-conjugated phalloidin (1:200, Sigma) were also included to visualize the nuclei and F-actin, respectively. For double staining, cells were sequentially stained with the different antibodies. Cells were mounted in DAKO mounting medium. Images were acquired by a confocal laser scanning microscope (Leica SP8 AOBS CLSM, Leica Microsystems, Heidelberg, Germany), equipped with an argon laser (488 nm), 2 He/Ne lasers (552 and 633 nm, respectively) and Leica Confocal Software. A 63×/1.25 oil immersion objective was used for 3-channel scanning (488 nm, 552 nm, 633nm). Images of single cells were acquired with similar gain settings and 15 cells were measured at each condition. To quantify the cellular distribution of protein or mRNA, i.e., cell body and primary processes and myelin sheets, the cells were segmented in 'in' (encompassing the cell body and primary processes, but excluding the nucleus) and 'out' (constituting the remaining processes and sheets) by selection by hand and in a blinded manner. Since OLGs grown *in vitro* do not form a continuous sheet covering the supporting glass coverslip, the area within the selections that did not hold cellular material was eliminated by a threshold to only visualize cellular material. The cellular distribution was defined as the ratio between fluorescent intensity between 'out' and 'in', i.e., a value above 1 corresponded to a relative enrichment in the myelin membrane.

Myelinated-cultures: Cultures were fixed in 4% PFA and incubated at RT in 0.5% TX-100 in 5% normal goat serum (NGS, Vector Laboratories, Burlingame, CA) for 60 min. After washing with PBS, cells were incubated for 2 hrs at RT with monoclonal rat anti-MBP (1:250, Serotec, Oxford, UK), and anti-NF-H antibodies (1:5000, EnCor Biotechnology Inc, Gainesville, FL) diluted in 2% NGS. Staining was visualized by an incubation for 60 min at RT with appropriate Alexa-conjugated secondary antibodies diluted in 2% NGS. Coverslips were mounted in Dako mounting media. All analyses were performed using a confocal laser scan microscope (Leica SP8 AOBS CLSM). The length of the myelin segment was determined by measuring MBP-positive segments that colocalized with NF-positive segments by using NeuriteTracer in ImageJ³⁰⁶, for at least 20 myelin segments per experimental condition in 3 independent experiments.

***In situ* hybridization**

OLGs were hybridized with 48 TMR labeled 20-nucleotide long probes designed against rat 14 kDa MBP, the major isoform present in rodent myelin²¹⁹. Briefly, cells were fixed in 4% PFA and opened with ethanol, incubated overnight at 37°C with 1 ng/μL probe mix in 10% formamide-containing hybridization buffer and washed with SSC (150mM NaCl, 15mM sodium citrate). Cells were subsequently blocked with BSA and incubation with primary anti-MBP and appropriate secondary antibodies as described above. Coverslips were mounted in Dako mounting medium and analyzed with a confocal laser scan microscope (Leica SP8 AOBS CLSM). For quantification of the cellular distribution see above.

Statistics

All data are represented as the mean \pm SD of at least three independent experiments. Statistical significance was calculated by a two-tailed Student's t-test for comparison between two means and by one-way ANOVA followed by a Dunnet's posttest when more than two means were compared to the control group (untreated cells). A p value of $p < 0.05$ was considered statistically significant.

RESULTS

TNF α treatment induces shortening of myelin segments in myelinated cultures

TNF α is prominently present in MS lesions, and within the CSF, the levels of TNF α correlate with disease activity^{21,279,282,283}. However, TNF α is also thought to be necessary for remyelination, and OPC proliferation in particular²⁹³. It is unknown whether TNF α has an effect on myelin membrane integrity. To assess such a potential effect, myelinated spinal cord cultures were employed. These spinal cord-derived cultures, plated on top of a feeding layer of astrocytes, represent an appropriate model system to study myelin maintenance, and approximately three weeks after plating, myelin segments can be clearly visualized by optical microscopy³⁰⁴. To examine the effect of TNF α on myelin stability, the myelinated cultures were exposed at 19-days *in vitro* (DIV) to the cytokine at a relatively low concentration of 10 ng/ml (approx. 100 U/ml). At 26 DIV, the cultures were fixed and stained with the neurofilament marker NF-H to visualize axons, and with the myelin marker MBP to visualize myelin segments. As shown in Figure 1, in TNF α -treated cultures, remarkable discontinuities between myelin segments (arrows) and in the length of MBP-positive myelin segments ('internodes') were apparent, the internodal length as such being diminished on average by

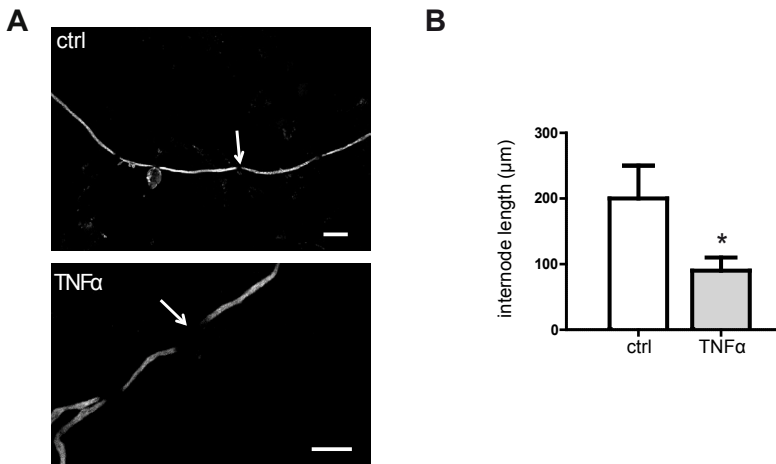


Figure 1. TNF α treatment induces shortening of myelin segments in myelinated cultures

