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Investigations towards protective treatments for demyelination

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List of abbreviations

APC	Adenomatous Polyposis Coli
ANOVA	Analysis of Variance
BBB	Blood-brain-barrier
BDNF	Brain-Derived Neurotrophic Factor
Brdu	Bromodeoxyuridine
bFGF	Basic Fibroblast Growth Factor
cDNA	Complementary Deoxyribonucleic Acid
CCL3	Chemokine (C-C motif) Ligand 3
CIS	Clinically Isolated Syndrome
COX-2	Cyclooxygenase 2
CNTF	Ciliary Neurotrophic Factor
CXCL	C-X-C chemokine ligand
DAPI	3,3´-Diaminobenzidine
DC	Dendritic Cell
DHODH	Dihydroorotate Dehydrogenase
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylfumarate
DMT	Disease Modifying Treatment
DMSO	Dimethyl Sulfoxide
EAE	Experimental Autoimmune Encephalomyelitis
ECH	Kelch-like Erythroid Cell-derived
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence-activated Cell Scanning
FBS	Fetal Bovine Serum
FGF2	Fibroblast Growth Factor 2
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GalC	Anti-galactocereboside
GDNF	Glial-cell-line-derived Neurotrophic Factor
GFAP	Glial Fibrillary Acidic Protein
HCAR2	Hydroxycarboxylic Acid Receptor 2
HCl	Hydrochloric Acid

HPRT-1	Hypoxanthine Phosphoribosyltransferase 1
IFN	Interferon
IGF	Insulin-like Growth Factors
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JAK	Janus Kinase
KEAP	Kelch-like ECH-associated Protein
LPC	Lysolecithin
LPS	Lipopolysaccharide
MBP	Myelin Basic Protein
MMF	Monomethylfumarate
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
MRC1	Mannose Receptor 1
NF-κB	Nuclear Factor-kappa B
NGF	Nerve Growth Factor
NK	Natural Killer Cell
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
Nrf2	Nuclear Factor (erythroid derived 2)-like 2
NQO1	NAD(P)H:Quinone Oxidoreductase 1
Olig-2	Oligodendrocyte Transcription Factor
OPC	Oligodendrocyte Precursor Cell
OSC	Organotypic Brain Slice Culture
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PDGFa	Platelet-Derived Growth Factor A
PFA	Paraformaldehyde
PLL	Poly-L-Lysine
PLP	Proteolipid Protein
PPMS	Primary Progressive Multiple Sclerosis
RA	Rheumatoid Arthritis
RRMS	Relapsing-remitting Multiple Sclerosis
SEM	Standard Error of the Mean

SPMS	Secondary Progressive Multiple Sclerosis
TMEV	Theiler’s Murine Encephalomyelitis Virus
TNF	Tumor Necrosis Factor
Th	T helper cell
qPCR	Quantitative Polymerase Chain Reaction

Untersuchungen zur protektiven Behandlung gegen die Demyelinisierung

Jessica Kronenberg

1. Zusammenfassung

Die Multiple Sklerose (MS) ist eine chronisch entzündliche, demyelinisierende Erkrankung des zentralen Nervensystems (ZNS). Trotz vorhandener medikamentöser Behandlungen ist die MS eine der häufigsten Ursachen für Behinderungen im jungen Erwachsenenalter. Obwohl die zugelassenen Therapien hauptsächlich immunmodulatorisch oder immunsupressiv auf Lymphozyten wirken, werden immer häufiger auch Effekte auf Zellen des ZNS untersucht. Zwei solcher Medikamente sind Teriflunomid und Dimethylfumarat. Beide sind bereits für die Behandlung der MS zugelassene Medikamente und Ziel dieser Arbeit war es, deren Wirkmechanismus auf Gliazellen zu untersuchen.

Der Wirkmechanismus von Dimethylfumarat (DMF) und des Metaboliten Monomethylfumarat (MMF) ist noch nicht vollständig geklärt. Derzeit werden pleiotrope Wirkungen diskutiert. Neben immunmodulatorischen Effekten auf T-Zellen, wird auch die Aktivierung von antioxidativen Genen im ZNS diskutiert. In dem ersten Teil dieser Arbeit wurde der Einfluss von DMF und MMF auf die Genregulation diverser Faktoren in Astrozyten und Mikroglia untersucht. Unsere Ergebnisse zeigen, dass weder DMF noch MMF einen Effekt auf Astrozyten haben. Im Gegensatz dazu führte eine Behandlung mit DMF, in nicht aktivierten und Lipopolysaccharide (LPS)-aktivierten Mikroglia, zur Hochregulierung des Wachstumsfaktors IGF-1 und des Mannose-Rezeptors der Makrophagen (MRC1). Überstände von diesen behandelten Mikroglia wurden gewonnen und deren Effekte auf die Proliferation und Differenzierung von Oligodendrozytenvorläuferzellen untersucht. Hierbei konnte gezeigt werden, dass Überstände von Mikroglia, die mit DMF oder MMF behandelt wurden, die Proliferation von Oligodendrozytenvorläuferzellen signifikant erhöhten. Damit implizieren unsere Ergebnisse, dass DMF und MMF keinen Effekt auf Astrozyten haben. Allerdings verschiebt DMF den Phänotyp von Mikroglia zu einem anti-inflammatorischen Phänotypen mit weiteren indirekten Effekten auf die Proliferation von Oligodendrozytenvorläuferzellen.

Im zweiten Teil dieser Arbeit wurden die Effekte von Teriflunomid auf die verschiedenen Phasen der Myelinisierung, De- und Remyelinisierung untersucht. Wie bei DMF ist auch der Wirkmechanismus von Teriflunomid noch nicht vollständig geklärt. Es wird vermutet, dass die Proliferation von aktivierten Lymphozyten durch die nicht-kompetitive, reversible Inhibition des mitochondrialen Enzyms Dihydroorotat-Dehydrogenase (DHODH), gehemmt wird. Neben Zellkultursystemen, stehen auch *ex vivo* Modelle wie Kulturen von organotypischen Gehirnschnitten (OSC) zur Verfügung, um Effekte von Medikamenten auf Gliazellen zu untersuchen. Unsere Ergebnisse zeigen, dass eine Behandlung mit Teriflunomid die Demyeliniserung in OSC signifikant verringerte. Dieser Effekt korrelierte mit einer verminderten Proliferation von Mikroglia. Andere direkte oder indirekte Effekte auf Oligodendrozyten konnten in diesem Zusammenhang weder in OSC noch in primären Zellen gezeigt werden. Dies impliziert, dass die positive Auswirkung von Teriflunomid während der Demyelinisierung auf einen anti-proliferierenden Effekt auf Mikroglia und nicht durch einen direkten Effekt auf Oligodendrozyten zurückzuführen ist.

Zusammenfassend lässt sich festhalten, dass sowohl DMF als auch Teriflunomid die Funktion von Gliazellen beeinflussen können. Um weiter die therapeutischen Maßnahmen zur Behandlung der MS zu verbessern, ist es von großer Bedeutung, solche Effekte genauer zu untersuchen.

Investigations towards a protective treatment for demyelination

Jessica Kronenberg

2. Summary

Multiple sclerosis (MS) is a demyelinating disease which is characterized by infiltration of peripheral lymphocytes into the central nervous system (CNS) leading to neuroinflammation, neurodegeneration, and axonal loss. MS is one of the leading causes for disabilities in young adults. Approved therapies mainly modify disease progression by immunomodulatory or immunosuppressive functions. Direct and indirect effects on glial cells are often poorly understood. To determine further possible modes of action and identify potential therapeutic targets we studied the effects of two drugs, dimethylfumarate (DMF) and teriflunomide, which are approved for the treatment of relapsing-remitting MS, on glial cells.

Fumaric acids are hypothesized to shift activated immune cells to an anti-inflammatory phenotype that is currently only poorly understood. To clarify the role of fumaric acids on primary rodent astrocytes and microglia, we investigated the gene expression profiles of neurotrophic factors, growth factors, and cytokines after stimulation with DMF and its active metabolite monomethylfumarate (MMF). Further secondary effects of supernatants from DMF or MMF pretreated microglia on proliferation and differentiation of oligodendrocytes were evaluated by immunohistochemical analysis. We could demonstrate that astrocytes changed their expression pattern of growth factors and cytokines upon inflammatory stimuli. However, neither DMF nor MMF treatment altered this expression. In contrast, DMF treatment of microglia demonstrated an upregulation of Insulin-like growth factors 1 (IGF-1) and mannose receptor 1 (MRC1) gene expression. Supernatants of DMF and MMF pretreated microglia further enhanced proliferation of oligodendrocyte precursor cells. Hence, the gained results imply that the proposed potential neuroprotective effect of fumaric acid is not mediated by neurotrophic factors released by astrocytes. However, DMF shifts microglia into an anti-inflammatory phenotype with further indirect effects on oligodendrocyte precursor proliferation.

In the second part of this thesis we aimed to elucidate the role of teriflunomide on glial cells using a brain slice culture system. In a former study we could already show that teriflunomide had anti-inflammatory and anti-proliferative effects on microglia in a mixed glial culture. We now aimed to specify the role of teriflunomide on glial cells

during different stages of de- and remyelination by immunohistochemical stainings. Therefore, we used organotypic brain slice cultures (OSC), which are the only available model to examine the processes of myelination, de- and remyelination *in vitro.* Further indirect effects of teriflunomide treated microglial supernatants on oligodendrocyte precursor proliferation and differentiation were examined with primary cell cultures. We showed that teriflunomide treatment resulted in reduced demyelination in the slice culture system, which correlated to the reduced microglial proliferation. However, other direct effects on oligodendrocytes or indirect effects of microglia supernatants in primary cell cultures were not observed. This suggests that teriflunomide exerts its positive impact on demyelination by anti-proliferative effects on microglia and not by a direct effect on oligodendrocytes.

Taken together, we could detect certain effects of both fumaric acids and teriflunomide on glial cells that might support the identification of new potential drug targets, which are needed to improve the efficacy of MS therapies.

3. Introduction

3.1 Multiple Sclerosis (MS)

Demyelinating diseases of the central nervous system (CNS), such as MS, are still a common cause for serious disabilities among young adults. MS is characterized besides demyelination by chronic inflammation, degeneration and neuronal loss. With an early disease onset between 20 and 40 years, a total of 2.5 million people are affected worldwide (Compston & Coles 2008; Lassmann et al. 2007; Ruano et al. 2016). Although most of the patients suffer from relapsing-remitting MS (RRMS), some patients have a primary progressive MS (PPMS), which is characterized by progressive worsening of neurologic functions from disease onset. Over the years even patients with RRMS can develop a secondary progressive course (SPMS) (Lublin et al. 2014).

One hallmark of the disease is the disruption of the blood-brain-barrier (BBB) which leads to an infiltration of peripheral immune cells into the CNS. These autoreactive T cells attack myelin proteins causing demyelination, loss of oligodendrocytes, microglia activation, astrogliosis, and axonal damage/loss (Compston & Coles 2008; Frischer et al. 2009). Axons are surrounded by myelin sheaths which act as an electric insulator and allow fast saltatory nerve conduction. By loss of oligodendrocytes, the myelin-producing cells of the CNS, axons lose their myelin sheaths due to demyelination. This results among others in impaired nerve conduction velocity (Olsen & Akirav 2015) and restricted trophic support from oligodendrocytes to neurons (Bradl & Lassman 2010). Demyelinated axons then undergo physiological changes, including morphological alteration, such as swelling or a beaded appearance (Coggan et al. 2015). Ultimately, demyelinated areas show axonal loss and neurodegeneration (reviewed in Alizadeh et al. 2015; Peterson & Fujinami 2007). However, demyelination is not always permanent as spontaneous remyelination often occurs. Remyelination is often incomplete in MS patients and has, thus, been suggested as a marker of disease progression (Franklin & Ffrench-Constant 2008). Reasons for failure of remyelination are a limited replenishment of myelinating oligodendrocytes, insufficient maturation of oligodendrocytes, or incomplete clearance of myelin debris. It is either the result of a lack of growth factors or can be induced by release of inhibitory factors from activated glial cells (reviewed in Alizadeh et al. 2015). Moreover, an immune response is manifested in terms of inflammation that can further cause continuous damage to oligodendrocytes and inhibit remyelination (Olsen & Akirav 2015). Therefore, in order

to study the cellular and molecular mechanisms of MS, as well as investigate the mode of action of drugs, several animal models as well as *in vitro* cell culture systems have been used in the past.

3.2 Glial cells and their role in de- and remyelination

Function and development of the brain depends on glial cells (astrocytes, microglia, oligodendrocytes) which promote survival and differentiation of neurons and play an essential role in CNS disorders (Burda & Sofroniew 2014; Pfrieger & Barres 1997). Oligodendrocytes are the myelinating glial cells of the CNS and their major role is to form myelin sheaths around axons. These sheaths act as an electrical isolation and ensure fast saltatory nerve conduction. Under pathological conditions, such as MS, oligodendrocytes undergo cellular death and subsequently demyelination occurs. For repair mechanisms, the differentiation of oligodendrocyte precursor cells (OPC) into mature myelinating oligodendrocytes is extremely important (Bradl & Lassmann 2010; Kotter et al. 2006). Delay in OPC differentiation, therefore, leads to slow or incomplete remyelination, as seen in the human CNS or animal models of MS (Baxi et al. 2017; Sim et al. 2002). Accordingly, effective recruitment, differentiation, and migration of OPC, as well as clearance of myelin debris are needed for successful remyelination. Activated microglia and astrocytes are mainly involved in these processes (Dulamea 2017; Keirstead & Blakemore 1999; Lampron et al. 2015).

Microglia are the innate immune cells of the CNS and play a dual role during inflammation. They have the ability to react quickly to pathological changes, such as injury, inflammation, or neurodegeneration. During demyelination microglia become activated and show a pro-inflammatory (M1-like) or an anti-inflammatory (M2-like) phenotype. The M1-like phenotype is associated with secretion of pro-inflammatory factors, nitrogen species, and reactive oxygen, which are correlated with diffuse axonal injury and secondary demyelination (Edwards et al. 2006; Kutzelnigg et al. 2005). In contrast, the M2-like phenotype is thought to enhance remyelination by phagocytosis of myelin debris and promoting oligodendrocytes differentiation (Armstrong et al. 2002; Napoli & Neumann 2010; Yuen et al. 2013). Thus the M1/M2-balance is important for complete remyelination (Mikita et al. 2011).

Astrocytes, similar to microglia, have a dual role during inflammation, de- and remyelination in MS (Minagar et al. 2002). They are the most abundant glial cells of the CNS and protect neurons by releasing neurotrophic factors such as glial-cell-line-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF) (Miyamoto et al. 2015; Oh & Yong 1996). Other protective effects during demyelination are the induction of apoptosis of infiltrating activated T cells and the release of anti-inflammatory cytokines (Gimsa et al. 2004; Moore et al. 2011). While astrocytes can support microglia and OPC recruitment, as well as their maturation to mature oligodendrocytes during remyelination (Maysami et al. 2006; Moore et al. 2011; Skripuletz et al. 2013), they also delay remyelination by glial scar formation. This reactive gliosis inhibits axonal growth and regeneration, and further hinders migration of OPC into demyelinated areas (Anderson et al. 2016; Fawcett & Asher 1999).

3.3 How to study glial cells?

To study glial cells, there is a large set of animal models, *in vitro* cell culture systems, and *ex vivo* systems available. Several different animal models are used to study cellular and molecular mechanisms of de- and remyelination. The most commonly used animal model is an autoimmune inflammatory model (experimental autoimmune encephalomyelitis, EAE) (Gold et al. 2000). Besides that, toxin-induced models of demyelination, e.g. the cuprizone model (Gudi et al. 2014; Matsushima & Morell 2001) or focal injection of lysolecithin (LPC) (Hall 1972), as well as virus induced models of demyelination (e.g. Theiler virus, TMEV) (Ulrich et al. 2006) are frequently used. Less suitable models are genetic models with mutations in myelin encoding genes (Ben-nun et al. 2014, Roach et al. 1985). Although animal models offer many possibilities to study the mode of action of drugs or general cellular and molecular mechanisms of MS, one big disadvantage remains, which is their complex interaction within the body. Furthermore, every model only partly mimics MS pathophysiology for mechanistic investigations of drugs.

Therefore, *in vitro* models are a necessary tool to understand cellular interaction. In MS, glial cells play a crucial role in the regulation of homeostasis during inflammation, de- and remyelination. Thus, mixed glial cell cultures, composed of astrocytes, oligodendrocytes and microglia, are often used (McCarthy & de Vellis 1980; Milner et al. 1996). According to well established protocols investigation of effects on isolated

single cell types can be easily studied. Even the reproduction of different stages of oligodendrocytes is possible. Since primary single cell cultures lack contact to neighboring cells and do not mimic the phase-dependencies of de- and remyelination, other methods are required.

Regarding this, other methods are necessary to investigate phases of de- and remyelination within intact cellular interaction, as well as reduction and refinement of animal models. Consequently, organotypic slice cultures (OSC) have been shown to be a helpful *ex vivo* tool to study molecular and cellular processes of the brain. With OSC, explanted slices of the brain are placed onto semipermeable cell culture inserts and are fed by medium on the bottom side of the membrane. This procedure provides reliable control of the extracellular environment, which is a huge advantage compared to animal models (Cho et al. 2007). Yet, another great advantage of OSC is the preservation of the three-dimensional brain architecture and the structural organization of the cells, reflecting the *in vivo* situation better than single cell cultures (Humpel 2015; Stoppini et al. 1991). Moreover, it is possible to induce demyelination by applying the bioactive lipid LPC in OSC of the cerebellum, a process followed by remyelination (Birgbauer et al. 2004). Hence, OSC provide a favorable model for studying processes of myelination, de- and remyelination (Birgbauer et al. 2004; Miron et al. 2010; Stoppini et al. 1991). In summary, OSC represent a model of intermediate complexity between *in vitro* cell cultures and *in vivo* models (Madill et al. 2016). They can be easily used for substance testing, like commonly used drugs, and offer an improved understanding of their mode of action throughout the different phases of myelination, de- and remyelination (Drexler et al. 2010; Sundstrom et al. 2005).

3.4 Disease-modifying treatments

The symptoms and clinical type of MS vary in patients, depending on the progression of the disease and location of demyelinated areas within the CNS. Therefore, it is challenging to find the right disease-modifying treatment (DMT) for the patient that reduces frequency of relapses and disease progression. During an acute relapse, patients are mainly treated with glucocorticosteroids, which are potent immunosuppressive and anti-inflammatory drugs (Kieseier & Hartung 2003). To prevent further disease-activity other DMTs should be initiated as early as possible. The first-line drugs are interferon (IFN)-β1a/b and glatiramer acetate. These immunomodulatory drugs reduce the relapse rate, but do not alter regeneration

processes in the CNS. Newer agents such as dimethylfumarate (DMF) and teriflunomide are also approved for first-line treatment. These oral DMTs showed already high efficacy in phase III clinical trials, which is primarily driven by suppressing the inflammatory response (Gasperini & Ruggieri 2009). However, many patients show disease activity and relapses under treatment with first-line drugs or deleterious side effects. In patients with highly active MS or in case of a treatment failure with first-line drugs, other second-line drugs such as fingolimod, natalizumab, or alemtuzumab are used. Furthermore, a switch to another first-line therapy might be considerable (Dörr & Paul 2015). Recently, the two drugs cladribine and ocrelizumab have also been approved for the treatment of MS (Table 1).

Optimization of drug treatment is a major challenge in MS therapy to stop disease progression. In this respect, the knowledge of the mode of action of these drugs is essential.

