Diploma thesis

Effects of siponimod on inflammation related astroglial gene expression
An experimental study

submitted by

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Graz, 26.01.2026

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Graz, 26.01.2026 Florian Stifter m.p.

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Zusammenfassung

Einführung

Siponimod ist ein Sphingosin 1 Phosphat (S1P) Rezeptor Modulator der für die Behandlung von sekundär-progressiver Multipler Sklerose (SP-MS) zugelassen ist. Es konnte gezeigt werden, dass die Behandlung mit Siponimod Zytokin induzierte kortikale Demyelinisierung in einem Ratten Modell verhinderte. Dieses Modell spiegelt menschliche kortikale Läsionen von progressiven Multiple Sklerose (MS) Formen und deren Hauptmerkmale wider. In der Behandlung von MS, ist der bekannte Hauptwirkmechanismus von Siponimod, dass es den Ausstrom von Lymphozyten aus den lympahtischen Organen und somit die Einwanderung in das zentrale Nervensystem (ZNS) behindert. Kortikale Läsionen zeigen jedoch kein gesteigertes inflammatorisches zelluläres Infiltrat. Das legt nahe, dass Siponimod diese Läsionen über einen anderen Wirkmechanismus beeinflusst und direkt an den ansässigen Gliazellen im ZNS wirken könnte. Daher war das Ziel dieser Diplomarbeit zu untersuchen, ob Siponimod astrogliale entzündungsbezogene Genexpression positiv beeinflusst und einen anti-entzündlichen Phänotyp fördert.

Methodik

Es wurde eine von Ratten stammende primäre gemischte Gliazellkultur die Astrozyten, Mikroglia und Oligodendrozyten beinhaltete, generiert. Nach zehn Tagen der Kultivierung, wurden die Kulturen in folgenden Konstellationen behandelt: 1) Siponimod + Zytokine (Interferon gamma (IFNγ) + Tumornekrosefaktor alpha (TNFα)), 2) Zytokine + Dimethylsulfoxid (DMSO), 3) Siponimod, 4) DMSO und 5) Naiv. Nach einer 20-stündigen Behandlung, wurden die Zellen geerntet und eine quantitative Echtzeit-Polymerase Kettenreaktion durchgeführt. Die Effekte von Siponimod auf die mRNA-Expression von zwölf entzüdungsbezogenen Genen wurde analysiert. Die relative Genexpression wurde mittels der $2^{-\Delta\Delta CT}$Methode unter Verwendung eines Referenz Genindex berechnet. Die statistische Analyse wurde gemäß der Datenverteilung unter Verwendung von parametrischen und nicht-parametrischen Verfahren durchgeführt.

Ergebnisse

Die alleinige Behandlung mit Siponimod reduzierte die mRNA-Expression von Komplementfaktor C3 (C3) und die gleichzeitige Behandlung mit Siponimod und

Zytokinen reduzierte die Expression von Prostglandinsynthase 2 (PTGS2) statistisch signifikant im Vergleich zu allen anderen experimentellen Gruppen. Die alleinige Behandlung mit Siponimod verringerte die Expression des ziliaren neurotrophen Faktor (CNTF) statistisch signifikant im Vergleich zu allen anderen Gruppen. DMSO beeinflusste die Genexpression in fünf aus elf untersuchten Genen. Die anderen Gene zeigten keine statistisch signifikanten Änderungen in ihrer Expression durch die Behandlung mit Siponimod.

Diskussion

Es konnte gezeigt werden, dass Siponimod die Genexpression von Gliazellen in den gemischten Gliazellkulturen beeinflusst. Siponimod zeigte einen anti-inflammatorischen Einfluss auf die Gliazellen in den gemischten Gliazellkulturen, indem es die Geneexpression von C3 und PTGS2 reduzierte und somit die Förderung eines anti-inflammatorisch Phänotyp. Daher konnte die Hypothese in zwei der untersuchten Gene bestätigt werden. Jedoch wurde mit der Reduzierung der Expression von CNTF ebenso ein pro-inflammatorischer Einfluss auf die Gliazellen in den gemischten Gliazellkulturen entdeckt. Des Weiteren wurde ein Einfluss von DMSO auf die mRNA-Expression der Gliazellen entdeckt, obwohl es in einer niedrigen Konzentration von 0.02% verwendet wurde. Daher sollten weitere detailliertere Studien durchgeführt werden, die den Einfluss von DMSO auf die Genexpression von Gliazellen, insbesondere in niedrigen Konzentrationen, untersuchen.