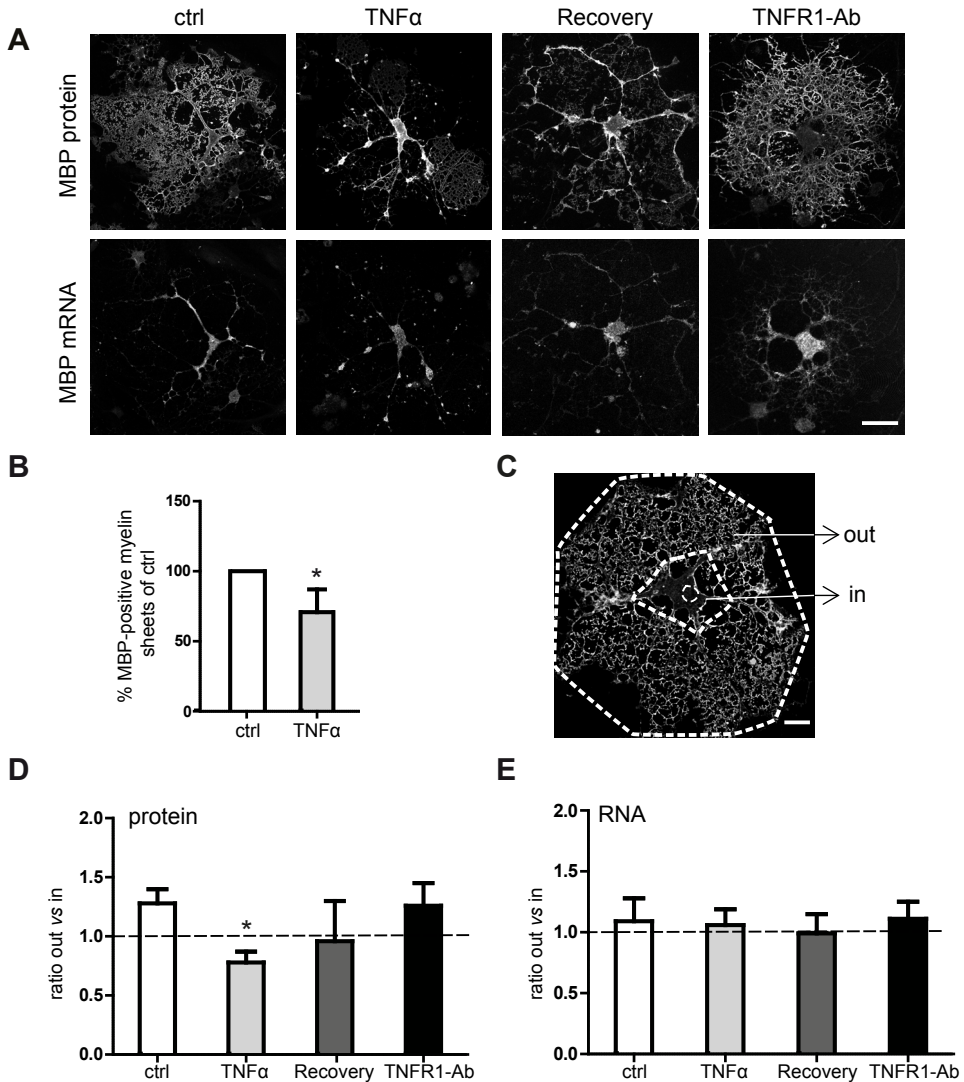
At 19 days *in vitro* (DIV) embryonic rat myelinated spinal cord cultures plated on a feeding layer of rat cortical astrocytes were untreated (ctrl) or treated with 10 ng/ml TNF α . **A)** Spinal cord cultures at 26 DIV were stained for MBP (myelin marker). Representative images are shown. Scale bar is 100 μ m. **B)** Quantitative analysis of the length of the myelin segments (internodal length). Bar represents mean + SD of 3 independent experiments. Statistical difference with untreated (ctrl) cells as assessed with a Student's t-test (* $p < 0.05$). Note that upon exposure of TNF α , the length of the MBP-positive myelin segments is significantly reduced.

approx. 50%, relative to untreated cultures. Hence, this finding suggests that the length of existing myelin segments is susceptible to remodeling, being shortened upon exposure to TNF α . In these cultures all cells, including neurons, astrocytes and oligodendrocytes, harbor receptors for TNF α ^{307,308}. Therefore, we next examined in OLG monocultures whether the effect of TNF α on myelin membrane remodeling is due to a direct and/or indirect effect on oligodendrocytes.

TNF α treatment induces a reversible redistribution of MBP protein from myelin membranes towards primary processes

OLGs grown in monoculture follow the same developmental pattern as in the presence of neurons, i.e., all the myelin components are expressed in a coordinated fashion and transported to the different subdomains in the myelin sheet^{9,32,222}. Therefore, mature OLGs represent a good *in vitro* model to study remodeling of myelin membranes. As direct exposure to TNF α may be toxic to OLGs in a concentration-dependent manner^{296,297}, we first exposed mature OLGs to different concentrations of TNF α ranging from 2 to 200 ng/ml. Cytotoxicity was determined by measuring the release of LDH into the culture medium, and cell viability with an MTT assay. At all concentrations tested, no significant effect of TNF α was apparent on LDH release and MTT activity relative to untreated cells (Suppl. Fig.1). Based on these findings, the concentration of 20 ng/ml of TNF α was used for all further experiments with OLG monocultures. To examine the effect of TNF α on myelin maintenance, mature OLGs were treated with TNF α , i.e., 7 days after initiating OPC differentiation, when the majority of the MBP-positive OLGs have synthesized a myelin sheet. As shown in Figure 2A, following 3 days of exposure to TNF α , MBP protein was mainly localized to the cell body and primary processes, whereas in untreated OLGs MBP's localization was more pronounced in the myelin sheets. Indeed, the number of cells positive for MBP that bear MBP-positive sheets, membranous structures between the cellular process, was reduced upon TNF α treatment as compared to untreated mature OLGs (Fig. 2B). Western blot and qPCR analysis showed no significant difference in both MBP protein and mRNA expression levels, respectively (Supp. Fig. 2), indicating that the observed difference in MBP localization is not due to degradation or altered synthesis. To reveal a potential enrichment of MBP protein expression in cell body and primary processes upon exposure of mature OLGs to TNF α , we developed a method to quantify the cellular distribution of MBP per cell. Given the irregular shape of OLGs, 3 circular lines were manually drawn per cell, i.e., one around the cell's nucleus, the second one at the interface of the end of the primary process and the start of the myelin sheet, and the third one around the outer edge of the myelin sheet (Fig. 2C). To determine the relative cellular distribution of MBP in each cell, the ratio between the fluorescent intensity of MBP in the myelin sheet, i.e., the area between the two outer circular lines and the fluorescence intensity of MBP in the cell body and the primary processes, i.e., the area between the two inner circular lines, was calculated. If the ratio exceeds a value of 1, MBP can be considered to be mainly localized to the outer myelin sheets and conversely, if the ratio was below 1, MBP is enriched in the cell body and primary processes. Applying this quantification method, we