Table 1: Disease modifying agents for the treatment of MS

	CIS	RRMS	SPMS	PPMS
Highly active		- Alemtuzumab - Fingolimod - Natalizumab - Cladribine - Ocrelizumab	- Interferon β - Mitoxantron	
Moderate course	- Glatiramer acetate - Interferon β	- DMF -Glatiramer acetate - Interferon β - Teriflunomide		- Ocrelizumab
Relapse	- Methylprednisolone plus - Plasma exchange/ immunoabsorption			

CIS: Clinically isolated syndrome; RRMS: Relapsing-remitting MS; SPSM: Secondary progressive MS; PPMS: Primary progressive MS

3.4.1 Fumaric acids

Fumaric acid esters have been used for the treatment of psoriasis for many years (Mrowietz et al. 1998). After oral intake, DMF (Tecfidera ®) is rapidly hydrolyzed to its metabolite monomethylfumarate (MMF). The mode of action is not yet fully understood. The immunomodulatory effect of DMF may be mediated by multiple mechanisms since pleiotropic biological effects have been observed. However, several studies have postulated an activation of nuclear factor (erythroid derived 2)-like2 (Nrf2). After exposure through oxidative stress, the increased expression of Nrf2 regulates gene expression of anti-inflammatory genes (Fig.1). In resting states Nrf2 is bound to Kelch-like erythroid cell-derived (ECH) associated protein-1 (KEAP1) in the cytoplasm. It has been shown that MMF binds to KEAP1 and facilitates the nuclear translocation of Nrf2, which results in upregulation of protective genes such as glutathione, NADPH: quinoline oxidoreductase-1 (NQO1) or aldo–keto reductase family 1 member B10 (Linker et al. 2011). In consequence, the translocation of NF-κB (nuclear factor κB) is inhibited and, thus, suppresses NF-κB-dependent transcription of pro-inflammatory cytokines (Kim & Vaziri 2010; Li et al. 2008; Wardyn et al. 2015).

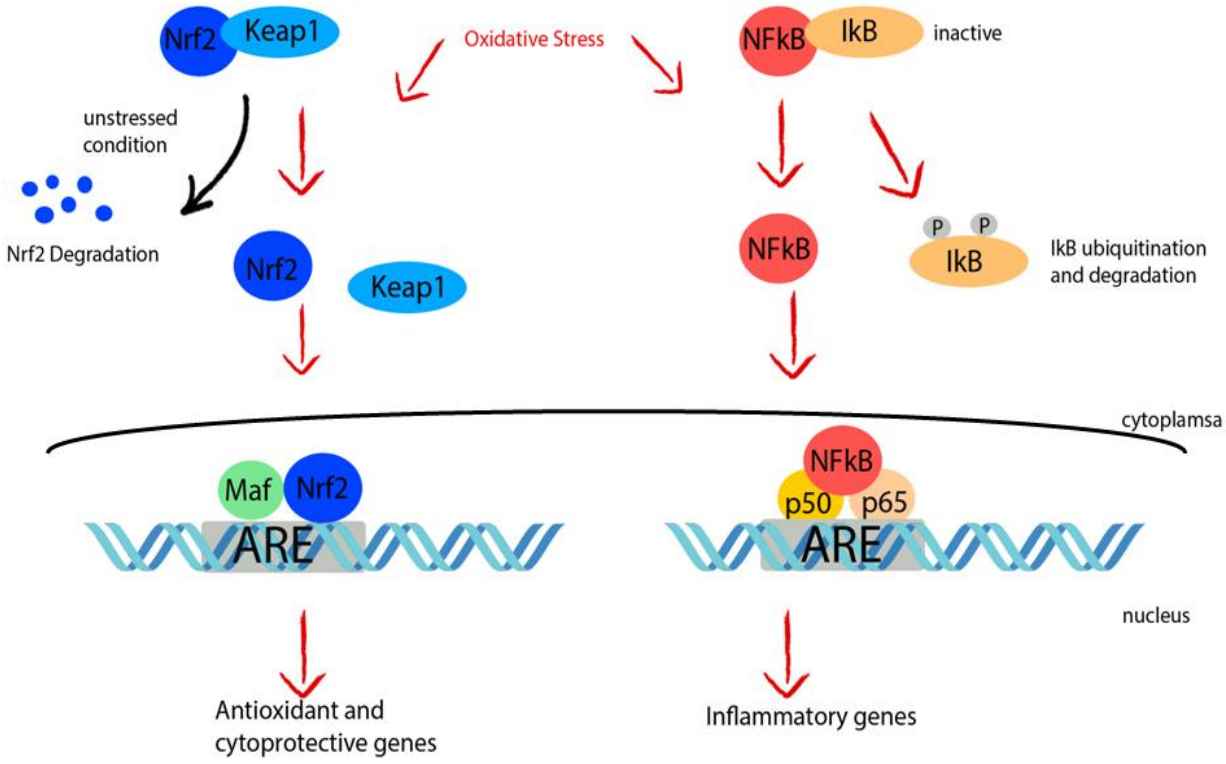

Figure 1: Nrf2 Pathway (modified from Kim & Vaziri 2010)

In the rodent model of myelin oligodendrocytes glycoprotein (MOG) induced EAE, treatment with DMF improved the clinical score of the animals. Additionally, reduced infiltration of microglia/macrophages, but not T cells, was found in inflammatory lesions (Schilling et al. 2006). Other *in vitro* studies have shown that MMF promotes the less inflammatory T helper (Th)-2 subset, rather than Th1 with increased interleukin (IL)-4, IL-5 and unaltered IFNγ as well as IL-2 production (de Jong et al. 1996). Similar effects have been seen in the regulation of dendritic cells (DC) and their influence on T cell response, which resulted in decreased immunological activity (Ghoreschi et al. 2011; Litjens et al. 2004; Zhu & Mrowietz 2001).

Newer studies focused on the *ex vivo* analysis of lymphocytes from MS patients. DMF therapy affected $CD8^+$ T cells, regulatory T cells and memory T cells, resulting in a shift towards an anti-inflammatory response (Berkovich & Weiner 2015; Gross et al. 2016; Longbrake et al. 2015). Even a modulation of the balance between pro- and anti-inflammatory B cell responses has been investigated by another group (Li et al. 2017). Recently, Kornberg et al. (2018) reported an effect of DMF and MMF on the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and thereby downregulation of aerobic glycolysis in activated myeloid and lymphoid cells. By and large, these results pointed out that the mode of action is seemed to be mainly driven by downregulation of pro-inflammatory responses of T cells and myeloid cells.

As DMF can enter the CNS, in relevant concentration, other neuroprotective effects on glial cells should be examined. There are only few data available which reveal this mechanism. In a co-culture model and a single cell culture with astrocytes and microglia lipopolysaccharide (LPS) induced production of IL-1ß, IL-6, tumor necrosis factor (TNF) α, as well as nitric oxide (NO) was reduced after DMF treatment. In addition, detoxifying enzymes were upregulated (Wierinckx et al. 2005; Wilms et al. 2010). Furthermore, DMF and MMF modulate microglia activation trough activation of the hydroxycarboxylic acid receptor 2 (HCAR2) towards an anti-inflammatory phenotype (Parodi et al. 2015). Direct effects of DMF on oligodendrocyte metabolism, which resulted in an enhanced antioxidant response and protection against oxidative stress, have also been reported (Huang et al. 2015). Regarding these findings, further studies are necessary to clarify the effect of DMF/MMF on glial cells.

3.4.2 Teriflunomide

Another oral immunomodulatory drug, teriflunomide (Aubagio®), has been approved for the treatment of RRMS since August 2013 in the EU (Chan et al. 2016). Teriflunomide is the active metabolite of leflunomide that was already used for the treatment of rheumatoid arthritis (RA) since 1998 (Rozman 1998). Besides the good efficacy and safety profile in RA, it has further been shown that leflunomide is very potent in treatments of several autoimmune diseases in animal models, such as organ transplantation or encephalitis (reviewed in Bartlett et al. 1991). First results in the EAE rat model presented that teriflunomide improved the outcome of clinical signs for EAE (Korn et al. 2004). A further study indicated similar results, including reduced demyelination, axonal loss, and inflammation in the relapsing-remitting Dark Agouti rat model of EAE (Merrill et al. 2009). These benefits are mediated due to reduced levels of infiltrating T cells, natural killer cells (NK), macrophages, and neutrophils, rather than a direct influence on cells of the CNS (Ringheim et al. 2013). The primary target of teriflunomide is the reversible and noncompetitive inhibition of the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH). This enzyme is required for the *de novo* pyrimidine synthesis by converting dihydroorotate into orotate, which is pivotal for the metabolism of highly proliferative cells such as activated lymphocytes (Bruneau et al. 1998; Rückemann et al. 1998; White et al. 2011). Inhibition of DHODH through teriflunomide had an immunosuppressive effect. In contrast, resting lymphocytes remain unaffected, because their pyrimidine synthesis relies on the salvage pathway that is DHODH independent (Fig.2) (Jameson 2002). The inhibitory effect can be abolished by exogenous addition of uridine (Korn et al. 2004; Li et al. 2013).

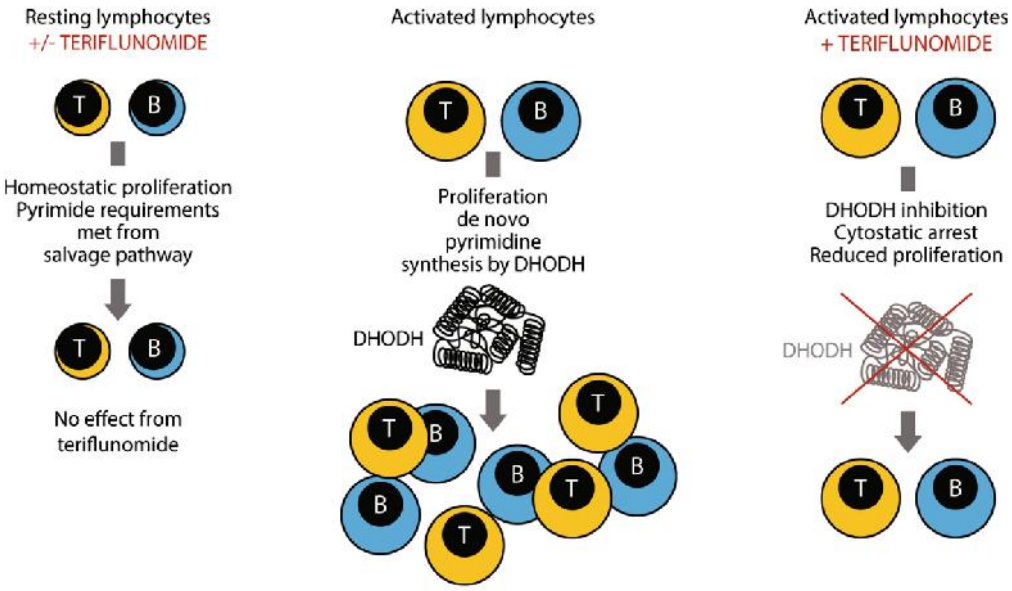

Figure 2: Teriflunomide´s presumed mode of action. Reprinted from (Bar-Or 2014), with permission from Elsevier

Interestingly, further DHODH independent or off-targets effects are reported in *in vitro* studies. For example, teriflunomide decreased the release of several pro-inflammatory cytokines from activated monocytes (Li et al. 2013). Inhibition of Janus Kinase 1 and 3 (JAK1/3) were also described as targets. These two tyrosine kinases are involved in the IL-2 secretion of T cells. Furthermore, teriflunomide inhibited the TNF-induced activation of NF-κB, which regulates gene expression of many pro-inflammatory cytokines (Manna et al. 2000). In rodent fibroblasts, the MEK/MAP pathway was inhibited, which suppressed activation of the inducible nitric oxide synthase (iNOS) (Korn et al. 2004). Finally, teriflunomide showed anti-inflammatory properties by inhibition of the accumulation of prostaglandin, which is an enzymatic product of cyclo-oxygenase-2 (COX-2) (Hamilton et al. 1999). Regarding these findings it is important to mention that these inhibitory effects were detected while using high concentrations of the drug (µM), compared to concentrations which are needed to block DHODH (nM) (reviewed in Claussen & Korn 2012).

However, the mode of action within the CNS is still unclear. Although, teriflunomide has only a low BBB penetration, approximately 1-2 % is found in the brain parenchyma (Kaplan 2015; Miller 2017). With oral treatment, a serum concentration of 20-60 mg/l is reached, which means that cells of the CNS may be exposed to a concentration of 0.2-0.6 mg/l teriflunomide *in vivo*, a concentration equivalent to approximately 1-3 µM. These concentrations are above the concentration level required for DHODH inhibition

(Limsakun & Menguy-Vacheron 2010; Wiese et al. 2013) and in the concentration range that influences T cells (Davis et al. 1996).

Recently, it has been shown that low concentrations of teriflunomide promote oligodendroglial cell differentiation and enhances *in vitro* myelination. A critical time frame was found, in which teriflunomide promotes differentiation of OPC into myelinating mature oligodendrocytes. Interestingly, higher concentrations decreased cell survival (Göttle et al. 2018). In a further study, it was demonstrated that microglia treated with teriflunomide showed higher expression of anti-inflammatory IL-10 after LPS treatment and reduced proliferation of microglia in mixed glial cell cultures (Wostradowski et al. 2016). Thus, investigation of the effect of teriflunomide on glial cells in a more complex model than isolated cell culture might clarify the beneficial mechanisms of the drug.

4. Aims

There are several approved disease modifying therapies for MS that modify the disease course. However, an ultimate therapy has not yet been found. Thus, it is highly relevant to determine off-target effects and identify new drug targets to improve efficacy of treatment. Two commonly used drugs are teriflunomide and fumaric acids that besides their immunomodulatory effects in the periphery may also have an impact on glial cells. Thus, the main objective of this thesis was to elucidate the role of teriflunomide and DMF, a fumaric acid ester, on glial cells in order to shed light into its mode of action and determine potential therapeutic targets.

In a first approach, the effects of fumaric acids on astrocytes and microglia were studied. The therapeutic mode of action of DMF, and its active metabolite MMF, is not yet fully understood. However, it is thought to be triggered through activation of the antioxidant Nrf2 pathway, as it has been described in several studies. Newer studies demonstrated that DMF shifts activated immune cells into an anti-inflammatory phenotype. Former reports suggest that the neuroprotective effect of DMF is exerted due to a reduced synthesis of pro-inflammatory factors in activated microglia and astrocytes. In order to assess this hypothesis, we performed gene expression analysis of neuroprotective factors, growth factors, as well as cytokines in activated and non-activated glial cells. As on the gene expression level, insulin-like growth factor (IGF-1) was upregulated in microglia pretreated with DMF, further ELISA and FACS analysis were performed to determine if the observed transcriptional changes also translate to protein levels. Phagocytosis, a hallmark of microglia activity, was also determined. Potential indirect effects of microglia on OPC proliferation and differentiation were evaluated by incubation with supernatants from microglia treated either with DMF or MMF.

In contrast to fumaric acids, the effect of teriflunomide on microglia was studied in OSC and primary cell cultures. OSC represents a helpful culture system to study the phases of de- and remyelination via LPC treatment because of its unique cell-cell architecture by closely mimicking the *in vivo* situation. The effects of teriflunomide on OPC proliferation and differentiation as well as microglial proliferation and astrocytes morphology were studied via immunohistochemical stainings during different stages of de- and remyelination in OSC. Further direct and indirect effects of teriflunomide on OPC proliferation and differentiation were evaluated in primary cell cultures.

5. Manuscript I

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Fumaric acids do not directly influence gene expression of neuroprotective factors in rodent astrocytes

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Author contributions

K.P., M.B., J.K., C.K.P. and M.S. designed research. M.B., J.K. and R.J. performed the experiments. K.P., M.B., J.K. and C.K.P. analyzed and interpreted the data. K.P., M.B., and J.K drafted the manuscript. M.S., T.S., R.P., and V.G. provided revision and support. All authors read and approved the manuscript.

5.1 Abstract

Background: Dimethylfumarate (DMF) has been approved for the treatment of relapsing remitting multiple sclerosis. However, the mode of action of DMF and its assumed active primary metabolite monomethylfumarate (MMF) is still not fully understood. Former reports suggest a neuroprotective effect of DMF mediated via astrocytes by reducing pro-inflammatory activation of these glial cells. We investigated potential direct effects of DMF and MMF on neuroprotective factors like neurotrophic factors and growth factors in astrocytes to elucidate further possible mechanisms of the mode of action of fumaric acids.

Methods: Primary rat astrocytes were pretreated *in vitro* with DMF or MMF and incubated with LPS or a mixture of IFN-γ plus IL-1β in order to simulate an inflammatory environment. The gene expression of neuroprotective factors such as neurotrophic factors (CNTF, BDNF, GDNF) and growth factors (NGF, PDGFa, FGF2) as well as cytokines (TNFα, IL-6, IL-1β) was examined by determining the transcription level with qPCR.

Results: The stimulation of astrocytes with either LPS or cytokines changed the expression profile of growth factors and pro-cytokines. However, the expression was not altered by neither DMF nor MMF.

Conclusions: There was no direct influence of fumaric acids on neuroprotective factors in primary rat astrocytes. This suggests that the proposed potential neuroprotective effect of fumaric acid is not mediated by direct stimulation of neurotrophic factors in astrocytes but is rather mediated by other pathways or indirect mechanisms via other glial cells and/or immune cells.

Key words: Glia, Astrocytes, Dimethylfumarate, Monomethylfumarate, Neuroprotection, Growth factors

5.2 Introduction

Multiple sclerosis (MS) is a chronic disease of the central nervous system (CNS) and is characterized by neuroinflammation, demyelination, and neuronal degeneration (Lassmann et al. 2007). It is a major cause of neurological disability in young adults (Hauser & Oksenberg et al. 2006). Fumaric acids are known to modulate the immune system and have been used in psoriasis treatment for many years. Many investigations examined immunomodulatory properties of DMF, and it is reported that fumaric acid is protective for neurons and glial cells and thus DMF is considered to be neuroprotective (Linker et al. 2011; Scannevin et al. 2012). Although the mode of action is not yet fully understood dimethylfumarate (DMF) has been approved for treatment of relapsing remitting multiple sclerosis. *In vitro* investigations demonstrated that DMF is hydrolyzed to its assumed bioactive primary metabolite monomethylfumarate (MMF) (Litjens et al. 2004a, b; Moharregh-Khiabani et al. 2009; Gold et al. 2012). Several investigations postulate a neuroprotective effect of DMF by inducing the nuclear factor (erythroid derived 2) like 2 (Nrf2) pathway and thus reducing toxic-oxidative stress (Ghoreschi et al. 2011; Williamson et al. 2012). In antigen presenting cells DMF stimulates type II dendritic cells (DC) which results in impaired secretion of pro-inflammatory interleukin (IL) 12 and IL-23 and increased production of the anti-inflammatory cytokine IL-10 (Ghoreschi et al. 2011). Furthermore, MMF induces the secretion of tumor necrosis factor alpha (TNFα) and anti-inflammatory IL-10 and IL-1RA in peripheral blood mononuclear cells (PBMC) *in vitro* (Asadullah et al. 1997). This indicates an inhibitory effect on inflammatory cells and a supporting impact on regulatory cells (Weiner 2009). However, there are only few experimental data available which explain the role of DMF and MMF within the CNS. Recent studies demonstrated a key role of astrocytes in the regulation of de- and remyelination in the CNS (Skripuletz et al. 2013). Therefore, it is of interest to investigate the influence of fumaric acids on astrocytes. There are reports on an anti-inflammatory effect of DMF on astrocytes by inhibiting pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), TNFα, IL-1β, and IL-6 (Wilms et al. 2010). To further elucidate the mode of action, we tested the hypothesis that DMF and MMF modulate the production of neurotrophic factors and growth factors in astrocytes *in vitro*. We thus analyzed the gene expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), platelet-derived growth factor subunit A (PDGFa), fibroblast growth factor 2 (FGF2) and ciliary neurotrophic factor

(CNTF) under the influence of DMF and MMF. Furthermore, we examined different time kinetics of pretreatment with fumaric acids and different types of LPS, cytokines, and exposition protocols as stimulators of astrocytes in order to simulate a suitable inflammatory situation in the CNS.