Abstract

Introduction

Siponimod is a sphingosine 1 phosphate (S1P) receptor modulator that is approved for the treatment of secondary progressive multiple sclerosis (SP-MS). It was shown that treatment with siponimod prevented cytokine induced cortical demyelination in a rat model. This rat model mimics human cortical lesions of progressive forms of multiple sclerosis (MS) and its main features. In the treatment of MS, the known main mechanism of action of siponimod is hindering the egress of lymphocytes out of the lymphoid organs and therefore the migration into the central nervous system (CNS). Cortical lesions, however, show no increased inflammatory cellular infiltrate. This suggests, that siponimod might influence these lesions through another mechanism of action and could act directly on the resident glial cells in the CNS. Therefore, the aim of this thesis was to investigate if siponimods influences astroglial inflammation related gene expression positively and promotes an anti-inflammatory phenotype.

Methods

A rat derived primary mixed glial cell (MGC) culture was generated which contained astrocytes, microglia and oligodendrocytes. After ten days of cultivation, the cultures were treated in the following constellations: 1) siponimod + cytokines (interferon gamma (IFNγ) + tumor necrosis factor alpha (TNFα)), 2) cytokines + dimethylsulfoxide (DMSO), 3) siponimod, 4) DMSO and 5) naive. After a 20-hour treatment, the cells were harvested and a quantitative real time-polymerase chain reaction (qRT-PCR) was performed. The effects of siponimod on the mRNA expression of twelve inflammation related genes was analysed. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method using a reference gene index. Statistical analysis was performed according to the data distribution using parametric or non-parametric methods.

Results

Sole siponimod treatment reduced the mRNA expression of complement component C3 (C3) and concomitant siponimod and cytokine treatment reduced the mRNA expression of prostaglandinsynthase 2 (PTGS2) statistically significant in

comparison to all other experimental groups. Sole siponimod treatment decreased the expression of ciliary neurotrophic factor (CNTF) statistically significant in comparison to all other groups. DMSO influenced gene expression in five out of eleven investigated genes. The other genes showed no statistically significant alterations in their expression through the treatment with siponimod.

Discussion

It was shown that siponimod influenced gene expression of glial cells in the MGC cultures. Siponimod showed an anti-inflammatory influence on the glial cells in the MGC cultures by reducing the gene expression of C3 and PTGS2 and thus the promotion of an anti-inflammatory phenotype. Therefore, the hypothesis was confirmed in two of the investigated genes. With the reduction in the expression of CNTF, however, there was also a pro-inflammatory influence on glial cells revealed. Furthermore, an influence of DMSO on the mRNA expression of glial cells was detected, despite being used at a low concentration of 0.02%. Therefore, more detailed studies should be conducted investigating the influence of DMSO on gene expression of glial cells, especially at low concentrations.

Table of contents

Abbreviations

MS	multiple sclerosis
CNS	central nervous system
HLA	human leukocyte antigen
EBV	Epstein-Barr Virus
IM	infective mononucleosis
OR	odds ratio
CIS	clinically isolated syndrome
DIS	dissemination in space
DIT	dissemination in time
RR-MS	relapsing-remitting MS
PP-MS	primary progressive MS
SP-MS	secondary progressive MS
MRI	magnetic resonance imaging
EAE	experimental autoimmune encephalitis
MOG	myelin-oligodendrocyte glycoprotein
$rMOG_{1-125}$	recombinant myelin oligodendrocyte glycoprotein
CD	cluster of differentiation
S1P	sphingosine-1-phosphate
S1P1-5	sphingosine-1-phosphate receptor subtypes 1-5
MGC	mixed glial cell
qRT-PCR	quantitative real time-polymerase chain reaction
LPS	lipopolysaccharide
DMEM	Dulbecco's Modified Eagle Medium
FBS	fetal bovine serum
IFNγ	interferon-gamma
TNFα	tumor necrosis factor alpha
DMSO	dimethylsulfoxide
D-PBS (-)	Dulbecco’s Phosphate Buffered Salin (-)
dd H2O	distilled and deionized water
DEPC-H2O	diethylpyrocarbonat treated-H2O
PCR	polymerase chain reaction
iNOS	inducible NO-synthase