confirmed our visual observations that in TNF α -treated mature OLGs, MBP is significantly enriched in the cell body and primary processes, whereas in untreated cells MBP mainly localized to the myelin sheet (Fig. 2D). Intriguingly, when the cells were allowed to recover for 24 hrs after 3 days of exposure to TNF α , MBP protein was prominently localized to the myelin sheets, and indistinguishable from untreated cells (Fig. 2A, D), indicating that the effect of TNF α is reversible. Moreover, the effect of TNF α was mediated by TNFR1, since a functional blocking antibody directed against TNFR1 counteracted the effect of



TNF α on MBP localization (Fig. 2A, D). Hence, these results suggest that the TNF α -mediated ectopic localization of MBP in the cell body and primary processes of mature OLGs might be a reversible reallocation of already synthesized MBP protein towards to the cell body and primary processes. Of interest, MBP mRNA was similarly distributed in untreated and TNF α -treated mature OLGs, being localized deep into the processes (Fig. 2A, E), indicating that the enrichment of MBP protein in cell body and primary processes upon TNF α treatment was likely not due to impaired MBP mRNA trafficking. Given the difference in localization pattern of MBP upon treatment with TNF α , we next examined the lateral membrane organization of MBP.

Galactosylceramide is enriched in TX-100-resistant microdomains upon TNF α treatment

In spite of its nature as a peripheral membrane protein, MBP does in fact associate with detergent resistant microdomains, termed 'lipid rafts', upon OLG maturation^{89,129}. Therefore, given the apparent redistribution of MBP following TNF α treatment, we next analyzed whether this redistribution of MBP is also reflected by a lateral reorganization of MBP's association with membrane microdomains. Previous findings have revealed that CHAPS-resistant membrane microdomains are prevalent in myelin membranes (reviewed in Ref. 26), while TX-100-resistant domains are more enriched at the cell body and primary processes⁷. Therefore, given the observed TNF α -induced redistribution of MBP from the sheet towards the cell body and primary processes, we first isolated membrane microdomains by TX-100 detergent extraction followed by OptiPrep density gradient centrifugation and Western blot analysis. Of the obtained 7 fractions, fractions 3-4 are considered as raft fractions, whereas TX-100-soluble proteins appear in fractions 6-7⁶². MBP is prominently present in TX-100-resistant membrane microdomains in both untreated and TNF α -treated mature OLGs

Figure 2. TNF α treatment induces a reversible redistribution of MBP protein towards primary processes in mature oligodendrocytes

Mature oligodendrocytes were left untreated (ctrl), treated with 20 ng/ml TNF α , or prior to treatment with TNF α pretreated with a blocking antibody against TNFR1 for 3 days, or allowed to recover from TNF α for 24 hrs (recovery). Cells were subjected to MBP mRNA probe labeling followed by immunocytochemistry for MBP protein. **A)** Representative confocal images of the localization of MBP protein and mRNA in the same cell. Scale bar is 20 μ m. **B)** Quantitative analysis of the number of MBP-positive cells bearing myelin-like membranes. Each bar represents the mean + SD of 3 independent experiments. In each experiment, the data of untreated cells was set at 100%. Statistical difference with untreated (ctrl) cells as assessed with an one sample t-test (* p<0.05). **C)** Representative example of the quantification method to analyze MBP protein localization within a cell (see Materials and Methods for details). For each cell the ratio of the intensity of the protein (or mRNA) in the outer myelin membrane ('out') vs the intensity of the protein in the cell body and primary processes ('in') is determined. A ratio above 1 indicates an enrichment in myelin-like membranes. Scale bar is 10 μ m. **D, E).** Quantitative analysis of the localization of MBP protein (D) and mRNA (E) as described at C. Each bar represents the mean + SD of 3 independent experiments. In each independent experiment 15 cells per condition were analyzed. Statistical difference with untreated (ctrl) cells as assessed with a one-way ANOVA (* p<0.05, Dunnet's posttest). Note that TNF α induces a reversible retraction of MBP protein from myelin sheet towards cell body and primary processes, likely by activation of TNFR1.

(Fig. 3A, B, $49.4\pm 13.0\%$ and $47.2\pm 5.2\%$ respectively). Similarly, irrespective of $\text{TNF}\alpha$ treatment, no change was apparent in the distribution of MBP in CHAPS-resistant microdomains (data not shown). Galactosylceramide (GalC), a lipid typically present at the extracellular leaflet of the lipid bilayer, including the myelin membrane, plays a major role in the lateral membrane association of MBP, localizing at the cytoplasmic face of the membrane^{80,86,89,91,309,310}. Remarkably, following $\text{TNF}\alpha$ treatment, GalC became relatively enriched in TX-100-resistant membrane microdomains (Fig. 3A, C, fractions 3-4), with a concomitant decrease in non-raft fractions (Fig. 3A, C, fractions 6-7). Interestingly, following $\text{TNF}\alpha$ treatment, the glycolipid appears to be clustered at the cell body plasma membrane and primary processes, as visualized with the anti-GalC antibody O1 (Fig. 4). Hence, $\text{TNF}\alpha$ alters the lateral organization of GalC, and induces its partial clustering, particularly in the