5.3 Material and Methods

Preparation and culture of astrocytes

Primary mixed glial cell cultures were prepared from neonatal Sprague-Dawley rats (P0-P3) as described previously (Kotsiari et al. 2010). Animals were maintained in the Central Animal Facility of the Hannover Medical School (MHH). All procedures were performed in compliance with the international guidelines on animal care and the review board for the care of animal subjects of the district government (Lower Saxony, Germany. Number: 2012/13). Brains were freed from meninges, the cerebellum, and the brain stem. Afterwards they were minced and further enzymatically dissociated by 0.1 % trypsin (Biochrom, Berlin, Germany) and 0.25 % DNase (Roche, Mannheim, Germany). The cells were then plated into culture flasks pre-coated with poly-l-lysine (PLL; Sigma-Aldrich, Hamburg, Germany). The flasks were filled up with medium consisting of Dulbecco´s Modified Eagle Medium (DMEM; Life Technologies Carlsbad, USA), 1 % penicillin/streptomycin (Sigma-Aldrich) and 10 % fetal bovine serum (FBS; Biochrom, Berlin, Germany). Cultures were incubated at 37 °C and 5 % CO_2.

Microglial cells were removed on day 7 by shaking at 37 °C for 45 min at 180 rpm on an orbital shaker (Edmund Bühler, Heching, Germany) and afterwards the medium was replaced. After resting for at least 2 h oligodendrocytes were removed by shaking at 37 °C at 180 rpm for 16-20 h. Supernatants, including oligodendrocyte precursor cells, were then removed and medium was replaced. 100 µM antimitotic arabinosylcytosine (Ara-C; Sigma-Aldrich) was added to each flask. Medium containing Ara-C was removed after 72 h, and the cells were washed with phosphate-buffered saline and harvested in trypsin/EDTA (0.05/0.02 %) solution (Biochrom). After counting, 3.0 x 10^5 cells were plated into 6-well plates. Astrocytes obtained following this protocol were referred to as highly enriched as demonstrated before (Prajeeth et al. 2017).

After a resting time of at least 4 days the cells were pretreated with 10 µM dimethylfumarate solution (DMF; Sigma-Aldrich) or 10 µM monomethylfumarate solution (MMF; Sigma-Aldrich) for 30 min or 24 h. In the control cultures the medium without MMF or DMF was changed accordingly. After 30 min or 24 h either 10 ng/ml lipopolysaccharide from *Escherichia coli* 055:B5 (LPS-E; Sigma-Aldrich), 100 ng/ml LPS-E, 10 ng/ml lipopolysaccharide from *Salmonella typhimurium* (LPS-S; Sigma-Aldrich), 100 ng/ml LPS-S, a cytokine mixture of 50 ng/ml interferon gamma (IFN-γ;

PeproTech, Rocky Hill, USA) and 10 ng/ml IL-1β (PeproTech), or medium were added. After 3, 6, 12, 24, and 48 h the supernatants and cells were collected.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Real-time quantitative polymerase chain reaction (qPCR) was performed for the genes NGF, BDNF, GDNF, PDGFa, FGF2 and CNTF. Ribonucleic acid (RNA) was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). Complementary deoxyribonucleic acid (cDNA) was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For qPCR analysis, the StepOne™ Real-Time PCR System and appropriate TaqMan assay (Applied Biosystems) were used (Table 1). The ΔΔCT method was used to determine differences in the expression between untreated and treated astrocytes. Gene expression was internally normalized against the housekeeping gene hypoxanthine-guanine-phosphoribosyl-transferase 1 (HPRT-1).

Table 1: Primer used for polymerase chain reaction

Gene	Gene expression assay number
NGF	Rn_01533872_m1
BNDF	Rn_00560868_m1
GDNF	Rn_00569510_m1
PDGFα	Rn_00709363_m1
FGF2	Rn_00570809_m1
CNTF	Rn_00755092_m1
Il-1ß	Rn_00580432_m1
IGF-1	Rn_00710306_m1
TNFα	Rn_99999017_m1
iNOS	Rn_00561646_m1
IL-6	Rn_01410330_m1
HPRT	Rn_01527840_m1

NGF nerve growth factor, *BDNF* brain-derived neurotrophic factor, *GDNF* glial cell line-derived neurotrophic factor, *PDGFα* platelet-derived growth factor subunit A, *FGF2* Basic fibroblast growth factor, *CNTF* ciliary neutrophic factor, *Il-1ß* interleukin 1-beta, *IGF-1* insulin-like growth factor 1, *TNFα* tumor necrosis factor, *iNOS* nitric oxide synthases, IL-6 interleukin 6, *HPRT* hypoxanthine-guanine-phosphoribosyl-transferase 1

Statistical analysis

All experiments were performed at least three times. GraphPad Prism version 5.02 was used for statistical analysis (GraphPad Software, Inc., La Jolla, CA, USA). One-way ANOVA followed by the Tukey's Multiple Comparison Test, or Bonferroni's Multiple Comparison Test for post hoc comparison was used for statistical analysis. Values are presented as the arithmetic means ± standard error of the mean (SEM). $P < 0.05$ was considered to indicate a statistically significant difference.

5.4 Results

DMF is biologically active and DMF and MMF are not toxic *in vitro*

First, we investigated a possible toxic effect of DMF and MMF on astrocytes *in vitro*. After an incubation of 24 h, 48 h, 72 h, and 96 h neither DMF (10 µM) nor MMF (10 µM) showed toxic effects on astrocytes *in vitro* (data not shown). It is well described that DMF reduces T cell counts *in vivo* and that DMF induces apoptosis of peripheral mononuclear blood cells (PBMC) *in vitro* (Treumer et al. 2003; Moharreg-Khiabani et al. 2010; Spencer et al. 2015; Gross et al. 2016). Therefore, we investigated effects of DMF (10 µM) on PBMC in our cell culture conditions and could demonstrate that DMF is biologically active *in vitro* (Supplemental data).

DMF and MMF have no effect on growth factor gene expression in activated astrocytes

The expression of the growth factors NGF, BDNF, GNDF, PDGFa, FGF2, and CNTF was measured in astrocytes after exposure to DMF (10 µM) and MMF (10 µM) for various time points. Except for an upregulation of FGF2 gene expression after 12 h of DMF treatment both DMF and MMF had no effect on unstimulated cells compared to control (Figure 1a). Thus, DMF and MMF did not modulate the expression of the tested growth factors in unstimulated astrocytes. In order to simulate an inflammatory environment as it may occur during an MS attack, astrocytes were stimulated with a mixture of cytokines (50 ng/ml IFN-γ and 10 ng/ml IL-1β) that are known to activate astrocytes (Chung & Benveniste 1990). Because in patients DMF treatment is given continuously with an MS attack occurring during DMF exposure the cells were pretreated with DMF (10 µM) or MMF (10 µM) for 24 h and were then activated by the cytokine mix. Astrocytes were harvested 3, 12, 24, and 48 h after activation (Figure 1b).

After cytokine stimulation gene expression of NGF and CNTF was significantly downregulated whereas gene expression of GDNF and PDGFa was increased. BDNF and FGF2 showed no measurable alterations. However, pretreatment with DMF or MMF for 24 h had no influence on any of these activation-mediated regulations.

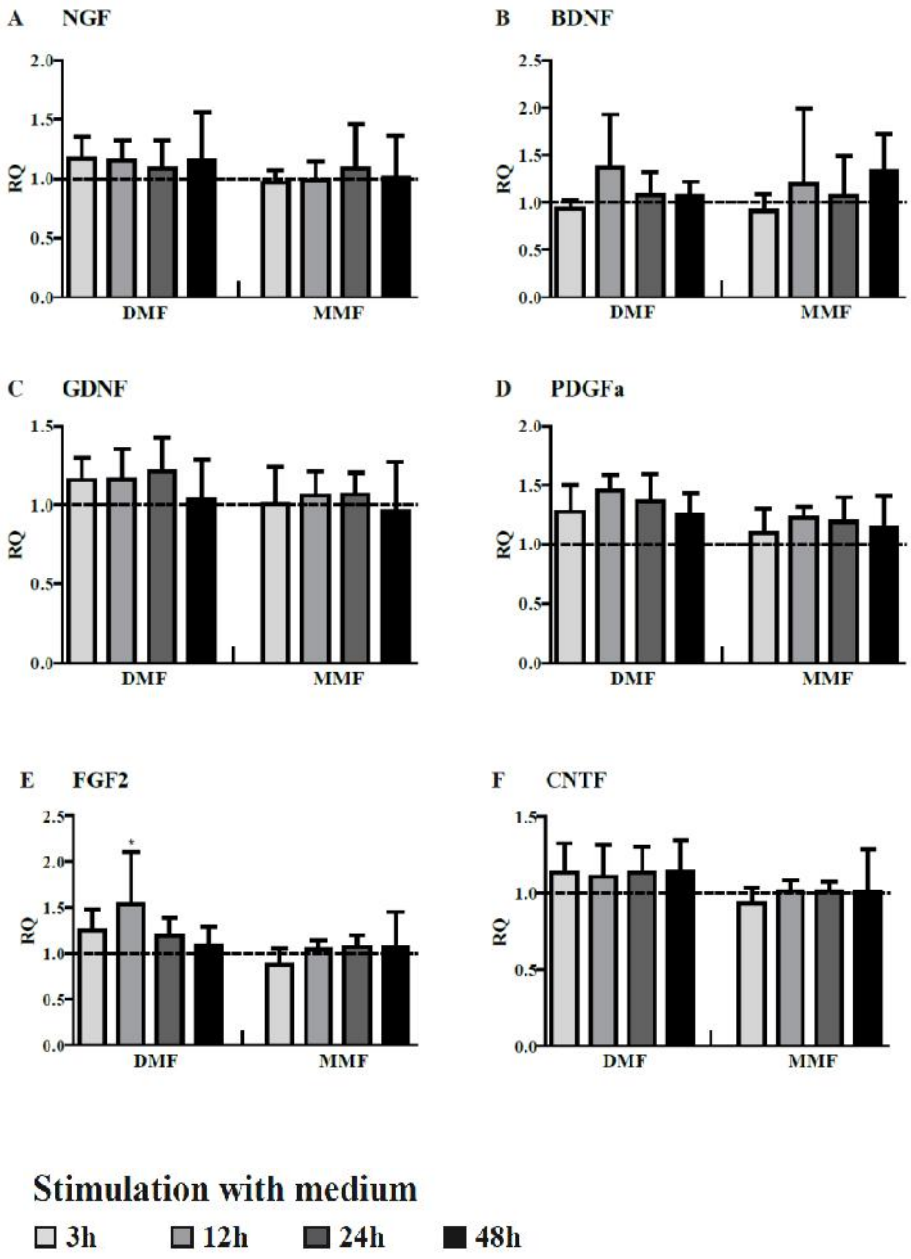


Figure 1a: Gene expression of neurotrophic factors and growth factors in astrocytes pretreated with DMF or MMF. Astrocytes were pretreated with medium, 10 µM DMF or 10 µM MMF for 24 h. Graphs show mRNA expression fold changes of NGF **(A)**, BDNF **(B)**, GDNF **(C)**, PDGFa **(D)**, FGF2 **(E)**, and CNTF **(F)** after 3, 12, 24 or 48 h compared to the control group (astrocytes only treated with medium) and normalized with HPRT-1 using the ΔΔCT method. Data are presented as the arithmetic means ± SEM of n = 3-6. Significant differences are marked by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

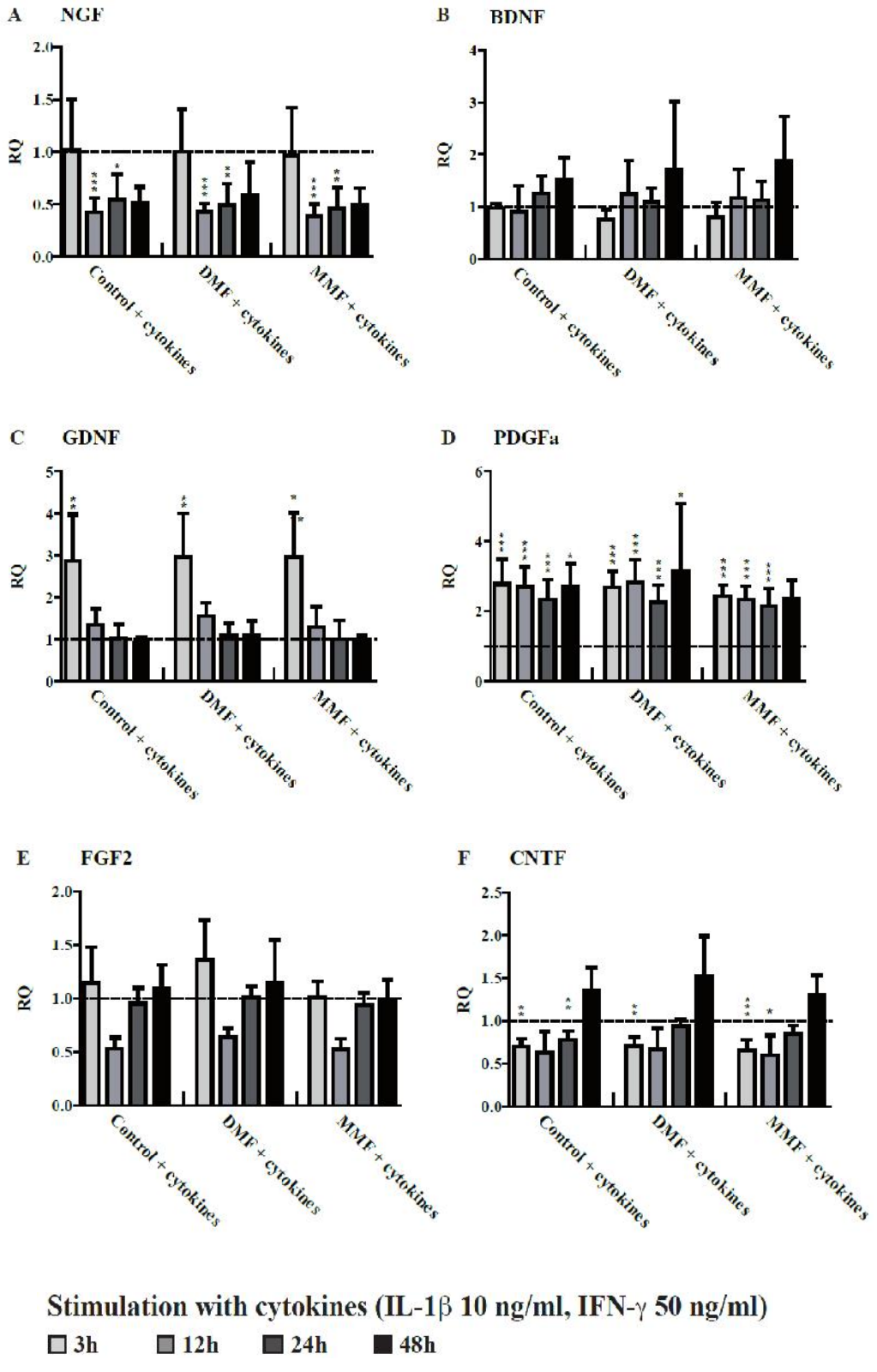


Figure 1b: Gene expression of neurotrophic factors and growth factors in astrocytes pretreated with DMF or MMF and stimulated with cytokines. Astrocytes were pretreated with medium, 10 µM DMF or 10 µM MMF for 24 h and afterwards stimulated with cytokines (50 ng/ml IFN-γ and 10 ng/ml IL-1β) for another 3, 12, 24 or 48 h. Graphs show mRNA expression fold changes of NGF **(A)**, BDNF **(B)**, GDNF **(C)**, PDGFa **(D)**, FGF2 **(E)**, and CNTF **(F)** compared to the control group (astrocytes only treated with medium) and normalized with HPRT-1 using the ΔΔCT method. Data are presented as the arithmetic means ± SEM of n = 3-6. Significant differences are marked by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

DMF and MMF have no effect on growth factor and cytokine expression in lipopolysaccharide stimulated astrocytes

In order to investigate another well-established inflammatory stimulus for astrocytes we used Lipopolysaccharides (LPS) as a ligand of the toll-like receptor 4 (TLR4) (Rosciszewski et al. 2017). In a previous publication LPS from Salmonella typhimurium was used to describe effects of DMF on microglia and astrocytes (Wilms et al. 2010). However, since LPS from different bacteria show a structural diversity (Heinrichs et al. 1998) and microglial TLR4 can differentiate between the class of LPS structures (Regen et al. 2011) we first wanted to determine potential effects of LPS from different bacteria sources. Hence, we investigated two different concentrations of LPS-E (lipopolysaccharide from Escherichia coli 055:B5) and LPS-S (lipopolysaccharide from Salmonella typhimurium) on cytokine, neurotrophic factor, and growth factor expression in astrocytes. Astrocytes were stimulated for 6 h with 10 ng/ml or 100 ng/ml of either LPS-E or LPS-S. Both LPS-E and LPS-S treatment led to a significant increase of gene expression of the pro-inflammatory mediator IL-1β, while there was a downregulated gene expression of the anti-inflammatory insulin-like growth factor 1 (IGF-1) and no change in FGF2 expression (Figure 2). There were no differences between different LPS sources and concentrations, so we decided to use LPS-E for further investigations.