Arg-1	arginase1
IGF-1	insulin like growth factor 1
C3	complement component C3
KCNJ10	potassium inwardly rectifying channel subfamily J member 10
BDNF	brain derived neurotrophic factor
TLR3	toll like receptor 3
CNTF	ciliary neurotrophic factor
IL6	interleukin 6
PTGS2	prostaglandinsynthase 2
IL10	interleukin 10
TRAF 5	tumor necrosis factor receptor associated factor 5
cDNA	complementary desoxy-ribonucleic acid
RT	reverse transcriptase
RT-	reverse transcriptase negative
CCNA2	cyclin A2
G6PD	glucose-6-phopshate dehydrogenase
GAPDH	glycerine-aldehyde-phosphate dehydrogenase
HPRT1	hypoxanthine-guanine phosphoribosyltransferase
TFRC	transferrin receptor coding gene
CT	cycle time
ANOVA	analyses of variance
HSD	honestly significant difference
Kir	inwardly rectifying potassium channel
OPC	oligodendrocyte precursor cell
COX2	cyclooxygenase-2
MPO	myelin-peroxidase
BW	bodyweight

List of figures

List of tables

1. Introduction

1.1 Multiple sclerosis

1.1.1 Multiple sclerosis - epidemiology and risk factors

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS) (1), evokes various neurological symptoms in patients (2) and presents itself with four different phenotypes (3). In the year 2020, globally estimated 2.8 million people suffered from multiple sclerosis, what equals a prevalence of 35.9 per 100 000 people (4). There was an increase in the estimated prevalence of MS on the average of 30 percent, between the years 2013 and 2020, in all regions of the world and globally women suffer twice often from MS then men (4). Risk factors for the pathogenesis of MS include genetic factors and certain environmental influences (5). People with relatives who are suffering from MS, have themselves a higher risk to develop the disease than people without relatives who have MS (6,7). The risk declines as the degree of kinship increases (7).

There was a positive association found between MS and certain alleles (HLA-DR2) of genes which code for the major histocompatibility complex (MHC) (8), more specifically for the HLA-DRB1*1501-DQB1*0602 haplotype of human leukocyte antigen (HLA) class II molecules (9). These findings were extended, as it was shown that the risk is most increased by the HLA-DRB1*15:01 allele, with an almost 4-times higher odds ratio (OR=3.92) to develop MS (10). Furthermore, it was analysed that the risk is also increased by other HLA alleles, and there were also protective HLA alleles in cohorts of European ancestry found (10). In an African population, the positive association with the DRB1*1501 allele was confirmed (11). Moreover, there is data that suggests that a homozygote HLA-DR2 haplotype increases MS susceptibility and the risk for a severe disease course (12). This data indicates that humans have a greater susceptibility to develop MS when they have certain alleles of HLA (10–12). This finding is consistent over different ethnical groups and regions (10,11), and it explains the increased familial risk.

One of the major environmental associated factors with MS, is an infection with the Epstein-Barr Virus (EBV) (13). A metanalysis revealed that the incidence rate of MS is 2.3 times higher after an infection with EBV and the manifestation as infective mononucleosis (IM), compared to EBV positive individuals without a manifestation

as IM (13). One large longitudinal study conducted in the US military, with over 10 million people involved, has shown that the risk of developing MS (955 MS cases) increased by 32-fold in young adults, the majority was under 20 years old, after an EBV infection with seroconversion (14).

These two risk factors were connected in a study, where it was shown that two distinct EBV glycoproteins evoked a significantly higher CD4 T cell immune response in HLA-DRB1*15:01 positive persons (homozygous) than in HLA-DRB1*15:01 negative individuals (15). This study combines these two risk factors and could deliver hints on a possible explanation why just specific individuals, who are genetically susceptible, develop MS after an EBV infection.

Furthermore, there are higher incidence regions of MS, like northern America and Europe and regions with lower incidence rate like African countries and south-east Asia (4). Another environmental factor, influencing MS risk, is migration to areas with a lower or higher incidence rate of the disease (16–18). It was shown that people who migrated before the age of 15 in a country with a lower incidence rate, decreased their risk of developing MS (16), whereas children of people who moved to a country with a higher incidence rate, had themselves a comparable incidence rate of developing MS as the resident population in this country (17). A recent study conducted, also confirmed that migration to a country with a high incidence rate leads to a significant increased hazard ratio for developing MS, when a greater timespan was spent there (e.g. 70% vs. 20%) and the immigration happened before the age of 15 (18). Furthermore, low Vitamin D serum levels are suggested to be also a risk factor for developing MS and high serum levels seem to be protective according to a longitudinal study (19). This observation was emphasized in participants before the age of 20, however, this effect was not found in African and Hispanic Americans (19). Another metanalysis confirmed this connection, where it was shown that there is an inverse association between genetically predisposed 25(OH)-D concentrations and incidence of MS (20). A further risk factor for developing MS, is the female sex with a incidence twice as high then men (4). Smoking could also lead to an increased possibility of developing MS, with an OR of 1.55 (conservative model) according to a metanalysis (21).