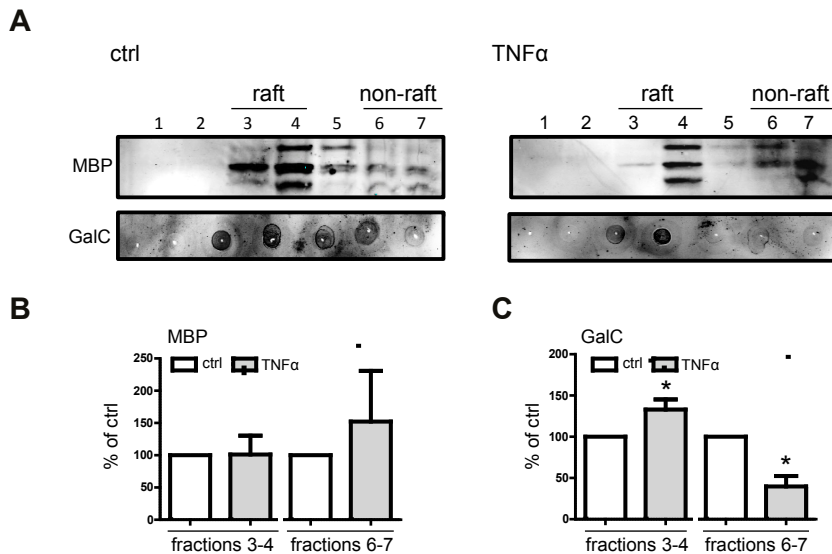


Figure 3. Galactosylceramide is enriched in TX-100-resistant microdomains upon $\text{TNF}\alpha$ treatment

Mature oligodendrocytes were left untreated (ctrl) or treated with 20 ng/ml $\text{TNF}\alpha$ for 3 days. The presence of MBP (A,B) and GalC (A,C) in membrane microdomains was analysed by TX-100 detergent extraction followed by OptiPrep density gradient centrifugation and Western (A,B) or dotblot (A,C) analysis. Representative blots of 3 independent experiments are shown. The total protein/lipid expression was calculated by adding the intensity of all the fractions. The protein/lipid percentage of each fraction was then calculated by dividing the protein intensity present in that fraction by total protein/lipid expression. Bar graphs of the pooled fraction percentage of (raft) fractions 3-4 and (non-raft) fractions 6-7 of MBP (B), GalC (C), relative to untreated cells (ctrl, set at 100% in each experiment) are shown. In untreated cells, $49.4\pm 13.0\%$ of MBP and $43.3\pm 21.2\%$ of GalC were present in fractions 3-4, and $25.8\pm 5.9\%$ of MBP and $43.7\pm 28.4\%$ of GalC in fractions 6-7. Statistical difference with untreated (ctrl) cells as assessed with a one sample t-test (* $p < 0.05$). Note that upon $\text{TNF}\alpha$ treatment GalC, but not MBP, is enriched in TX-100-resistant membrane microdomains, while a contaminant significant decrease in the non-raft fractions was noticed.

processes, which seems to reflect the appearance of MBP at similar conditions (cf. Fig. 2A). However, these findings still do not explain the underlying mechanism as to why MBP in mature OLGs redistributes from myelin membranes towards the cell body and primary processes upon exposure to $\text{TNF}\alpha$.

$\text{TNF}\alpha$ treatment disrupts the actin cytoskeleton in mature oligodendrocytes

To further examine the underlying mechanism of the redistribution of MBP upon $\text{TNF}\alpha$ treatment, we considered a potential involvement of the cytoskeleton of the cell. Previous studies have shown that $\text{TNF}\alpha$ can induce changes in cytoskeletal structures^{311–313}, and that MBP interacts with the cytoskeleton^{90,314,315}. Also, a connection between raft localization and the cytoskeleton has been proposed³¹⁶. In OLGs, the cytoskeleton consists of tubulin and actin filaments, while it lacks intermediate filaments³¹⁷. In control mature OLGs, actin filaments, visualized with fluorescently-labeled phalloidin, are predominantly present in the processes as well-structured long filaments (Fig. 5A). In $\text{TNF}\alpha$ -treated cells, this well-structured pattern of actin filaments is lost, revealing a diffuse distribution of apparently smaller filaments in secondary and tertiary process, but remarkably not in the cell body and primary processes. Quantitative analysis showed that the number of cells with this diffuse distribution pattern of actin filaments was increased in $\text{TNF}\alpha$ -treated cells as compared to untreated mature OLGs, which was reversed 24 hrs after $\text{TNF}\alpha$ withdrawal (Fig. 5A, B), indicating that the actin cytoskeleton is not irreversibly disturbed. Importantly, in both untreated and recovered cells MBP and actin co-localized. The (partial) co-localization was lost when mature OLGs were exposed to $\text{TNF}\alpha$, indicating an uncoupling of MBP and

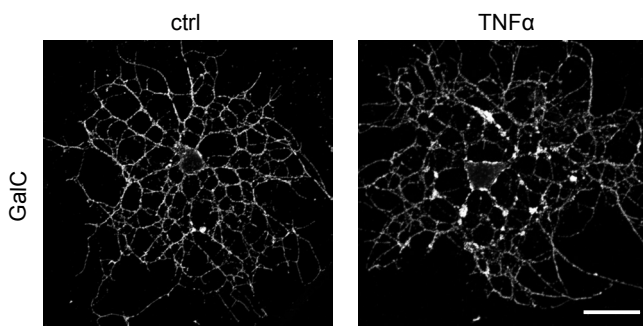


Figure 4. $\text{TNF}\alpha$ treatment alters the localization of galactosylceramide

Mature oligodendrocytes were left untreated (ctrl), or treated with 20 ng/ml $\text{TNF}\alpha$. After 3 days, the surface localization of GalC was assessed using immunocytochemistry on live cells (O1 antibody). Scale bar is 20 μm . Note that upon $\text{TNF}\alpha$ treatment GalC appears as clusters at the plasma membrane of the cell body and primary processes.

the actin cytoskeleton. Immunocytochemical analysis of the tubulin cytoskeleton using an antibody against β -tubulin revealed no apparent difference in the localization of tubulin