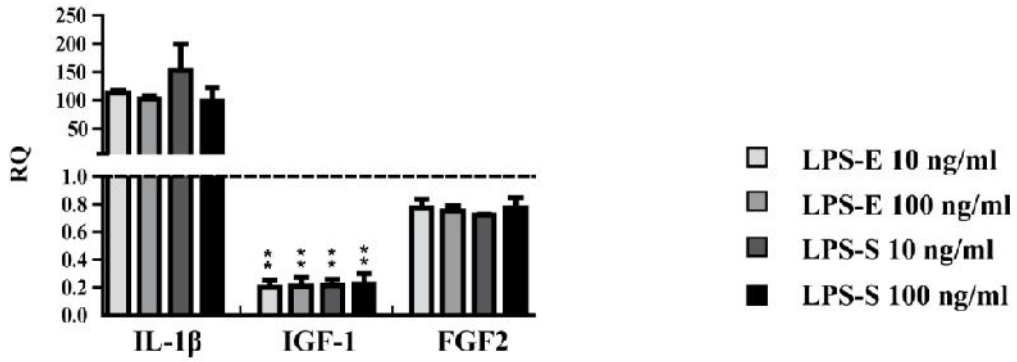
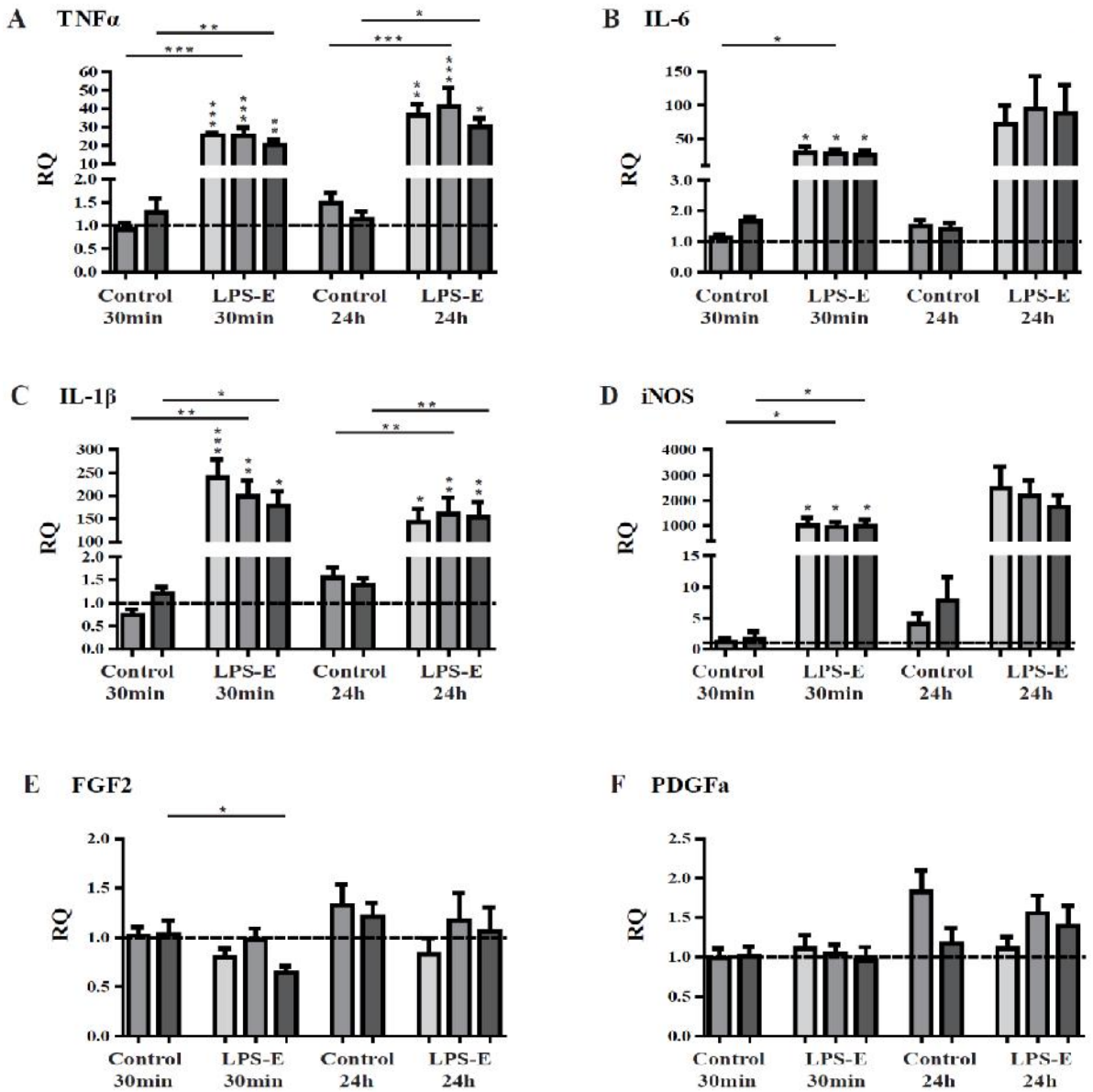


Figure 2: Effect of different types and concentrations of LPS on gene expression of pro-inflammatory IL-1β and growth factors IGF-1 and FGF2. Astrocytes were stimulated for 6 h with 10 ng/ml or 100 ng/ml of either LPS-E (lipopolysaccharide from *Escherichia coli* 055:B5) or LPS-S (lipopolysaccharide from *Salmonella typhimurium*). Graphs show mRNA expression fold changes of IL-1β, IGF-1, and FGF2 compared to the control group (astrocytes only treated with medium) and normalized with HPRT-1 using the ΔΔCT method. Data are presented as the arithmetic means ± SEM of n = 4. Significant differences are marked by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

In analogy to the cytokine stimulation of astrocytes we pretreated primary rat astrocytes with DMF (10 µM) or MMF (10 µM) for 30 min and 24 h, respectively. This was followed by a stimulation with LPS-E (10 ng/ml) for another 6 h. Afterwards we studied the expression of TNFα, IL-6, IL-1β, iNOS, FGF2, PDGFa, and CNTF by qPCR (Figure 3). Although LPS had a strong effect on the gene expression in astrocytes, there were no effects of DMF or MMF detectable at both pretreatment periods on the expression of the factors mentioned above.



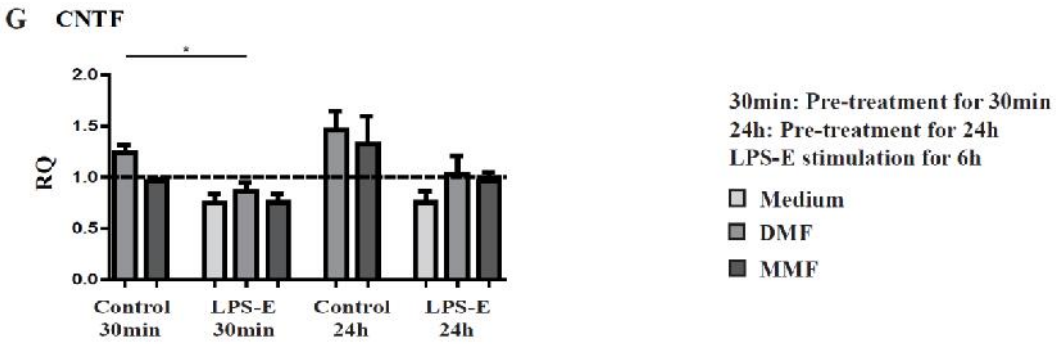


Figure 3: Gene expression of pro-inflammatory cytokines and growth factors in astrocytes pretreated with DMF or MMF and stimulated with LPS. Astrocytes were pretreated with medium, 10 µM DMF or 10 µM MMF for 30 min or 24 h and afterwards stimulated with 10 ng/ml LPS-E (lipopolysaccharide from *Escherichia coli* 055:B5). Graphs show mRNA expression fold changes of TNFα **(A)**, IL-6 (**B)**, IL-1β **(C)**, iNOS **(D)**, FGF2 **(E)**, PDGFa **(F)** and CNTF **(G)** compared to the control group (astrocytes only treated with medium) and normalized with HPRT-1 using the ΔΔCT method. Data are presented as the arithmetic means ± SEM of n = 4. Significant differences are marked by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

5.5 Discussion

The purpose of the present study was to further elucidate the mode of action of DMF and MMF on astrocytes. Hence, we investigated the influence of DMF and MMF on the gene expression of cytokines, growth factors, and neurotrophic factors in primary rat astrocytes. We used the most likely *in vivo* concentration after oral intake of DMF (10 µM) (Wilms et al. 2010; Rostami-Yazdi et al. 2009) and for MMF a comparable concentration to the maximal MMF concentration detected in serum of healthy subjects (10 µM) (Litjens et al. 2004a). Previous publications described that DMF induces apoptosis of peripheral blood mononuclear cells (PBMC) *in vitro* (Moharregh-Khiabani et al. 2010; Stoof et al. 2001). As a proof of principal, we used a similar concentration on PBMC that also showed an effect on the inhibition of PBMC proliferation comparable to published data (Supplemental figure 1). For a suitable simulation of a representative environment, e.g. an MS relapse during the treatment with DMF, we pretreated the cells with DMF or MMF for 24 h and then stimulated them with the cytokines IFN-γ (50 ng/ml) and IL-1β (10 ng/ml) for different periods of time (3, 12, 24, and 48 hours). Analysis of qPCR demonstrated that after cytokine activation expression of GDNF and PDGFa was upregulated while expression of NGF and CNTF was downregulated. There was no regulation of BDNF and FGF2. However, for all factors there was no further modulation by DMF or MMF. Thus, all measured alterations can be ascribed to the cytokine stimulation, irrespective of DMF or MMF pretreatment.

We further investigated the influence of bacteria-derived LPS to simulate inflammation since previous reports have illustrated that DMF decreases the synthesis of pro-inflammatory factors such as TNFα, IL-6, IL-1β, and iNOS in LPS stimulated astrocytes (Wilms et al. 2010). LPS is widely used to simulate an inflammation on target cells (Kipp et al. 2008). In previous investigations LPS from different bacterial sources was used and in different concentrations (Sawada et al. 1998; Xiang et al. 2014). In order to determine possible concentration- and/or bacteria-dependent effects we compared the gene expression of cytokines and growth factors in astrocytes treated with two different concentrations, 10 ng/ml and 100 ng/ml, of two different types of LPS derived from *Escherichia coli* 055:B5 and from *Salmonella typhimurium*. In all approaches, the results showed an increased expression of the pro-inflammatory mediator IL-1β as well as a downregulation of the anti-inflammatory factor IGF-1 compared to control. The expression of FGF2 was unchanged in all experimental groups. Thus, the effects of

LPS in different concentrations derived from different bacteria seem to be comparable when applied to astrocytes without any other stimulus.

In previous studies, different protocols of pretreatment with DMF were used, from no pretreatment before simulated inflammation (Lin et al. 2011) to 30 min of pretreatment. We investigated two different periods of pretreatment, 30 min and 24 h, before stimulation with LPS for another 6 h. Gene expression of cytokines, neurotrophic factors, and growth factors were analyzed by qPCR and demonstrated no effects apart from the LPS-induced changes.

We were not able to reproduce the effects described by Wilms et al. (2010) that DMF pretreated astrocytes stimulated by LPS induce a significant downregulation of mRNA synthesis for IL-1β, IL-6, and TNFα, and a moderate reduction of mRNA synthesis for iNOS in astrocytes. In our experiments there was a significant increase after LPS stimulation but DMF and MMF had no influence on either pro-inflammatory factors, neurotrophic factors, or on growth factors. Applying the same statistical analysis as Wilms et al. we set the results of not pretreated LPS stimulated cells as 100 % but this did not change our findings (data not shown). These divergent results could be due to different protocols for the preparation of the cells. Since we put extra attention on the purity of our astrocyte cultures, we may have had less microglia contaminations that could either directly or indirectly affect the results.

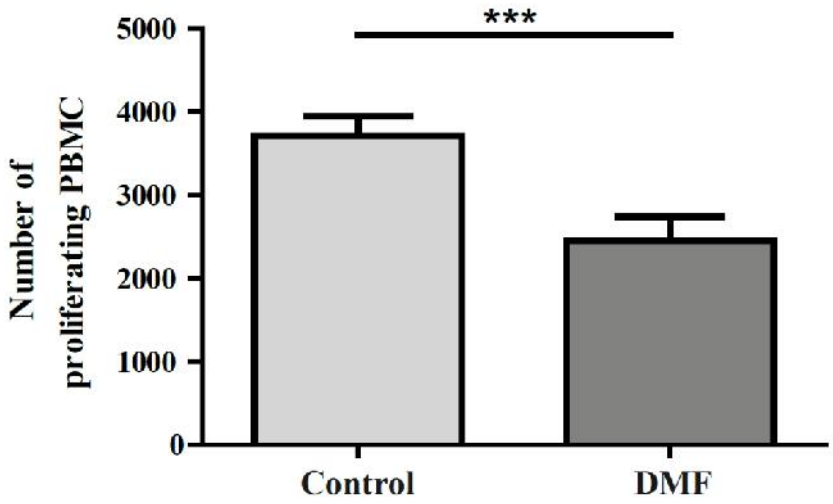
Although DMF has been proven as an effective oral MS therapy the immunomodulatory influence of the drug is still not fully understood and arguments need perpetual adjustments to new findings. Exemplary, former investigations claimed Nrf2 as the pivotal pathway for a possible neuroprotective action of DMF (Linker et al. 2011). However, recent studies with Nrf2-deficient ($Nrf2^{-/-}$) mice demonstrated a new perspective on the impotence of Nrf2 (Brennan et al. 2016) as oral DMF uptake revealed similar effects in $Nrf2^{-/-}$ and wild-type mice (Schulze-Topphoff et al. 2016). Similarly, DMF treatment of mice that were experimentally demyelinated did not lead to a protection of oligodendrocytes (Moharregh-Khiabani et al. 2010). DMF has been confirmed to have an impact on different types of cells including T cells as one main target. Studies demonstrated that under oral therapy with DMF CD4+ cells including pro-inflammatory Th1 cells as well as CD8+ T cells are reduced whereas Th2 cells are increased (Gross et al. 2016; Longbrake et al. 2015; Wu et al. 2017). Th1 cells activate astrocytes and microglia via pro-inflammatory cytokines, and thus induce myelin phagocytosis (Prajeeth et al. 2017, 2018). The effect of DMF on astrocytes is most

likely indirectly mediated by reduction of Th1 cells, followed by reduced astrocyte activation. Hence, beside the mentioned indirect involvement of astrocytes there might be no direct influence of DMF or MMF on neurotrophic factors and growth factors in astrocytes as an additional mode of action of fumaric acids. In former investigations MMF induced *in vitro* effects only at higher concentrations than found in serum of patients after intake of 120 mg DMF (one Fumaderm® tablet) (Rostami-Yazdi et al. 2009). The MMF concentration used in this study might have been too low to induce effects on astrocytes *in vitro* but was the highest realistic *in vivo* concentration in the CNS.

In summary, our results indicate that neither DMF nor MMF directly affect gene expression levels of pro-inflammatory factors, neurotrophic factors, and growth factors in astrocytes, irrespective of the time of pretreatment with fumaric acids or the mechanism of astrocyte stimulation.

5.6 Supplementary Data

Proliferation of CD3 activated PBMC was significantly inhibited after treatment with DMF. The number of newly generated cells was decreased by 33 %. Not activated PBMC showed no differences in proliferation after DMF treatment compared to control (Supplemental figure 1). Thus, the selected DMF concentration had the expected effect in our culture conditions.



Supplementary Figure 1: ***Proliferation of PBMC.*** PBMC from healthy human donors were either activated by plate-bound anti-CD3 mAb or remained not activated and were treated with either control (medium, methanol and PBS) or 10 µg/ml DMF for 48 h. Cells were then incubated with ^{3}H-thymidine for another 24 h. Radioactive incorporation was measured as counts per minute (cpm). Data are presented as the arithmetic means ± SEM (n = 10). Differences between the groups were analyzed by one-way-ANOVA followed by Tukey's post hoc test and are indicated by asterisks (***p < 0.001).

Preparation and culture of peripheral blood monocytic cells

Heparinized blood was taken from healthy human donors. Peripheral blood monocytic cells (PBMC) were isolated using a ficoll gradient centrifugation (Biocoll, Berlin, Germany) according to standard laboratory protocol. Afterwards, PBMC were washed with phosphate buffered saline (PBS) and centrifuged twice. Cells were resuspended in Roswell Park Memorial Institute (RPMI) medium 1640 (supplemented with 10% fetal bovine serum) (Biochrome, Berlin, Germany). 1.1 x 10^{6} cells per well were plated in 96-well plates pre-coated with anti-CD3 monoclonal antibodies (OKT3, purified from hybridoma supernatant).

Proliferation assay

PBMC were treated with either control consisting of resolvent or with 1 µg/ml dimethylfumarate solution for 48 h. Cells were incubated at 37 °C and 5 % CO_2. On day 3 ^{3}H-thymidine (Hartman analytics, Braunschweig, Germany) was added and cells were incubated for another 24 h. As marker for proliferation incorporated radioactivity was measured as counts per minute (cpm) by a scintillation beta-counter (Perkin Elmer, Rodgau, Germany).

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Conflict of interests

This study was partly funded by Biogen Corporation.

Ethical approval / Informed consent

The investigation of human peripheral blood monocytic cells (PBMC) was approved by the local ethics committee of the Hannover Medical School (Number: 1322-2010). All clinical investigations have been conducted according to Declaration of Helsinki.

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6. Manuscript II

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Fumaric acids directly influence gene expression of neuroprotective factors in rodent microglia

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Author contributions

J.K., K.P., M.B., C.K.P. and M.S. designed research. M.B. and J.K. performed the experiments. Apart from this S.H. and A.P. performed some of the experiments. J.K., M.B. and K.P analyzed and interpreted the data. C.K.P. helped to design FACS analysis and interpretation of data. J.K., M.B., and K.P drafted the manuscript. M.S., T.S., R.P., and P.S provided revision and support. All authors read and approved the manuscript.

6.1 Abstract

Dimethylfumarate (DMF) has been approved for treatment of relapsing-remitting multiple sclerosis. The mode of action of DMF and its assumed active primary metabolite monomethylfumarate (MMF) is still not fully understood, notably for brain resident cells. Therefore, we investigated potential direct effects of DMF and MMF on microglia and indirect effects on oligodendrocytes. Primary rat microglia were differentiated into M1-like, M2-like and M0 phenotypes and treated *in vitro* with DMF or MMF. The gene expression of pro-inflammatory and anti-inflammatory factors such as growth factors (IGF-1), interleukins (IL-10, IL-1β), chemokines (CCl3, CXCL-10) as well as cytokines (TGF-1β, TNFα), iNOS, and the mannose receptor (MRC1) was examined by determining the transcription level with qPCR and on the protein level by ELISA and FACS analysis. Furthermore, microglia function was determined by phagocytosis assays and indirect effects on oligodendroglial proliferation and differentiation. DMF treatment of M0 and M1-like polarized microglia demonstrated upregulation of gene expression for IGF-1 and MRC1, but not on the protein level. While the phagocytic activity remained unchanged, DMF and MMF treated microglia supernatants led to an enhanced proliferation of oligodendrocyte precursor cells (OPC). These results suggest that DMF has anti-inflammatory effects on microglia which may results in enhanced proliferation of OPC.

Keywords: Microglia, Dimethylfumarate, Monomethylfumarate, IGF-1

7. Manuscript III

Under preparation

Teriflunomide impairs demyelination of organotypic brain slice cultures

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Author contributions

J.K performed all experiments, acquisition of data, analysis and interpretation of data. J.K. and V.G. contribute to scientific design. Apart from this, S.H. and R.H. performed some of the experiments. J.K. drafted the manuscript. M.S. and V.G. provided revision and support. All authors read and approved the manuscript.

7.1 Abstract

Background: Teriflunomide has been proven to be effective in the therapy of multiple sclerosis (MS). Its main mechanism of action is thought to be the inhibition of proliferation of activated lymphocytes by selective inhibition of the enzyme dihydrooroate dehydrogenase (DHODH). We now aimed to clarify the role of teriflunomide on glial cells in an organotypic cerebellar slice culture system (OSC) during different stages of de- and remyelination.

Methods: Organotypic cerebellar slice cultures were cultivated from 10-day-old mice and left to fully myelinate for another 7 days. Demyelination was induced by lysolecithin and was followed by immunohistochemistry against myelin basic protein. Oligodendrocyte precursor proliferation and differentiation, microglia proliferation, and astrocyte morphology were investigated by immunohistochemistry during the course of de- and spontaneous remyelination. In order to further investigate glia-glia interactions primary cultured glial cells were used.

Results: Teriflunomide treatment of organotypic slice cultures during demyelination resulted in reduced myelin degradation, while we observed no effect on myelination or remyelination. Treatment during demyelination inhibited proliferation of microglia, however, indirect effects of teriflunomide treated microglia were not observed on oligodendrocyte precursor cells *in vitro*.

Conclusions: Our results suggest, that teriflunomide may be protective for myelin degradation which is possibly caused by an anti-proliferative effect on microglia.

Keywords: Multiple sclerosis, Teriflunomide, Organotypic cerebellar slice culture, Microglial, Astrocytes, Oligodendrocytes

7.2 Introduction

Teriflunomide (Aubagio®) has been approved since August 2012 in the EU as treatment for relapsing-remitting multiple sclerosis (RRMS). Its primary mode of action consists of reversible and noncompetitive inhibition of the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) (Bar-Or et al. 2014, Elder et al. 1997, Williamson et al. 1995). The enzyme DHODH is required for the *de novo* pyrimidine synthesis of proliferating lymphocytes. Thereby teriflunomide reduces the number of activated peripheral T and B lymphocytes which can infiltrate into the central nervous system (CNS). Resting lymphocytes rely on DHODH independent salvage pathways for pyrimidine synthesis and thus remain unaffected by teriflunomide treatment (Aly et al. 2017, Rückemann et al. 1998). Moreover, another DHODH independent mechanism, the inhibition of cytokine release that was not reversed by addition of uridine, has been shown (Li et al. 2013). Although teriflunomide has only a low blood-brain-barrier penetration, approximately 1-2 % of the serum concentration is found in the brain parenchyma, which corresponds to a concentration of at least 2.5-4.1 µM (Limsakun et al. 2010; Kaplan et al. 2015, Miller 2017). This implies that cells of the CNS, such as microglia or oligodendrocytes, may be exposed to and modulated by teriflunomide treatment. In fact, it has been shown that teriflunomide promotes oligodendroglial cell differentiation and enhances *in vitro* myelination. Hereby, a critical time frame was found, in which teriflunomide promotes differentiation of oligodendrocyte precursor cells (OPC) to myelinating mature oligodendrocytes (Göttle et al. 2018). In a former study, we could demonstrate that microglia treated with teriflunomide had an increased expression of the anti-inflammatory interleukin-10 (IL-10) after lipopolysaccharide (LPS) treatment and a reduced proliferation in mixed glial cell cultures (Wostradowski et al. 2016). In addition, teriflunomide decreased the release of several pro-inflammatory cytokines from activated monocytes in a DHODH-independent mechanism (Li et al. 2013). These results suggest that teriflunomide may modulate de- or remyelination due to its anti-proliferative and anti-inflammatory effects on microglia or by direct stimulation of OPC maturation.

In this study we investigated the effect of teriflunomide treatment on myelination, demyelination and remyelination in organotypic slice cultures (OSC), a widely used system to study de- and remyelination *in vitro* with the advantage that the complex cytoarchitecture of the CNS is preserved and cell to cell interaction reflects a situation

which is close to the *in vivo* environment (Harrer et al. 2009; Cho et al. 2007; Birgbauer et al. 2004).

7.3 Material and Methods

Organotypic brain slice culture

Organotypic brain slice cultures were prepared as previously described (Stoppini et al. 1991). Hence, 350 µm thick parasagittal slices of the cerebellum from postnatal 10-day-old (P10) C57BL/6JHanZtm mice were cut using a vibratome (Leica VT1000 S Vibrating blade microtome). Slices were cultured on Millicell-CM culture inserts (Millipore, Darmstadt, Germany) in medium containing 50 % minimum essential medium (Invitrogen, Carlsbad, CA), 25 % hank´s balanced salt solution (Lonza, Verviers, Belgium), 25 % horse serum (Invitrogen, Carlsbad, CA), 1 % penicillin/streptomycin, 6.5 mg/ml glucose (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA, USA) for a maximum of 14 days at 37 °C and 5 % CO2. Medium was changed every 2-3 days.

Pharmacological treatment of organotypic brain slice culture

Teriflunomide from a stock solution (stock: 10 mM, dissolved in dimethyl sulfoxide (DMSO); Genzyme, Waltham, MA; USA) was directly diluted in culture medium and changed every 2-3 days. For the myelination study, cultures were treated for five subsequent days with 25 µM of teriflunomide. For the de- and remyelination study, cultures were demyelinated with lysolecithin (0.5 mg/ml, LPC) for 15-17 h after 7 days-in-vitro (DIV). To determine the effect of teriflunomide on demyelination, slices were incubated with 3 µM, 10 µM or 25 µM from 6 DIV until 9 or 11 DIV. For the remyelination study, cultures were treated with 25 µM of teriflunomide from 9 DIV until 12 or 14 DIV (Fig. 1).

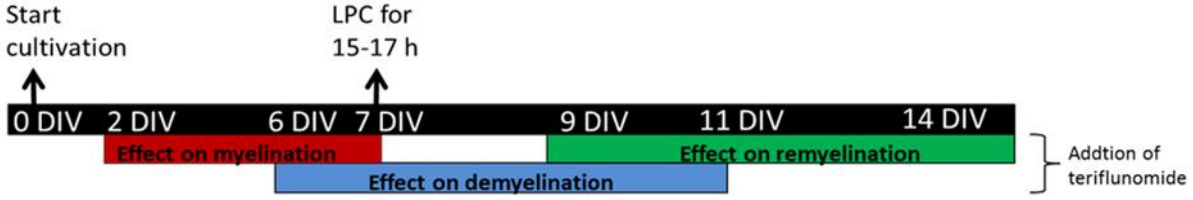


Figure 1: Pharmacological treatment of OSCs. Experimental setup. OSCs from 10-day-old mice were prepared and exposed to 3, 10 or 25 µM teriflunomide at indicated time points until fixation. LPC: Lysolecithin

Whole mount immunohistochemistry

Slices were fixed for 1-2 h in 4 % paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), and permeabilized for 1-2 h in 0.6 % Triton X-100 (Serva, Heidelberg, Germany). After blocking with 0.3 % Triton X-100 + 5 % normal goat serum (NGS) for at least 4 h, the slices were incubated with primary antibody diluted in 0.3 % Triton X-100 for 48 h by 4 °C. After washing three times with PBS, slices were incubated with secondary antibody diluted in 0.3 % Triton X-100 overnight by 4°C. Slices were washed tree times with PBS and finally mounted on glass slides.

The following primary antibodies were used: oligodendrocyte transcription factor 2 (Olig-2, polyclonal rabbit, 1:500, Millipore), myelin basic protein (MBP, 1:500, Biolegend, San Diego, USA), Glial fibrillary acidic protein (GFAP, 1:200, Millipore, Burlington, USA), Iba-1 (1:200, Wako, Neuss, Germany), Ki-67 (1:100, BD Pharmingen, San Jose, USA). Secondary antibodies were goat anti-mouse Alexa-555 and goat anti- rabbit Alexa-488 (all from Invitrogen). The amount of MPB staining was measured by calculating the percentage of colour intensity per 20x objective image using ImageJ Software (Fiji, U. S. National Institutes of Health, Bethesda, Maryland). The number of Olig-2, Ki-67, Iba-1 and APC immunopositive cells were counted in a blinded fashion from three individual images per slice.

Mixed glia cell cultures

For all experiments, neonatal Sprague-Dawley rats P0-P3 were used to prepare primary mixed glial cell cultures as previously described (Heckers et al. 2017). Therefore, brains were freed from meninges, choroid plexus, and brain stem. Afterwards they were minced and further enzymatically dissociated with 0.1 % trypsin (Biochrom, Berlin, Germany) and 0.25 % DNase (Roche, Mannheim, Germany). The cells were then plated into culture flasks pre-coated with poly-l-lysine (PLL; Sigma-Aldrich, Hamburg, Germany), filled with Dulbecco´s Modified Eagle Medium (DMEM; Life Technologies Carlsbad, USA) supplemented with 1 % penicillin/streptomycin (Sigma-Aldrich Hamburg, Germany) and 10 % fetal bovine serum (FBS; Biochrom, Berlin, Germany). Cultures were kept until use at 37 °C and 5 % CO2.

Microglial cells were isolated on day 7 by shaking at 37 °C for 45 min at 180 rpm on an orbital shaker (Edmund Bühler, Heching, Germany) and afterwards 300.000 cells were seeded on 12 well plates (Sarstedt, Nümbrecht, Germany). Microglia were

incubated overnight at 37 °C, 5 % CO2 and at the following day pretreated with 3 µM, 10 µM or 25 µM teriflunomide for 16 hours followed by further stimulation with IL-4 (20 ng/ml; Peprotech, Hamburg, Germany) for another 10 h. After washing with PBS medium was changed to serum free culture medium for further 16 h. Microglial supernatants were then harvested and kept at -80 °C until use.

After resting for at least 2 h, oligodendrocytes were isolated by shaking at 160 rpm for 16-20 h. Supernatants were collected, centrifuged, and cells were then transferred into an uncoated flask for 30 min at 37°C to reduce contamination of astrocytes and microglia. 80.000 cells were plated on PLL coated 12 mm glass coverslips and cultured in proliferation or differentiation medium for 24 h followed by incubation for another 48 h with teriflunomide treated microglia supernatant. In another approach, oligodendrocytes were incubated with 3 µM, 10 µM or 25 µM of teriflunomide with or without IL-4. Proliferation medium consisted of KnockOut™ DMEM/F-12 supplemented with GlutaMAX™, StemPro supplement, 20 ng/ml EGF, 20 ng/ml human FGF, 10 ng/ml PDGF-AA (all from Thermo Fisher Scientific, Osterode, Germany). Neurobasal® medium supplemented with GlutaMAX™, B-27 supplement (all from Thermo Fisher Scientific, Osterode, Germany), and 30 ng/ml T3 (Sigma-Aldrich, Hamburg, Germany) was used for oligodendrocytes differentiation. To collect astrocytes supernatant, cells were incubated with cytosine arabinoside (Ara-C; 8 µM; Sigma Aldrich, St. Louis, MO, USA) for 72 h. Then astrocytes were further incubated for 16 h with DMEM without serum. Cell culture supernatant were harvested and kept by -80 °C until use.

Treatment of oligodendrocytes

To evaluate the direct effect of teriflunomide on oligodendrocytes, cells were plated and allowed to proliferate or differentiate in normal culture medium for 24 h. Oligodendrocytes were then directly incubated with 3 µM, 10 µM, or 25 µM teriflunomide in culture medium for another 48 h. To determine if microglia treated with teriflunomide influence differentiation or proliferation of OPC, cells were then incubated with a ratio of 1:3 of defined culture media supplemented with pretreated microglia supernatants devoid of growth factors for another 48 h. To investigate whether supernatant from untreated astrocytes influences OPC proliferation, OPC were then incubated with a ratio of 1:3 of defined culture medium supplemented with untreated

astrocytes supernatants devoid of growth factors with addition of 3 µM, 10 µM, or 25 µM teriflunomide for further 48 h.

Cells were then fixed and stained as described in the following. To determine the differentiation index of mature oligodendrocytes to OPC, primary cells were incubated with anti-A2B5 (hybridoma supernatant, clone 105, European Collection of Cell Cultures) and anti-galactocereboside (GalC, hybridomasupernatant, clone IC-07, European Collection of CellCultures) supernatants for 30 min at 37 °C. After fixation with 4 % PFA, cells were incubated with the secondary antibodies AlexaFluor 488 goat anti-mouse IgG3 and AlexaFluor 555 goat anti-mouse IgMµ 1:500 (Thermo Fisher Scientific).

To investigate the percentage of proliferating OPC, cells were incubated for 3 h with 10 µM Bromodeoxyuridine (BrdU, Roche, Indianapolis, USA). Cultures were washed with PBS and incubated with anti-A2B5 supernatant. After fixation with 4 % PFA, cells were permeabilized with methanol at -20°C and DNA was denaturized with 2 M HCl at 37 °C (Roth, Karlsruhe, Germany). Cells were then neutralized with 0.1 M borate buffer pH 8.5 and stained with anti-BrdU 1:100 (BrdU, Roche, Indianapolis, USA) and incubated with secondary antibodies AlexaFluor 555 goat anti-mouse IgMµ and AlexaFluor 488 goat anti-mouse IgG 1:500 (Thermo Fisher Scientific).

Statistical analysis

All experiments were performed at least four times. GraphPad Prism version 5.02 was used for statistical analysis (GraphPad Software, Inc., La Jolla, CA, USA). Kruskal-Wallis test followed by Dunns post test was used for statistical analysis. Values are presented as arithmetic means ± standard error of the mean (SEM). $P < 0.05$ was considered to indicate a statistically significant difference.

7.4 Results

Effect of teriflunomide on myelination

To investigate the effect of teriflunomide on myelination of OSC, slices were treated for three or five subsequent days with 25 µM teriflunomide, starting at 2 DIV (Fig. 1). Slices were then analyzed immunohistochemically by MBP staining at 5 DIV and 7 DIV (Fig. 2). Teriflunomide treatment did not affect the amount of MBP staining which suggest that teriflunomide did not influences OSC myelination.

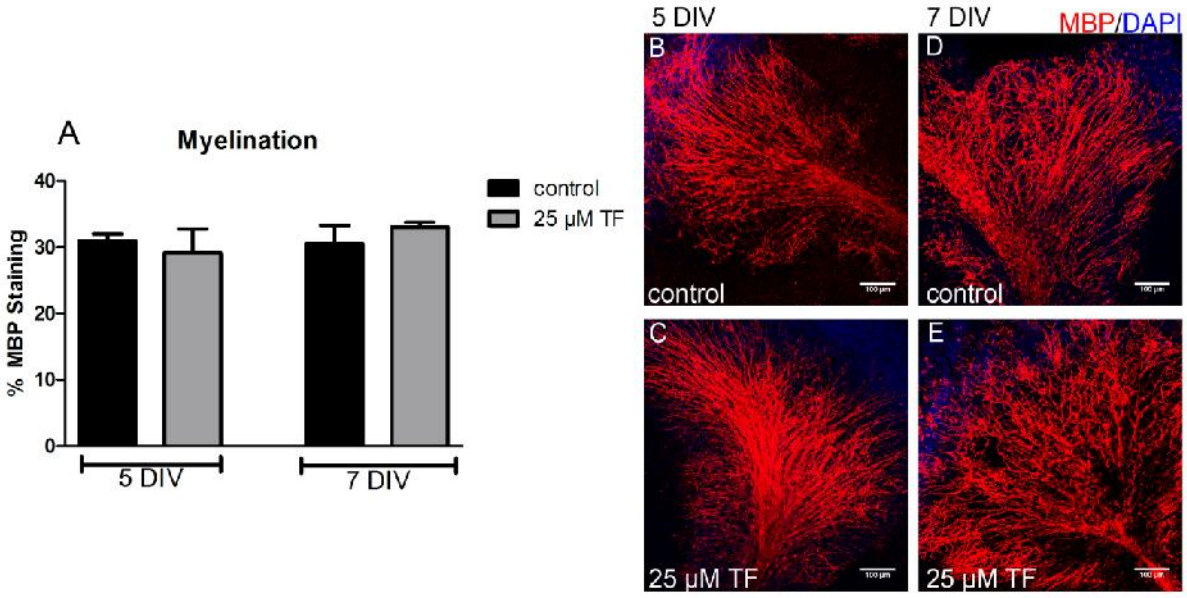


Figure 2: Effect of teriflunomide on myelination of OSCs. OSCs were treated with 25 µM teriflunomide at 2 DIV until fixation to determine its effect on myelination. Quantification of the amount of MBP showed no effect in cultures treated with 25 µM teriflunomide **(A)**. Representative images of slices at 5 DIV and 7 DIV stained for MBP **(B-E)**. Data are represented as mean ± SEM. Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test (n=4). *Scale bar 100 µM.* DIV: Days-in-vitro, TF: Teriflunomide

Effect of teriflunomide on demyelination

It has been shown that lysolecithin (LPC) causes focal demyelination when injected into the spinal cord or cerebellar peduncle *in vivo* or applied to OSC (Birgbauer et al. 2004). Following 7 DIV to allow recovery from slicing and permit final myelination, slices were treated with 0.5 mg/ml LPC for 15-17 h. Two days post-LPC (9 DIV) the myelin marker MBP was significantly decreased compared to control. Application of 25 µM teriflunomide, starting one day before LPC treatment until fixation at 9 DIV, led to a significant increase of MBP (Fig. 3a, b). The amount of MBP was not increased, when slices were incubated with 3 µM or 10 µM teriflunomide (Fig. 3c). After four days in normal medium, there was spontaneous remyelination and a recovery of MBP

expression in LPC treated slices. As we did not see a demyelination in slices treated with 25 µM teriflunomide the amount of MBP remained unchanged over the time (Fig. 3b).

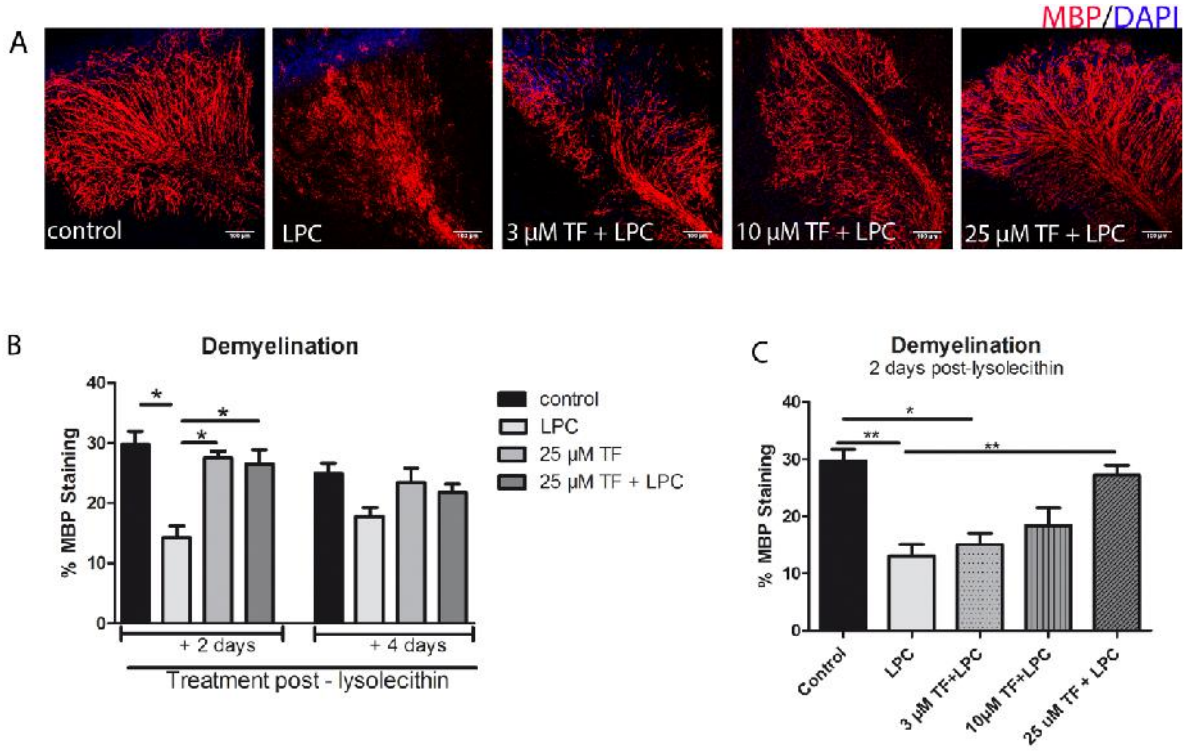


Figure 3: Effect of teriflunomide on demyelination of OSCs. OSCs were treated with 3, 10, or 25 µM teriflunomide starting at 6 DIV until fixation to evaluate its effect on demyelination. Representative images of slices at two days post-LPC stained for MBP **(A)**. Two days post-LPC the amount of MBP was significantly increased in slices treated additionally with 25 µM teriflunomide as compared to the LPC control. Four days post-LPC a significant remyelination of LPC treated slices was observed **(B)**. Lower concentrations of teriflunomide had no significant effect on demyelination **(C)**. Data are represented as mean ± SEM (n= 6-9). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test (*p < 0.05; ** p < 0.01). *Scale bar 100 µM*. TF: Teriflunomide. LPC: Lysolecithin

Effect of teriflunomide on oligodendroglial cells in OSC

To evaluate the proliferation of OPC, a double staining for Olig-2 (marker for oligodendroglial) and Ki-67 (proliferation marker) was performed at one, two, and four days post-LPC. At all indicated time points the number of proliferating OPC was unaffected by LPC or teriflunomide treatment (25 µM) in the grey matter (data not shown). One day post-LPC the number of proliferating OPC was unaffected by LPC or teriflunomide treatment in the white matter. These numbers increased after two days post-LPC and were significantly upregulated when slices were incubated with LPC alone or in combination with teriflunomide. At four days post-LPC the number of double

positive cells was diminished for all conditions, whereas LPC and teriflunomide treated slices showed a slight but not significantly increased number of proliferating OPC (Fig. 4). Maturation of oligodendrocytes was determined by a double staining for Olig-2 and protein adenomatous polyposis coli (APC). Two days post-LPC the number of double positive mature oligodendrocyte (APC+/Olig-2+) was downregulated after LPC treatment. After four days the number of double positive cells after LPC treatment reached control levels. Teriflunomide did not alter these cell numbers (Fig. 5).