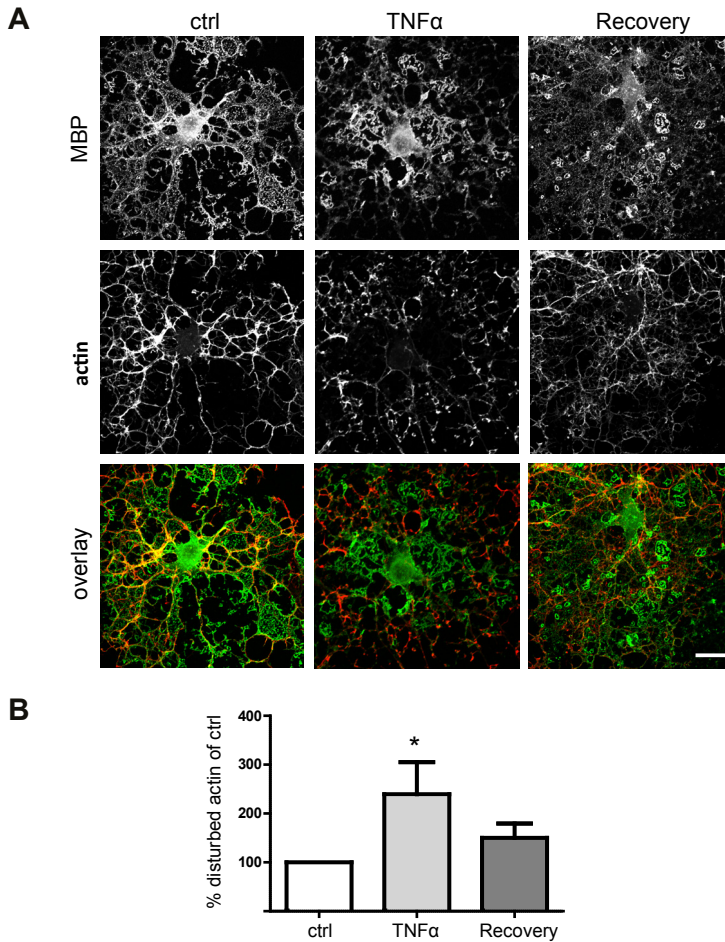


Figure 5. TNF α treatment disrupts the actin cytoskeleton in mature oligodendrocytes

Mature oligodendrocytes were left untreated (ctrl), treated with 20 ng/ml TNF α for 3 days, or allowed to recover from TNF α for 24 hrs (recovery). Cells were subjected to MBP immunocytochemistry (green), followed by visualization of actin filaments with TRITC-conjugated phalloidin. Representative confocal images (**A**) are shown. Scale bar is 20 μ m. The appearance of the actin cytoskeleton of at least 100 cells was scored and characterized as organized or disorganized (**B**). Each bar represents the mean + SD of 3 independent experiments. In each experiment, the data of untreated cells was set at 100%. Statistical difference with untreated (ctrl) cells as assessed with a one-way ANOVA (* $p < 0.05$, Dunnett's posttest). Note that upon TNF α treatment the actin cytoskeleton is disordered into apparently smaller filaments in secondary and tertiary processes.

(Suppl. Fig 3); tubulin is primarily present in the primary processes, in both TNF α -treated and untreated cells. Therefore, these results indicate that TNF α reversibly disrupts the actin, but not tubulin cytoskeleton in mature OLGs. Given the uncoupling of MBP and actin upon TNF α treatment and the fact that actin may serve as a stabilizer of distinct membrane microdomains^{318,319}, we next examined whether the lateral movement of MBP towards the cell body and primary processes was actin-dependent upon TNF α treatment.

TNF α treatment alters MBP's microdomain association from actin-dependent to actin-independent

Previous studies revealed that detergent fractionation at 4°C may create artefacts with regard to a protein's distribution in the gradient, particularly when it associates with the cytoskeleton. To examine whether the TNF α -mediated change in the cellular localization of MBP was actin-dependent, we applied a detergent-free raft isolation method that separates actin-dependent and actin-independent membrane microdomains³⁰⁵. Upon detergent-free OptiPrep gradient fractionation, fractions 1-2 are the very light fractions and represent membrane microdomains with a high lipid to protein ratio. The association of proteins in these membrane microdomains is sensitive to latrunculin B treatment³⁰⁵, and fractions 1-2 are therefore considered as actin-dependent rafts. Fractions 3-4 also represent membrane microdomains, given the high lipid to protein ratio, and are characterized as actin-independent membrane microdomains. Proteins that are not present in membrane microdomains appear in fractions 7-9. As shown in Figure 6A, in control mature OLGs, approx. 55% of the MBP fraction resided in fractions 1-2, i.e., in actin-dependent membrane microdomains. By contrast, TNF α treatment significantly reduced the actin-dependent membrane microdomain association of MBP (Fig. 6B). In fact, MBP became associated with actin-independent membrane microdomains, given its abundant and increased presence in fractions 3-4 (Fig. 6A, B). Furthermore, TNF α treatment also reduced the levels of actin and GalC in fractions 1-2 (Fig. 6A, C, D) without a concomitant increase in fractions 3-4, suggesting a redistribution to non-raft membranes. Hence, TNF α treatment perturbed the integrity of the actin cytoskeleton in mature OLGs, which correlates with a segregation of MBP from actin-dependent to actin-independent membrane microdomains. In addition to its function as a molecular membrane glue, MBP also acts as a molecular barrier for proteins to enter the myelin membranes. Therefore, we next examined functional consequences of TNF α -induced reallocation of MBP with regard to the localization of other myelin proteins.

TNF α treatment interferes with the localization of myelin proteins PLP and CNP

Proteolipid protein (PLP) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) are myelin specific proteins, showing a distinct localization in myelin; PLP is present in compact myelin, facilitating the apposition of the extracellular leaflets of the different myelin membrane layers^{31,320}, whereas CNP is abundantly present in non-compact myelin, and has been identified as a factor that delays myelin compaction during development¹¹⁷. In untreated

cultured mature OLGs, PLP is present in the cell body, primary processes and the myelin sheets, while CNP expression is more restricted to the cell body and primary processes (Fig. 7A, B). Following cellular treatment with TNF α for three days, PLP mainly localizes to the cell body and primary processes, while the protein is nearly absent from the myelin sheet (Fig. 7A, C). In contrast, CNP was more evenly distributed in the cells following TNF α exposure (Fig. 7B, D). Hence, TNF α treatment perturbs the organized structure of myelin specific proteins within mature OLGs, and therefore likely the compaction, which might allow for remodeling and shortening of existing myelin membranes, as illustrated in Figure 1.