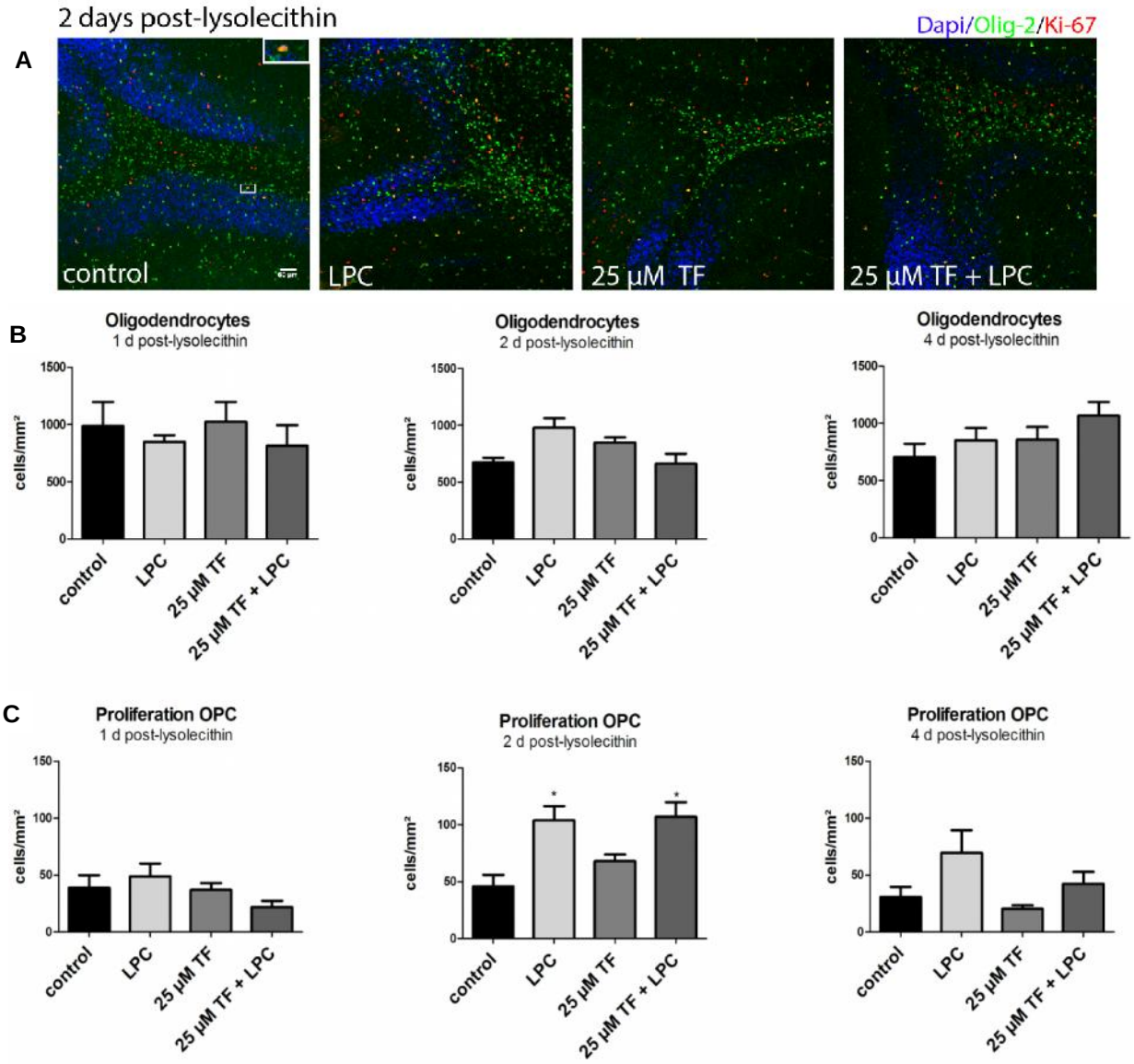


Figure 4: Effect of teriflunomide on OPC proliferation. Effect of teriflunomide on proliferation of OPC during de- and remyelination. Representative images of slices at two days post-LPC treatment stained against Olig-2 and Ki-67 **(A)**. Absolute numbers of Olig-2 positive **(B)** and Olig-2/Ki-67 double positive cells **(C)** were counted in white matter after one, two, and four days post-LPC. Proliferation of oligodendrocytes was significantly increased two days post-LPC, both after LPC treatment alone or in combination with 25 µM teriflunomide. Data are represented as mean ± SEM (n=5-8). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test (*p < 0.05). *Scale bar: 100 µM*. TF: Teriflunomide. LPC: Lysolecithin

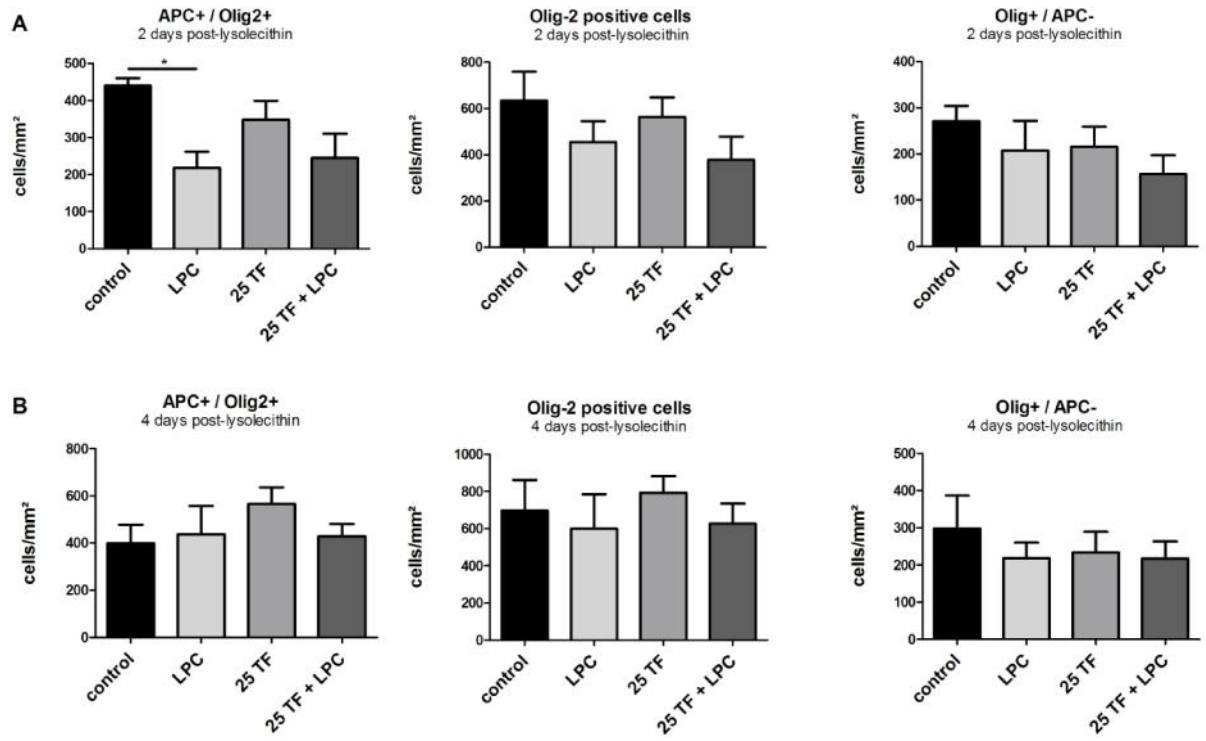


Figure 5: Effect of teriflunomide on oligodendrocytes maturation in OSCs. Effect of teriflunomide on oligodendrocytes maturation during demyelination was determined by a double staining for Olig-2 and APC. Two days post-LPC the number of double positive cells (APC+/Olig-2+) are downregulated after LPC **(A)**. Four days post-LPC the numbers of double positive cells (APC+/Olig-2+) are unaffected after LPC or teriflunomide treatment **(B)**. Data are represented as mean ± SEM (n=4-5). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test (*$p < 0.05$). TF: Teriflunomide. LPC: Lysolecithin

Effect of teriflunomide on microglia and astrocytes during demyelination

In order to evaluate the effects of teriflunomide on glial cell reactions during demyelination, effects on astrocytes and microglia were investigated by immunohistochemical staining. At one day post-LPC the morphology of astrocytes was changed in LPC treated cultures, indicating swelling of astrocytes. Teriflunomide treated cultures showed less swelling of astrocytes (Fig. 6). In control slices mainly normal morphology was seen.

The proliferation of microglia was determined by Iba-1 and Ki-67 double staining. Two days post-LPC the total number and of proliferating microglia was increased after LPC treatment. Even after two further days the number of proliferating microglia was still increased in this condition. Treatment with teriflunomide did not alter the number of microglia compared to control (Fig.7).

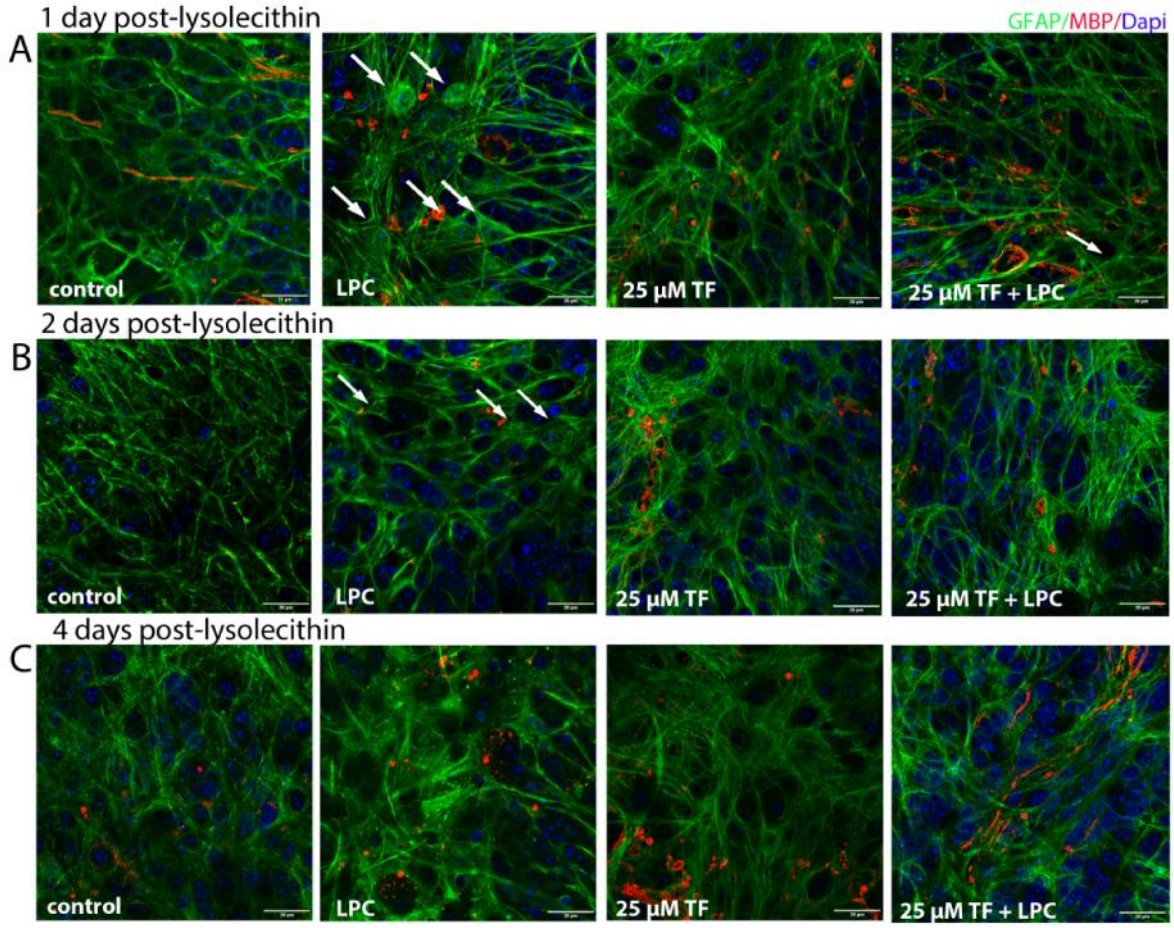
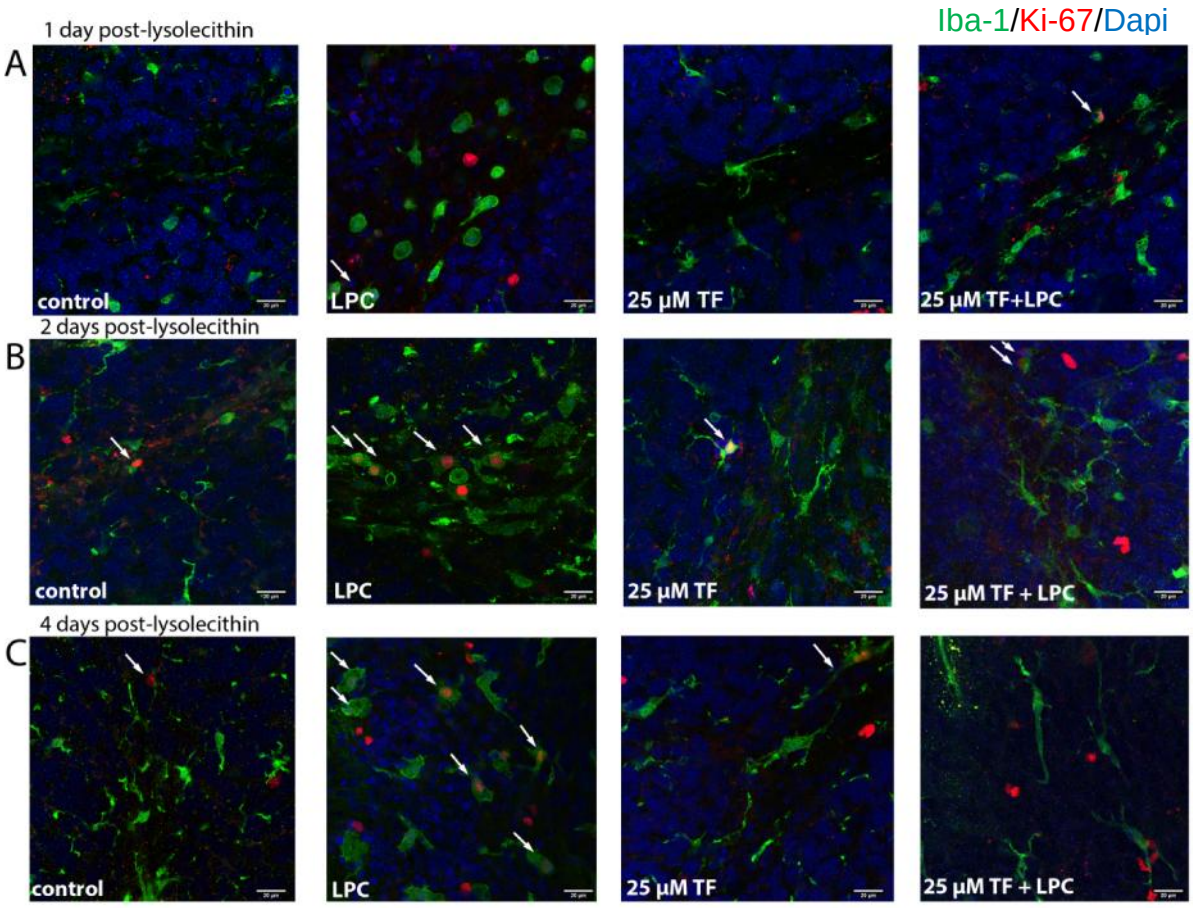


Figure 6: Effect of teriflunomide on astrocytes in OSCs. Effect of teriflunomide on astrocytes during demyelination. Representative images of GFAP stained slices at one day **(A)**, two **(B)** and three days **(C)** after LPC treatment. Arrows indicate morphological changes of astrocytes which were diminished after treatment with 25 µM teriflunomide. *Scale bar: 20 µM*. TF: Teriflunomide. LPC: Lysolecithin


Iba-1/Ki-67/Dapi
1 day post-lysolecithin
A
control
LPC
25 μM TF
25 μM TF+LPC
2 days post-lysolecithin
B
control
LPC
25 μM TF
25 μM TF + LPC
4 days post-lysolecithin
C
control
LPC
25 μM TF
25 μM TF + LPC

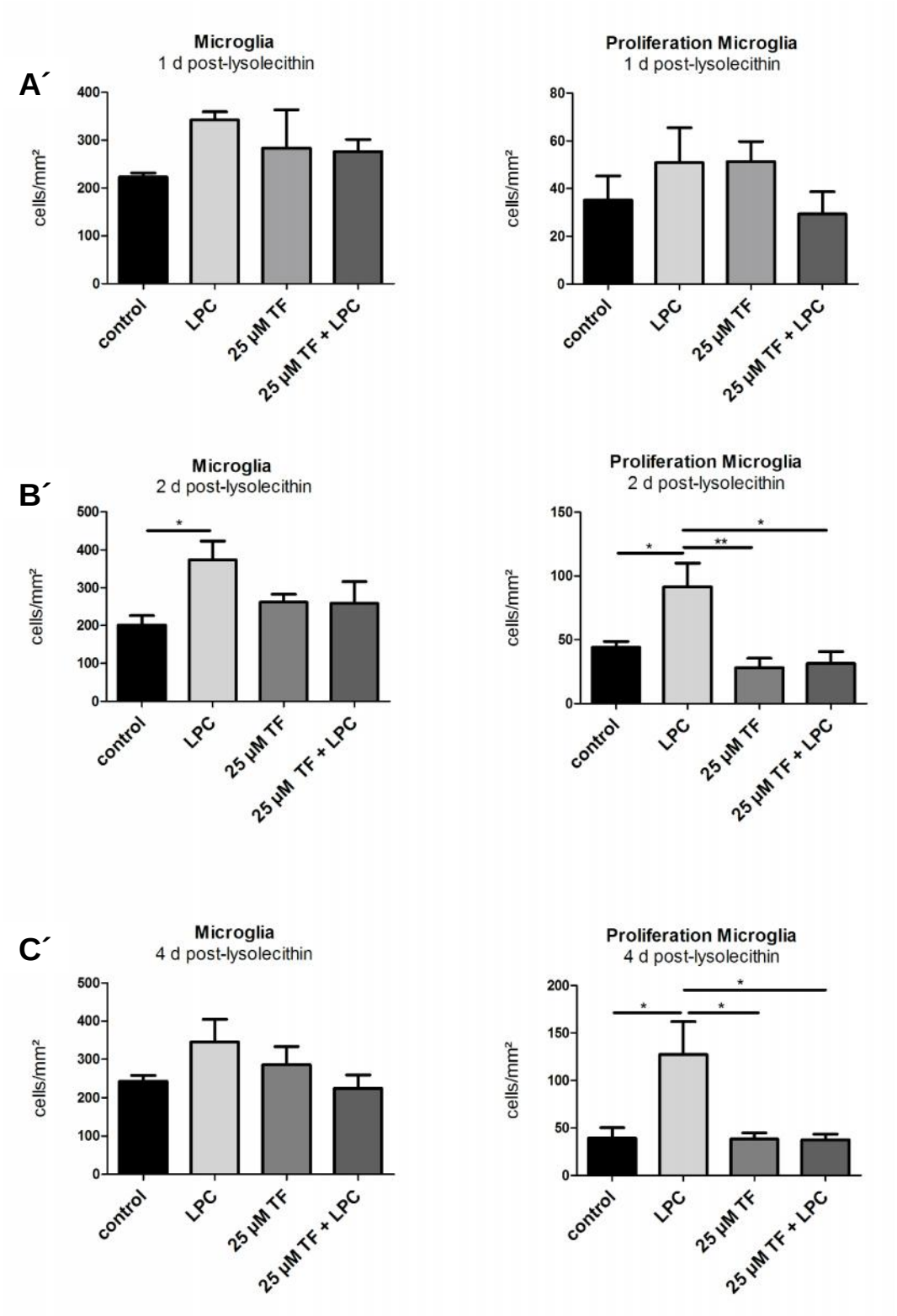


Fig. 7 Effect of teriflunomide on microglia proliferation in OSCs. Proliferation of microglia was determined by Iba-1 and Ki-67 double staining. OSCs were treated with 25 µM teriflunomide at 6 DIV (see Fig.1), and slices were fixed at one **(A, A')**, two **(B, B´)** and four **(C, C´)** days post-LPC. Arrows indicate Iba-1 and Ki-67 double positive cells. Data are presented as mean ± SEM (n=4-8). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test (*p < 0.05). *Scale bar: 20 µM.* TF: Teriflunomide, LPC: Lysolecithin

Effect of teriflunomide on remyelination

To assess the effect of teriflunomide on remyelination of OSC, slices were incubated with teriflunomide from two days post-LPC until fixation at five (12 DIV) or seven (14 DIV) days post-LPC. The amount of MBP was measured to calculate the influence on remyelination. Teriflunomide treatment did not alter the amount of MBP staining, which suggests that teriflunomide did not influence remyelination in the OSC (Fig.8).

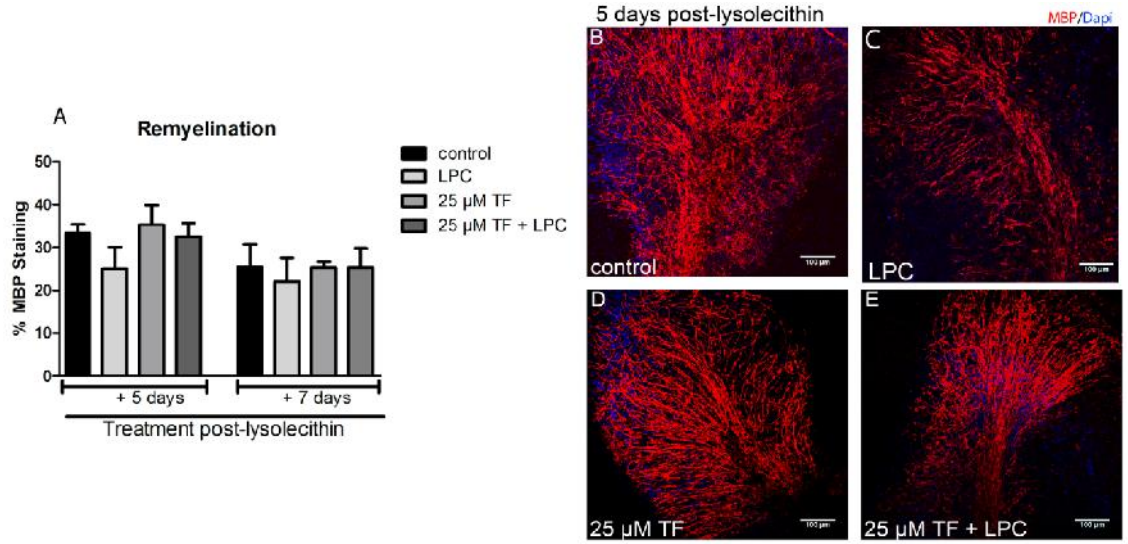


Figure 8. Effect of teriflunomide on remyelination of OSCs. Quantification of MBP showed no effect of 25 µM teriflunomide **(A)**. Representative images of slices at five days post-LPC treatment stained against MBP **(B-E)**. Data are represented as mean ± SEM (n=4-5). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test. *Scale bar 100 µM*. TF: Teriflunomide, LPC: Lysolecithin.

Effect of supernatants from teriflunomide treated microglia on oligodendrocytes

We further tested the effect of teriflunomide (3 µM, 10 µM, and 25 µM) treated microglia on differentiation and proliferation of OPC *in vitro*. Differentiation of OPCs was determined by staining for A2B5 (immature OPCs) and GalC (mature Oligodendrocytes) after treatment with different microglia conditioned media for 48 h. Absolute numbers of A2B5 and GalC positive cells remained unchanged after incubation with supernatants from teriflunomide treated microglia (Fig. 9). Proliferation of OPC was evaluated with BrdU incubation for 3 h after incubation of cells with supernatants from teriflunomide treated microglia for 48 h. Proliferation remained unchanged (Fig. 10).

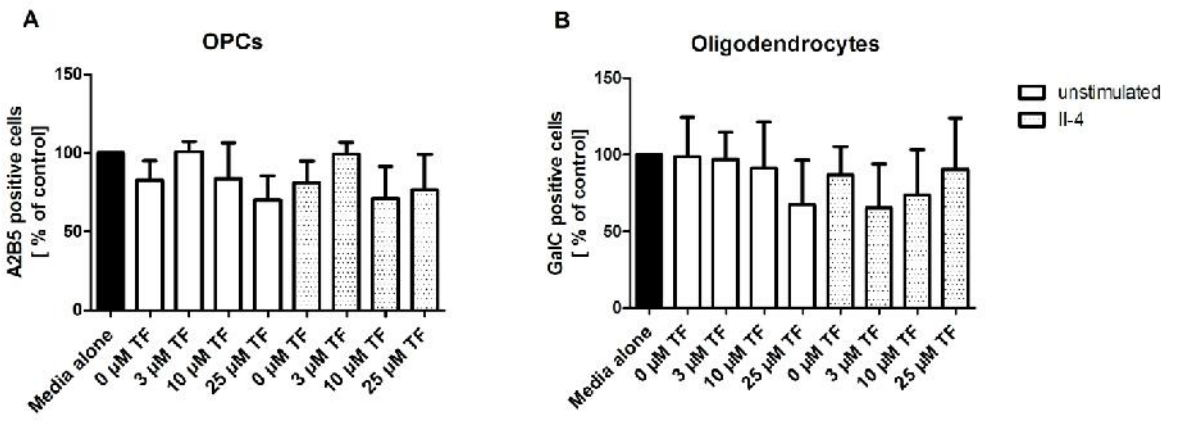


Figure. 9 Effect of supernatants from teriflunomide treated microglia on oligodendrocytes. Proliferation of A2B5$^+$ OPCs **(A)** and ratio of GalC$^+$ cells to A2B5$^+$ OPCs **(B)** were determined after incubation of oligodendroglial cultures with supernatants from microglia treated with 3, 10, or 25 µM teriflunomide. Data are presented as mean ± SEM (n=4-8). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test. TF: Teriflunomide.

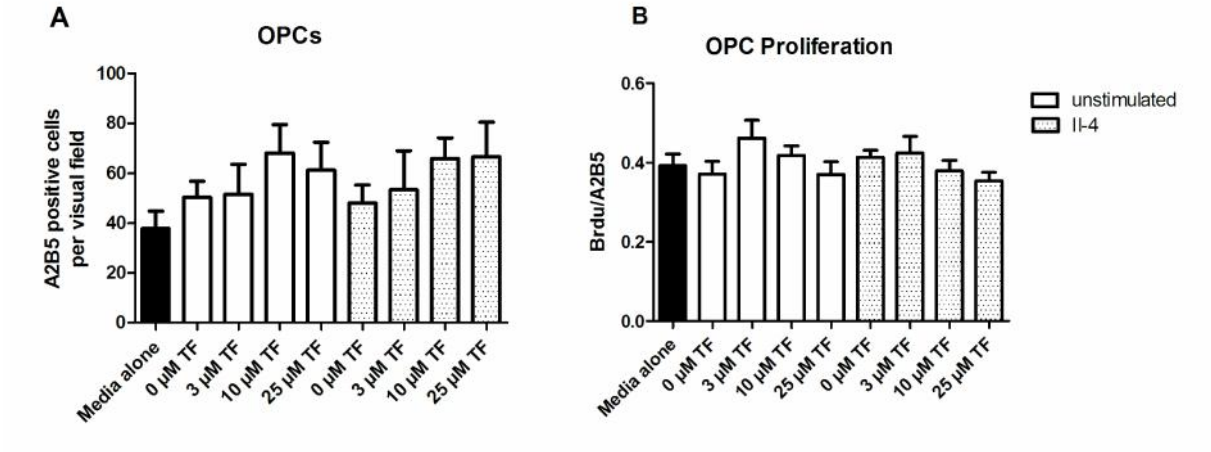


Figure. 10 Effect of supernatant from teriflunomide treated microglia on oligodendrocytes. Proliferation of A2B5$^+$ OPCs **(A)** and proliferation index **(B)** were determined after incubation of oligodendroglial cultures with supernatants from microglia treated with 3, 10, or 25 µM teriflunomide. Data are presented as mean ± SEM (n=4-8). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test. TF: Teriflunomide

Effect of teriflunomide on primary oligodendrocytes

As we detected an effect of teriflunomide on MBP during demyelination in OSC, we further wanted to elucidate possible direct effects of teriflunomide on oligodendrocytes. Therefore, 3 µM, 10 µM or 25 µM teriflunomide was given for 48 h to the oligodendrocyte culture medium. As we investigated also effects of M2 derived microglia supernatants IL-4 was added to the culture. Again, differentiation was determined with staining for A2B5 and GalC and proliferation with staining for BrdU. As shown in Fig.11 the proliferation index was not changed after teriflunomide treatment, but absolute numbers of A2B5 positive cells decreased significantly after

incubation with 10 µM or 25 µM teriflunomide (Fig.11 A-F). The total number of A2B5 and GalC did not change after teriflunomide treatment but the differentiation index calculated by the ratio of GalC/A2B5 significantly increased after incubation with IL-4 and 3 µM, 10 µM, or 25 µM teriflunomide (Fig. 11 G-L).

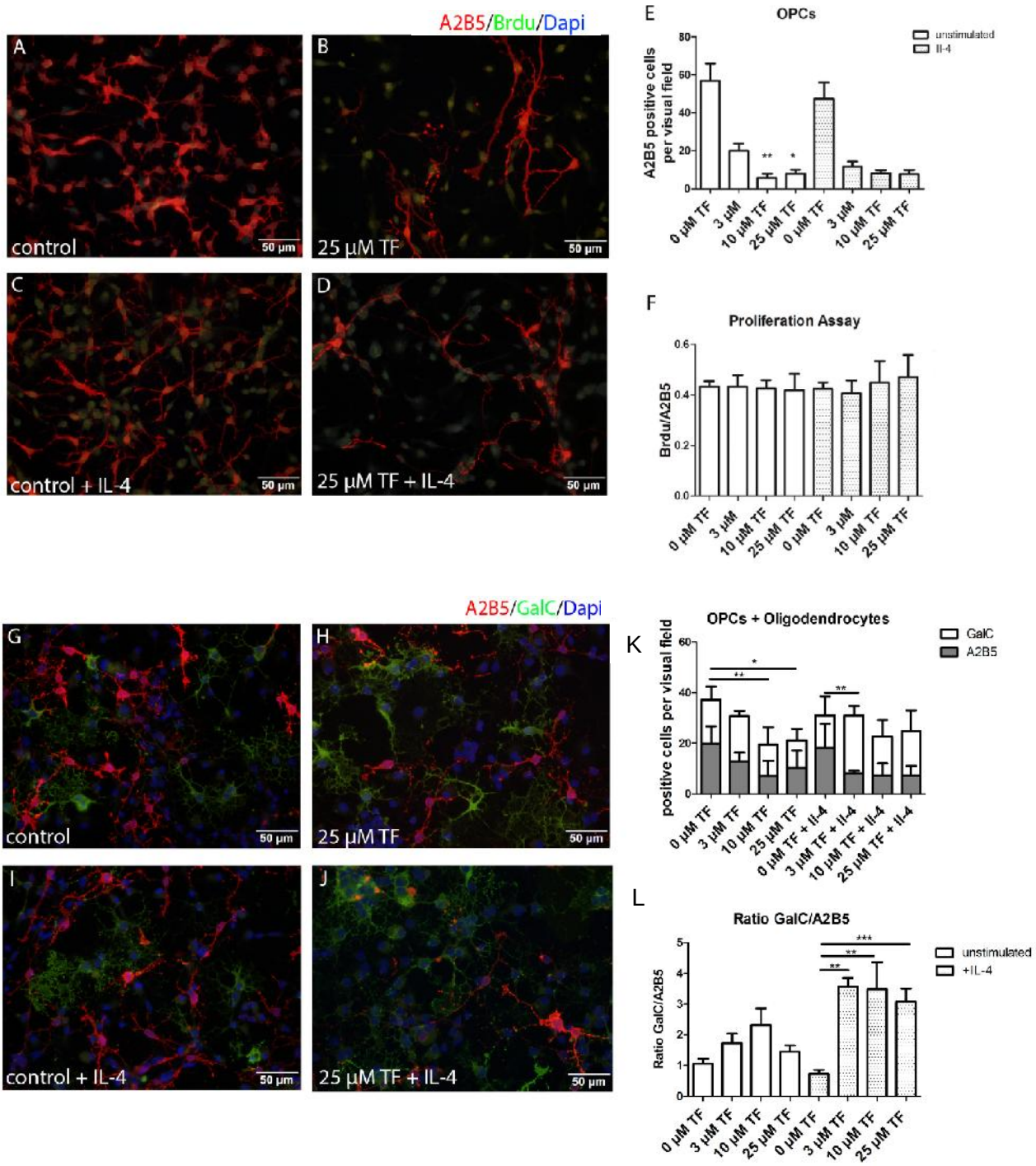
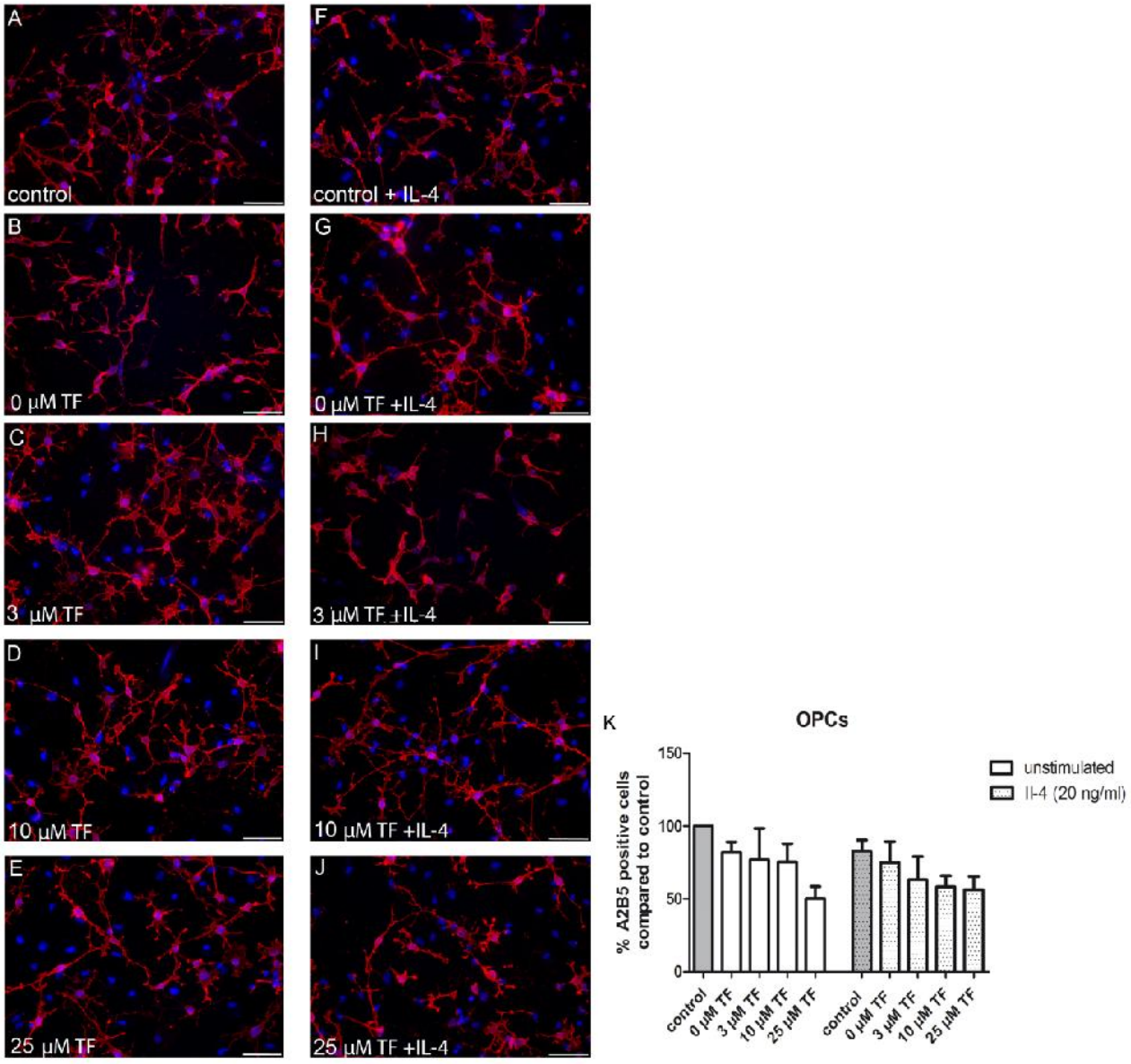


Figure. 11 Effect of teriflunomide on primary oligodendroglial cells. Primary OPC were treated with 3, 10, or 25 µM teriflunomide for 48 h. Proliferation of A2B5$^+$ OPCs **(A-F)** and ratio of GalC$^+$ cells to A2B5$^+$ OPCs **(G-L)** were determined by immunhistochemical staining. Data are presented as mean ± SEM (n=5-10). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test. (*p < 0.05; **p < 0.01; ***p < 0.001). TF: Teriflunomide

Effect of astrocyte supernatants

As we observed a decline of A2B5 positive OPC after direct treatment of cells with 3 µM, 10 µM or 25 µM teriflunomide, we wanted to elucidate, if normal astrocytic supernatant could abolish this effect. After addition of supernatant from astrocytes the number of A2B5 positive oligodendrocytes increased (additional file 1: Figure S1). These results indicate that astrocytes release protective factors. Therefore, apoptosis of oligodendroglial cells was protected in the slice culture system.