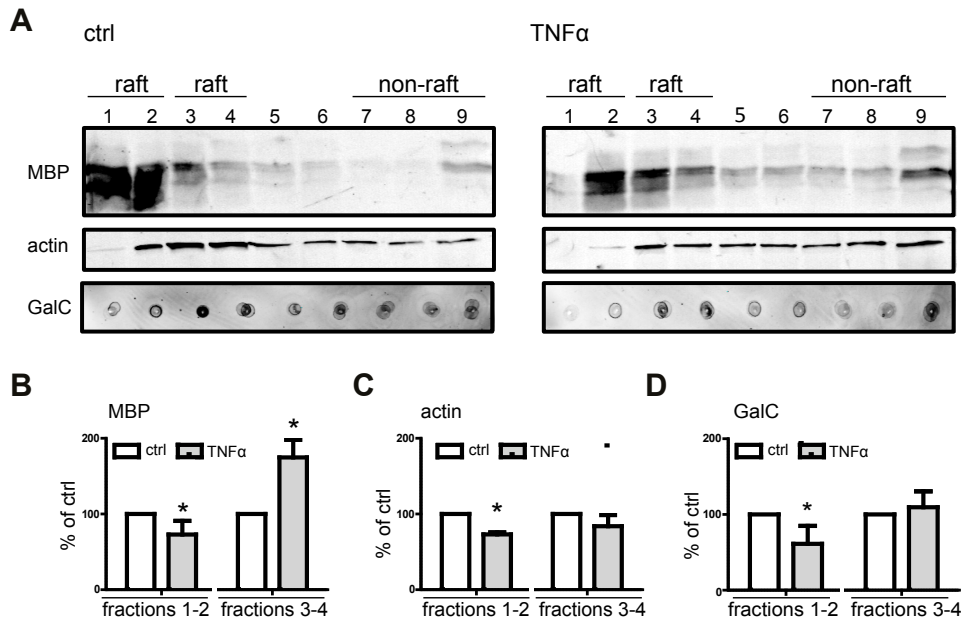


Figure 6. TNF α treatment alters MBP's microdomain association from actin-dependent to actin-independent

Mature oligodendrocytes were left untreated (ctrl) or treated with 20 ng/ml TNF α for 3 days. The presence of proteins (A-C, MBP, actin) and GalC (A,D) in membrane microdomains isolated in a detergent-free manner in combination with OptiPrep density gradient centrifugation, and Western (MBP, actin) or dotblot (GalC) analysis. Representative blots of 4-5 independent experiments are shown. The total protein/lipid expression was calculated by adding the intensity of all the fractions. The protein/lipid percentage of each fraction was then calculated by dividing the protein intensity present in that fraction by total protein/lipid expression. Bar graphs of the pooled fraction percentage of actin-dependent raft fractions 1-2 and actin-independent raft fractions 3-4 of MBP (B), actin (C) and GalC (D) relative to untreated cells (ctrl, set at 100% in each experiment) are shown. In untreated cells, 56.0 \pm 12.1% of MBP, 27.9 \pm 20.9% of actin and 23.2 \pm 4.7% of GalC were present in fractions 1-2, and 19.3 \pm 0.6% of MBP, 25.1 \pm 3.7% of actin and 29.5 \pm 4.4% of GalC in fractions 3-4. Statistical difference with untreated (ctrl) cells as assessed with a one sample t-test (* p<0.05). Note that upon TNF α treatment MBP redistributed from actin-independent membrane microdomains (A, B, fractions 1-2) to actin-dependent membrane microdomains (A, B, fractions 3-4).

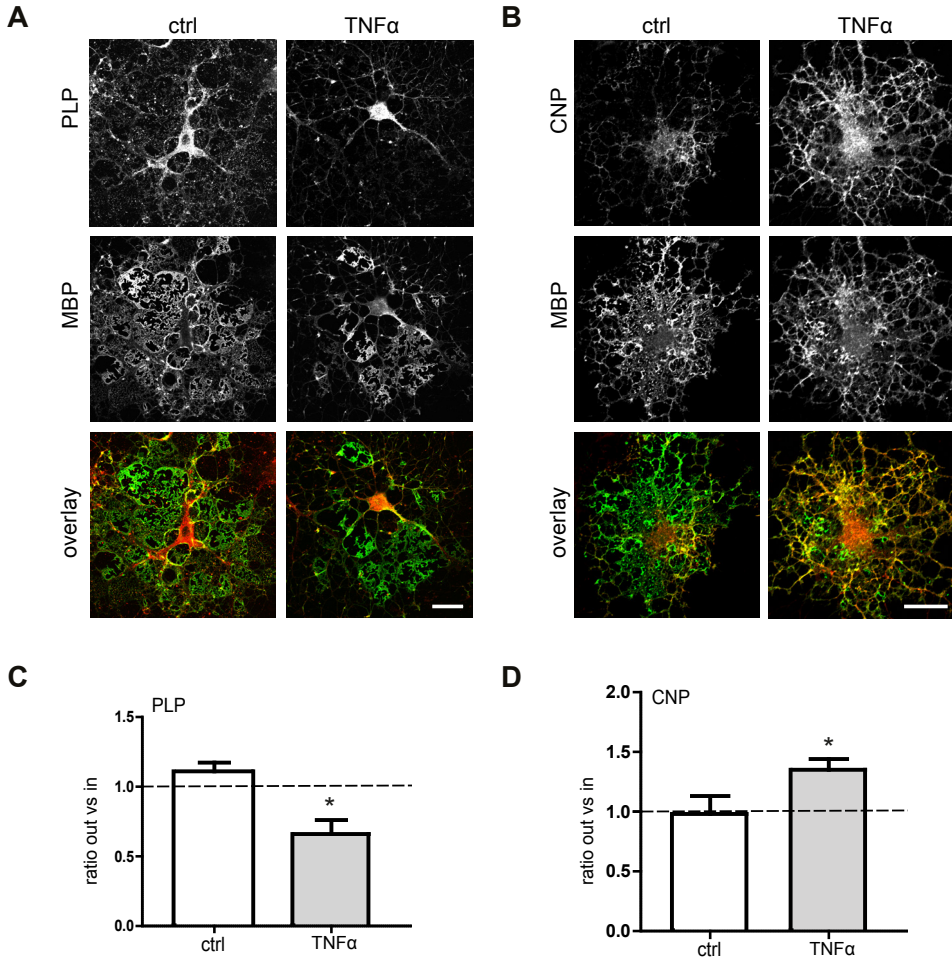


Figure 7. TNF α treatment interferes with the localization of myelin proteins PLP and CNP