Additional file 1: Figure S1. Effect of supernatant from astrocytes on teriflunomide treated oligodendrocytes. Numbers of A2B5$^+$ OPCs were determined after incubation of oligodendroglial cultures with supernatants from unstimulated astrocytes and astrocytes treated with 3, 10 or 25 µM teriflunomide. Data are represented as mean ± SEM (n=5). Statistical analysis was performed using Kruskal-Wallis test followed by Dunns post test.

7.5 Discussion

Teriflunomide is thought to act mainly on activated lymphocytes and thus this has been the focus of research in the past. The inhibition of DHODH in activated lymphocytes by teriflunomide reduces their proliferation, whereas resting lymphocytes mainly remain unaffected. The inhibition of DHODH requires an effective concentration range of nM, whereas several other DHOHD independent effects are reported *in vitro* using concentrations in the µM range (Elder et al.1997). This includes inhibition of protein kinase Jak1 and Jak3 in a murine T cell line and inhibition of COX-2 functions, as well as altering cytokine production (reviewed in Claussen 2012). Moreover, we have previously shown an anti-proliferative and anti-inflammatory effect on microglia in a mixed glia cell culture system after treatment with teriflunomide (Wostradowski et al. 2016). Furthermore Göttle et al. (2018) showed, that under defined stimulation periods teriflunomide treatment may enhance oligodendrocytes differentiation.

Therefore, the aim of this study was to elucidate the effects of teriflunomide glial reactions during myelination, demyelination and remyelination in OSCs. We demonstrated that myelination and remyelination in OSCs were not affected after treatment with teriflunomide, but demyelination was significantly reduced after incubation with 25 µM teriflunomide.

Lower concentrations of 3 µM and 10 µM did not exert this effect. Thus, the effective concentration may be slightly higher than what is reached in MS patients (Hopkins 2017, Rakhila 2011). Göttle et al. (2018) have demonstrated that teriflunomide may enhance OPC differentiation *in vitro*. However, this was only the case in an experimental setting with pulsed treatment. We could not recapitulate this effect on OPC during myelination and remyelination, however, in our experimental setting the treatment with teriflunomide was continuous which is probably closer to the physiological situation, where teriflunomide is given continuously to patients with MS.

In contrast, we could demonstrate that teriflunomide has a myelin protective effect when demyelination is induced by LPC in the OSC. The mechanism is not yet clear and may involve indirect effects via microglia and astrocytes.

The role of activated microglia during de- and remyelination is not fully understood, but it has been shown that activated microglia clear myelin debris by enhanced phagocytosis and release of cytokines as well as growth factors that may stimulate OPC differentiation (Voss et al. 2012). On the other hand, microglia can also release

cytotoxins and pro-inflammatory factors which contribute to oligodendrocyte damage (Remington 2007).

In our study we detected an upregulation of microglial proliferation after LPC treatment and addition of teriflunomide abolished this effect. Similar results were obtained in a former study where a lower teriflunomide concentration of 5 µM reduced the proliferation of microglia in mixed glial cell cultures (Wostradowski et al. 2016). Furthermore, microglial density was decreased upon teriflunomide treatment in both, a TMEV mouse model and a mouse model of traumatic brain injury, indicating less inflammation (Pol et al. 2018, Prabhakara et al. 2018).

In addition, effects on astrocytes are reported as well. In activated and teriflunomide pretreated astrocytes production of pro-inflammatory factors such as TNFα are reduced and protected their survival upon H_2O_2 induced cytotoxicity (Elding et al. 2017). Therefore, we investigated if teriflunomide could affect astrocyte morphology by GFAP immunostainings. The morphology was altered after LPC treatment, but not after simultaneous treatment with teriflunomide. This LPC induced alteration might compensate astrocyte swelling which is observed in different models of brain injuries (Reinehr 2007). Thus, teriflunomide treatment of OSC seemed to result in less activated astrocytes.

Indirect effects of teriflunomide pretreated microglia were then tested in primary oligodendrocyte cultures. Supernatants derived from teriflunomide pretreated microglia did not alter proliferation and differentiation of OPC. Determining direct effects of teriflunomide on OPC, we could demonstrate a significant decrease of A2B5 positive OPC, with no effect on proliferation. Göttle et al (2018) showed that apoptotic oligodendrocytes were increased even under a low concentration of 1 µM teriflunomide, suggesting that this impaired cell survival is caused by induction of pyrimidine stress. In our experiment this impact was abolished after addition of supernatants from astrocytes, implying that these effects are probably not present in OSCs or *in vivo* with a constant astrocytic support.

Taken together, our study demonstrates that teriflunomide may be protective for myelin degradation in LPC induced demyelination in OSC. This effect seemed to be mediated by inhibition of microglia proliferation and is not mediated by a direct impact on OPC differentiation or proliferation. This effect on glial cells might be relevant for the treatment of demyelinating disease such as MS. However, further studies need to

clarify the protective effect of teriflunomide on mature oligodendrocytes and this effect *in vivo*.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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8. Comprehensive discussion

Currently used MS therapeutics are targeting lymphocytes which consequently induce immunosuppressive or immunomodulatory mechanisms. In order to clarify their effects on glial cells, it is important to further elucidate their mode of action, and study off-target effects. In the present studies, we specifically investigated the effects of fumaric acids and teriflunomide on glial cells. Both drugs are approved for the treatment of MS, as they exert immunomodulatory properties. However, their specific effects on glial cells still remain to be demonstrated (Bar-Or et al. 2014; Linker et al. 2011).

Fumaric acids have been used in psoriasis treatment for many years. Beneficial effects in MS patients have been suggested by pleiotropic effects of DMF, and its presumed active metabolite MMF (Prosperini & Pontecorvo et al. 2016). Regarding glial cells, a decreased expression of several pro-inflammatory factors in activated microglia and astrocytes after DMF treatment was reported (Wilms et al. 2010). Hence, the aim of the first study was to clarify the role of fumaric acids on both astrocytes and microglia. It is well described that DMF reduces T cell counts *in vivo* and induces apoptosis of peripheral blood mononuclear cells (PBMCs) *in vitro*. Thus, as a proof of principal, we could show that similar DMF concentration inhibited proliferation of PBMCs. Once efficacy concentration was found, several functions of primary rat astrocytes and microglia in activated and non-activated cells were studied, upon treatment with appropriate concentration of DMF and MMF.

Astrocytes have been described to play a dual role, both protective and deleterious, during the processes of de- and remyelination. Once activated, they release several pro-inflammatory cytokines which support the process of demyelination (reviewed in Nair et al. 2008). To induce microglia or astrocyte activation the widely used endotoxin LPS was added to the cultures (Nakamura et al. 1999). In this study we demonstrated that LPS-activated astrocytes led to changed expression profiles of growth factors and cytokines, but neither DMF nor MMF altered these gene expression patterns. For example, after cytokine activation gene expression of GDNF and PDGFα was upregulated, while expression of NGF and CNTF was downregulated. To investigate the effects of DMF treatment and LPS stimulation, we used two different periods of pretreatment (30 min, 24 h) with two different LPS concentrations derived from two different bacteria. Surprisingly, even with this approach we could not detect any effect upon DMF or MMF application. Contrary to this observation, previous studies have

shown that DMF pretreatment lead to downregulation of IL-1β, IL-6 and TNFα in LPS-activated astrocytes and microglia (Wilms et al. 2010). Similar anti-inflammatory effects, as the reduced expression of the inducible form of nitric oxide synthase (NOS2) in astrocytes after DMF treatment, have also been described by other groups (Lin et al. 2011; Schmidt & Dringen 2010). However, similar results could not be reproduced. A possible explanation could be the different preparation methods of mixed glial cultures as well as the isolation techniques of primary cells and the used DMF doses. Similar to astrocytes, the function of microglia within MS lesions is currently highly debated. During inflammation, microglia are rapidly activated and thereby several factors essential for remyelination are released (Miron et al. 2013; Napoli & Neumann 2010). Contrary, microglia secrete several soluble factors e.g. TNFα that promote a pro-inflammatory environment. Therefore, polarization of microglia is divided in M1-like (pro-inflammatory) and M2-like (anti-inflammatory) phenotype. In our study IGF-1 and MRC-1 gene expression was significantly upregulated in microglia after DMF treatment under pro-inflammatory conditions. As IGF-1 is a mediator of cell growth and differentiation of OPCs that enhances myelin production, upregulation of IGF-1 might promote remyelination (Gveric et al. 2002; Shahbazi et al. 2017). Therefore, we analyzed the amount of IGF-1 protein, which was released into the cell culture supernatant, by ELISA assay, and the regulation of IGF-1 in microglia cells by FACS analysis. Interestingly, the protein level was not altered as measured by ELISA assay, but IGF-1 was slightly upregulated in samples evaluated by FACS analysis after DMF and MMF treatment. Leading us to the conclusion that measured protein level of IGF-1 did not correlate with the observed upregulation seen on the gene expression level. This discrepancy might be due to a variety of post-transcriptional mechanisms, which still have to be defined (Greenbaum et al. 2003). Within this study the focus was also laid on phagocytosis of microglia. Phagocytosis is one of the hallmarks of these cells, as this process is essential for the removal of damaged tissue and thus stimulates remyelination (Napoli & Neumann, 2010). In the present study, functional analysis of phagocytic activity of microglia was not triggered by DMF or MMF pretreatment. It is interesting to note, that in a previous study, it was found that higher concentrations of DMF induced microglial phagocytosis in an Nrf2-dependent manner (Lastres-Becker et al. 2016). Suggesting that a wider concentration window of DMF and MMF should be applied in future studies.

In a further step, OPCs were incubated with DMF or MMF pretreated microglia supernatants to examine possible secondary effects of theses supernatants. Incubation of OPCs with DMF and MMF pretreated M0 and M2-like microglia supernatants significantly enhanced their proliferation but did not impact their differentiation. It has already been shown by Moore et al. (2014) that human fetal M2-like microglia supernatants do not increase the total number of O4+ or GalC+ positive oligodendrocytes after two days. In contrast, M1-like supernatants decreased the total number of O4+ or GalC+ positive cells. In our cultures, incubation with supernatants from M1-like microglia diminished the number of OPCs strongly, thus rendering the evaluation of both differentiation and proliferation impossible.

Taken together, since we found that DMF has anti-inflammatory effects on microglia which may results in enhanced proliferation of OPCs, further studies are needed to clarify the pleiotropic effects of DMF. Beyond its described neuroprotective effect through the Nrf2-dependent anti-oxidative pathway, recent studies showed that DMF led to GAPDH inhibition which then reduces the level of aerobic glycolysis in myeloid and lymphoid human cells. This inhibition resulted in down-modulated immune responses (Kronberg et al. 2018). Regardless of the absolute number of lymphocytes, DMF influenced the immunophenotype of circulating T cells in MS patients in a positive manner (Longbrake et al. 2015). Thus, it could be speculated that results directly obtained from MS patient are more relevant to uncover the mode of action of DMF, rather than reported neuroprotective effects via the Nrf2 pathway. Nevertheless, DMF penetrate into the CNS (Penner et al. 2016), thus, it is highly relevant to investigate possible neuroprotective effects. Regarding the presented results, it can be concluded that fumaric acids potential neuroprotective effects are not mediated by astrocytes, but rather by shifting microglia into an anti-inflammatory phenotype that might have a positive influence on inflammatory conditions. Finally, as a secondary effect DMF and MMF pretreated microglia supernatants enhance the proliferation of OPCs.

Besides DMF, the oral immunomodulatory drug teriflunomide is frequently used for the treatment of RRMS. Therefore, the aim of the second study was to assess the impact of teriflunomide during different stages of myelination, de- and remyelination in an organotypic cerebellar slice culture system. In 2013, teriflunomide has been approved for the treatment of RRMS (Mullard 2013). Its main effect is mediated by the inhibition of the mitochondrial enzyme DHODH, whereby the proliferation of activated

lymphocytes is reduced (Bar-Or et al. 2014). Besides that, a small percentage of teriflunomide, which crosses the BBB, reaches the CNS and possible modulates the function of glial cells (Limsakun & Menguy-Vacheron 2010; Tallantyre et al. 2008). In a previous study we demonstrated the anti-proliferative effects of teriflunomide on microglia and that it increased the release of the anti-inflammatory factor IL-10 in activated cells (Wostradowski et al. 2016). These findings strengthen the hypothesis that teriflunomide could exert a direct effect on glial cells.

In order to further investigate the effects of teriflunomide on myelination, de- and remyelination of CNS cells, we used OSCs. The use of OSCs has the advantage that the normal three-dimensional cytoarchitecture is preserved and therefore enables the study of cell to cell interaction. Although the mode of action is not clear, treatment with LPC leads to a significant demyelination (Birgbauer et al. 2004). The effects of LPC treatments on oligodendrocytes, astrocytes and microglia were evaluated by immunohistochemical stainings. With stainings against different oligodendrocyte markers (APC, Olig-2) we were able to show that the number of APC positive mature oligodendrocytes was diminished, while the number of Olig-2 positive cells remained unaffected, although their proliferation was enhanced. Furthermore, we showed that microglia proliferate in respond to LPC treatment. This implies that, LPC either induces myelin and membrane degradation of mature oligodendrocytes or actively triggers microglia destruction (Birgbauer et al. 2004; Miron et al. 2010; Woodruff & Franklin 1999).

To study the effect of teriflunomide in OSC, teriflunomide was added to the medium at indicated time points (see Fig.1; Manuscript III). Our results demonstrated that teriflunomide had no impact on myelination or remyelination. However, demyelination was significantly diminished after incubation with 25 µM teriflunomide, but lower concentrations (3 µM, 10 µM) had no effect. Previous pharmacokinetic studies in rats have shown that cells of the CNS are exposed to 2.5-4.1 µM. Thus, the *in vivo* relevance of the used concentration of 25 µM teriflunomide is unclear. In addition to that, other effects such as suppression of TNFα were only observed with high teriflunomide concentrations (Xu et al. 1995; 1996). Nevertheless, Göttle et al. (2018) demonstrated that both timepoint of treatment and concentration are crucial factors regarding the regenerative potential of oligodendrocytes. Thus, it can be speculated that treatment of OSC with lower concentration (e.g. 3 µM, 10 µM) at early DIV could result in a positive impact on LPC-induced demyelination.

Two days post-LPC the number of proliferating OPC was upregulated after LPC treatment, whereas the number of mature oligodendrocytes was downregulated. Surprisingly, simultaneous incubation with teriflunomide did not alter this effect. Regarding this observation, it has been previously shown that in TMEV-infected mice the density and differentiation of oligodendroglial cells are not increased after teriflunomide treatment (Pol et al. 2018). This correlated with our study since teriflunomide treatment did not result in neither an enhanced proliferation nor maturation of oligodendrocytes in OSCs during demyelination. By determining direct effects of teriflunomide in *in vitro* oligodendrocytes, we were able to demonstrate a significant reduction of A2B5$^+$ OPC, with no obvious effects on proliferation. Trophic support of supernatants derived from astrocytes abolished this effect, which was indicated by an increased number of A2B5$^+$ OPC. Consequently, we did not observe a reduction of oligodendrocytes in OSC and it is unlikely that this effect occurs *in vivo*. Besides this, the morphology of astrocytes in OSC was studied with GFAP stainings. Astrocyte morphology was altered after LPC treatment which might be due to swelling of astrocytes. This phenomena is often observed in different models of brain injuries (Häussinger et al. 2000; Kimelberg 2005). Simultaneous treatment with 25 µM teriflunomide abolished this effect. Another study showed that in activated and teriflunomide pretreated astrocytes production of pro-inflammatory factors such as TNFα are reduced and prolonged the survival of astrocytes upon H_2O_2 induced cytotoxicity (Edling et al. 2017). In addition, Miljkovic et al. (2001) demonstrated that NO production is decreased in LPS/IFNγ activated astrocytes, suggesting that astrocytes were less activated in teriflunomide treated OSC. However, the extent of which this corresponds to a diminished demyelination must be further elucidated.

Apart from the effects on oligodendrocytes and astrocytes, we also examined the impact of teriflunomide on microglia. It is well known that during demyelination microglia become easily activated and start to proliferate. In line with this observation, we detected an increase of microglial proliferation after LPC treatment of OSCs. In contrast to this, addition of teriflunomide neutralized this effect. Furthermore, teriflunomide treatment modulated microglia activation by upregulating IL-10 gene expression and reducing proliferation of microglia (Korn et al. 2004; Wostradowski et al. 2016). Thus, it can be speculated that in OSC, LPC-induced demyelination leads to microglia proliferation and enhanced phagocytosis of myelin debris, as well as remyelination. Treatment with 25 µM teriflunomide led to inhibition of microglia

proliferation which could be mediated by blocking the DHODH enzyme (Fox et al. 1999). This inhibition reflects a reduction in microglial cell activation (Figuera-Losada et al. 2014) upon treatment, indicating that teriflunomide shifts microglia profile into an anti-inflammatory phenotype. This is supported by recent studies which have shown that teriflunomide decreased microglial density in both, a TMEV-infected mouse model and rat model of traumatic brain injury, leading to less neuroinflammation (Pol et al. 2018; Prabhakara et al. 2018). Even neurotoxicity was reduced in a co-culture system of microglia with HIV-transduced monocytic cells (Ambrosius et al. 2017). Recently, Groh et al. (2018) investigated different teriflunomide treatment regimes in a myelin proteolipid protein 1(PLP1) mutant mouse model with both, preventive treatment and therapeutic treatment. Preventive treatment started before neuronal loss and led to a non-permanent reduction of microglial cells in optic nerves of PLP1 mutant mice. In contrast to that, therapeutic treatment started when neuronal loss was already progressed. This treatment regime failed to reduce microglial cell number. As preventive treatment with teriflunomide improves histopathology and clinical deficits in this model, MS patients might benefit from early administration of teriflunomide at the appearance of first clinical symptoms.

Since teriflunomide reduced the proliferation of microglia in LPC treated OSC, further secondary effects of teriflunomide treated microglia were tested in primary OPC. Surprisingly, proliferation and differentiation of oligodendrocytes were not altered after treatment with supernatants derived from microglia pretreated with teriflunomide. In conclusion, our study showed that teriflunomide enhanced demyelination which correlated with decreased proliferation of microglia, as well as reduced activation of astrocytes. However, a direct impact on OPC differentiation or proliferation could not be shown. Considering these results, teriflunomide may be protective for LPC induced myelin degradation. This effect seemed to be mediated by the inhibition of microglia proliferation, rather than a direct impact on OPC proliferation or differentiation.

The aim of this thesis was to clarify the role of teriflunomide and fumaric acids on glial cells in order to gain more knowledge on the mode of action of these drugs. Additionally, we highlighted the advantage of the OSC model to investigate the processes of myelination, de- and remyelination *in vitro*. Our findings demonstrated that fumaric acids do not modulate gene expression profile of astrocytes. However, DMF had anti-inflammatory effects on microglia which further enhanced proliferation of OPC. Second, we showed that teriflunomide could also exerted it´s mode of action

on glial cells by inhibiting microglial proliferation. In OSC, this effect might be the reason for a reduced demyelination upon teriflunomide treatment. The current findings are highly relevant to uncover the mode of action of current treatment options for MS patients. However, further studies are necessary to clarify the precise role *in vivo* of DMF and teriflunomide specifically on cells within the CNS.

9. References

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Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled

“Investigations towards protective treatments for demyelination”

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution: Department of Neurology, Hannover Medical School

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affrim the above statements to be complete and true to the best of my knowledge.

Date, Signature

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