Mature oligodendrocytes were left untreated (ctrl) or treated with 20 ng/ml TNF α for 3 days. Cells were subjected to double immunocytochemistry for MBP (green) and either PLP (**A, C**, red) or CNP (**B, D**, red). Representative confocal images are shown. Scale bar is 20 μ m. Quantitative analysis of the localization of PLP (**C**) and CNP (**D**) was performed as described in Materials and Methods (see also Fig. 1C). Each bar represents the mean + SD of 3 independent experiments. In each independent experiment 15 cells per condition were analyzed. Note that TNF α induces a retraction of PLP from myelin membranes towards primary processes, while CNP was more evenly distributed upon TNF α treatment.

DISCUSSION

Upon CNS demyelination, extrinsic factors such as extracellular matrix proteins and pro-inflammatory cytokines contribute to tissue repair. However, their persistent presence may be detrimental as exemplified by MS, where OLG apoptosis, demyelination and impaired remyelination may occur. In this context, we examined the effect of pro-inflammatory cytokine TNF α on myelin membrane integrity and stability. Our findings revealed that long-term treatment with TNF α induced a remarkable decrease in the length of myelin segments, and a lateral redistribution of MBP from the sheet towards cell body and primary processes in cultured mature OLGs. A similar enrichment of MBP in OLG cell bodies was reported following treatment of neuron-OLG co-cultures with TNF α ³²¹. In mature OLGs, TNF α treatment resulted in a perturbed actin cytoskeleton along with the dissociation of MBP from actin-dependent membrane microdomains. The effect of TNF α was not cytotoxic, reversible upon TNF α withdrawal, and likely mediated by interaction of the cytokine with TNFR1. Indirectly, our data also support the notion that MBP may act as a molecular barrier for other myelin proteins ³² given that TNF α exposure caused a concomitant redistribution of PLP towards primary processes and a reallocation of CNP towards the myelin sheets. The *in vivo* significance of these data may relate to a beneficiary effect of transiently present TNF α during remyelination, providing the necessary plasticity to existing myelin membranes, allowing the intercalation of newly formed myelin segments and paranodes to be 'reconstructed'.

Upon TNF α treatment, the length of the MBP-positive internodes significantly decreased in myelinated cultures, indicating that the internodes permit remodeling of existing myelin segments when necessary. In this context it was recently demonstrated that in the adult brain myelin segments may remodel and that the length of internodes shorten with age ³²². To shorten myelin segments, myelin decompaction has to occur, which requires a reallocation of myelin proteins and lipids. The present study suggests that the presence of TNF α might be a key feature in triggering and/or facilitating such a decompaction. Thus, in mature OLGs the presence of TNF α caused a lateral dislocation of MBP from myelin membranes towards the cell body and primary processes. The concomitant redistribution of MBP from actin-dependent to actin-independent membrane microdomains may be instrumental in the overall mechanism of decompaction, its occurrence being supported by an altered localization of other myelin components, such as PLP and CNP, presumably reflecting the relief of MBP's barrier function ³².

It remains to be determined whether the perturbation of the actin cytoskeleton is a direct effect of TNF α treatment or a secondary response of MBP's lateral redistribution to actin-independent membrane microdomains. A direct effect of TNF α on actin filaments has been observed in other cell types ^{311–313}. Furthermore, upon actin cytoskeleton disruption by cytochalasin B, both MBP and GalC are mislocalized ^{79,80}. A TNF α -mediated disruption of the actin filaments may thus lead to an uncoupling of MBP and the actin cytoskeleton, resulting in the lateral redistribution of MBP to actin-independent membrane microdomains, and the

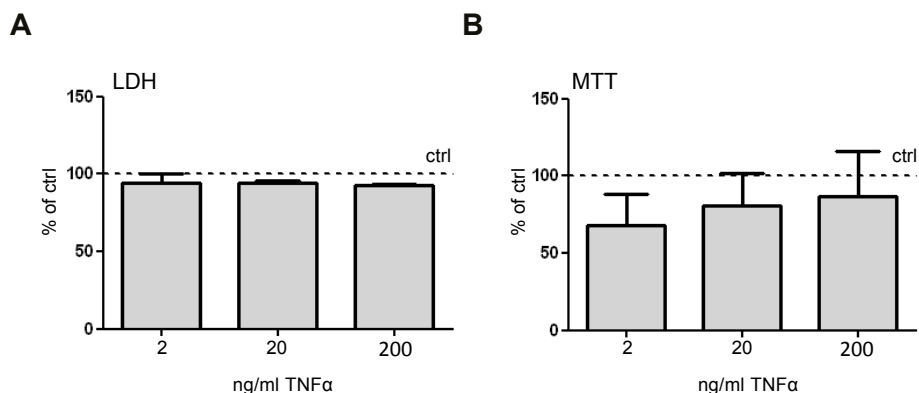
retraction of MBP towards cell body and primary processes. As a consequence, GalC might redistribute to TX-100-resistant membrane microdomains. Indeed, alterations in the lipid environment of MBP at the inner leaflet may result in clustering of GalC at the extracellular leaflet of the membrane³⁰⁹. In favor of a secondary, i.e., indirect effect of TNF α treatment on a perturbed actin cytoskeleton is the observation that MBP interacts with the actin cytoskeleton at the membrane surface⁹⁰, and that OLGs, in the absence of functional MBP, display a punctuated actin cytoskeleton⁸¹. Furthermore, MBP can undergo various posttranslational modifications, including methylation, phosphorylation, and deimination²⁰⁴, the latter two being able to modulate MBP-mediated assembly of actin⁹⁰. Thus, phosphorylation and deimination of MBP may decrease the ability of MBP to link actin to the membrane surface. In fact, we have noticed a slight decrease in the total levels of phosphorylated MBP upon long-term TNF α treatment (our unpublished observations). However, whether TNF α altered the deimination, or induced other reported posttranslational modifications of MBP remains to be determined. It is also possible that TNF α treatment leads to a segregation of GalC into TX-100-resistant membrane microdomains. This redistribution of GalC at the extracellular leaflet of the membrane may mediate a concomitant redistribution of MBP at the inner leaflet¹⁵, thus resulting in uncoupling of MBP and the actin cytoskeleton, and hence a perturbed actin cytoskeleton. Indeed, GalC plays a major role in the lateral membrane localization of MBP, i.e., antibody-mediated clustering of GalC alters the distribution of MBP and the actin cytoskeleton⁸⁰. Clearly, these considerations on the exact scenario of events upon TNF α treatment leading to myelin membrane shortening warrants further investigations.

Upon injury in healthy CNS, TNF α is only transiently secreted, and at those conditions its role is likely beneficiary for TNFR2-mediated OPC proliferation in relation to remyelination²⁹³ and, as shown here, TNFR1-mediated remodeling of existing myelin segments. However, in MS and other inflammatory demyelinating diseases, the level of TNF α is persistently increased³²³. Upon 'natural' remyelination, myelin sheaths are still in the process of internodal elongation during axonal enwrapment and compaction¹¹⁷. However, at disease conditions, reflected by the persistent presence of TNF α , the described lateral reallocation of myelin components in conjunction with the perturbed actin cytoskeleton might preclude the compaction of newly formed myelin sheaths, thus contributing to remyelination failure when TNF α is present persistently. Indeed, our preliminary results suggest that TNF α treatment of developing OLGs and myelinating cultures for 14 days exert similar effects as shown here for mature OLGs and myelinated cultures, further emphasizing the necessity to reduce TNF α -actions at later stages of the remyelination process.

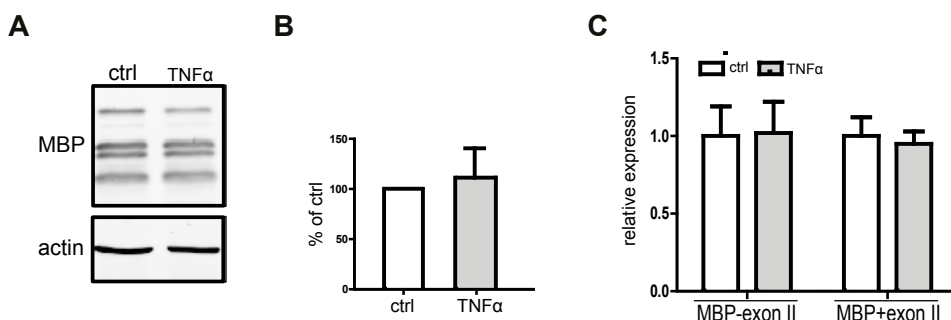
ACKNOWLEDGMENTS

We gratefully acknowledge Karin Klappe and Bich Nguyen for expert technical assistance. This work was supported by grants from the Dutch MS Research Foundation ('Stichting MS Research', W.B., M.B., D.H.), and the Foundation 'Jan Kornelis de Cock'. Parts of this work was performed at the UMCG Microscopy and Imaging Center (UMIC), sponsored by NWO grant 175-010-2009-023.

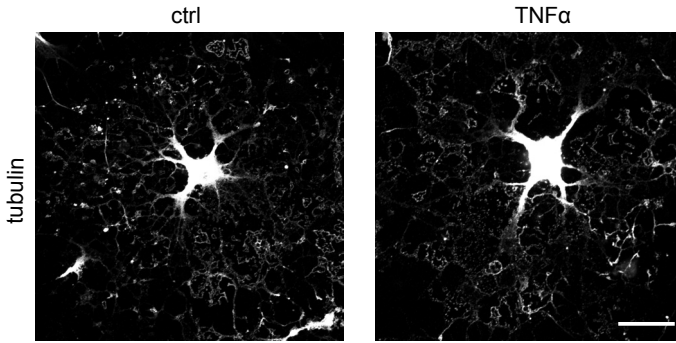
SUPPLEMENTARY FIGURES

**Supplementary Figure 1. TNF α is not toxic to mature oligodendrocytes**

Mature oligodendrocytes were left untreated (ctrl), or treated with 2, 20 or 200 ng/ml TNF α . After 3 days, LDH (**A**) and MTT (**B**) assays were performed. Each bar represent the mean + SD of the relative cytotoxicity (A) and viability (B) to untreated (ctrl) cells. Statistical analysis were performed with an one sample t-test.

**Supplementary Figure 2. TNF α treatment does not alter MBP protein and mRNA levels**

Mature oligodendrocytes were left untreated (ctrl), or treated with 20 ng/ml TNF α for 3 days. **A, B**) Cell lysates were analysed for protein levels of MBP and actin. Representative blots are shown (A). Expression of MBP, as a ratio of actin, was quantified relative to that of untreated cells (ctrl), which were set at 100% in each experiment (B). Each bar represent the mean + SD of 3 independent experiments. Statistical analysis were performed with an one sample t-test. **C**) Cells were subjected to real time qPCR analysis using specific primers for MBP isoforms with and without exon-II. mRNA expression was normalized to the house-keeping genes HMBS and HPRT1. Bars depict mean + SD of 3 independent experiments. Statistical analysis were performed with a one sample t-test. Note that upon 3 days exposure to TNF α the protein and mRNA levels of all MBP postnatal isoforms remain unaltered.



Supplementary Figure 3. TNF α treatment appears not to affect the tubulin cytoskeleton

Mature oligodendrocytes were left untreated (ctrl), or treated with 20 ng/ml TNF α . After 3 days, the tubulin cytoskeleton was visualized by immunocytochemistry (anti- β -tubulin). Scale bar is 20 μ m.