Taking all these literature into account, it can be concluded that MS seems to be an example for a disease where there has to be a genetical predisposition, like certain

HLA alleles (10–12) or the female sex (4), combined with environmental risk factors, exposed in adolescence, like an EBV infection (14) with a manifestation as IM (13), and low Vit-D serum levels (19) or the habit of smoking (21) which seem to be more present in high incidence regions (16–18), which might subsequently lead to the outbreak of the disease.

1.1.2 Multiple sclerosis – phenotypes and diagnostic criteria

In the clinical context MS presents itself with four different phenotypes (3). The clinically isolated syndrome (CIS) can be the first presentation of MS, however, which does not yet fulfil all diagnostic criteria as dissemination in space (DIS) and/or dissemination in time (DIT) (22). At the time it meets all the diagnostic criteria, it gets classified as relapsing-remitting MS (RR-MS) (3). If the diagnosis is MS and there is a steady progression in disease course from the beginning, it should be classified as primary progressive MS (PP-MS) (3). If there is a progression of disease by an initial relapsing course, it should be classified as secondary progressive MS (SP-MS) (3). The disease progression should be minimally assessed once a year (3). An assessment of the activity should also be conducted at least annually, to classify these phenotypes into active or inactive forms (3). Active phenotypes are defined by showing new or enlarging correlates in the magnetic resonance imaging (MRI) and/or clinical signs of neurological dysfunctions (3).

Regardless the phenotype, MS presents itself clinically with different symptoms, which include fatigue, spasticity, voiding disorders, motor symptoms, pain, cognitive dysfunction and depression, ordered by descending frequency (2). In PP-MS and SP-MS spasticity, voiding disorders, fatigue and motor problems are the most common symptoms (2).

For the diagnosis of MS, the 2017 McDonald criteria are used (table 1). To meet these diagnostic criteria, lesions in the CNS, confirmed clinically or with MRI, disseminated in space (DIS) and disseminated in time (DIT) are demanded (22). To fulfil the diagnostic criteria of DIT, liquor specific oligoclonal bands are also considered to confirm diagnosis in some cases (22).

Table 1: 2017 McDonald criteria for the diagnosis of MS in patients with an attack at the onset *(22)*

Clinical attacks	Number of lesions	Additional data needed for diagnosis of MS
≥2	≥2	None
≥2	1	None
≥2	1	DIS (clinical attack or MRI lesion)
1	≥2	DIT (clinical attack, MRI or CSF specific oligoclonal bands)
1	1	DIT and DIS

If a person meets these criteria and there is no other pathology or cause found to explain the symptoms and lesions, the diagnosis of MS should be made (22). For the diagnosis of the PP-MS there are different criteria (table 2). There needs to be a clinical progression for one year, confirmed retrospectively or prospectively, regardless of clinical relapses and two of the following criteria (22):

Table 2: 2017 McDonald criteria for the diagnosis of MS in patients with progression from the beginning of the disease *(22)*

≥1 T2-hyperintense MRI lesions in ≥1 of these brain regions: periventricular, cortical, juxtacortical, infratentorial
≥2 T2-hyperintense MRI lesions in the spinal cord
The verification of CSF – specific oligoclonal bands

Regarding SP-MS, no standardised definition has prevailed. The currently most precise definition of SP-MS is from Lorscheider et al. (23) (table 3), where the diagnosis is made of following criteria:

Table 3: Diagnostic criteria by Lorscheider et al. for the diagnosis of SP-MS *(23)*

Disability progression by 1 step on the Expanded Disability Status Scale (EDSS) in patients with EDSS ≤ 5.5 in the absence of a relapse
Disability progression by 0.5 EDSS steps in patients with EDSS ≥ 6 in the absence of a relapse
+ a minimum EDSS score of 4 and pyramidal functional system (FS) score of 2, and confirmed progression over ≥ 3 months, including confirmation within the leading FS

Depending on these clinical phenotypes of the disease there are different histopathological findings. These findings present themselves as focal demyelinating lesions in the white matter, as a diffuse injury of the normal appearing white matter with a diffuse inflammatory reaction or as demyelinating cortical lesions in the grey matter (24). The focal demyelinating plaques in the white matter are present in all stages of the disease, but they differ in activity between the phenotypes (24). In patients with RR-MS focal active white matter lesions with an inflammatory cellular infiltrate, consisting of macrophages and lymphomonocytes with subsequent demyelination (25), predominate, whereas in PP-MS and SP-MS, there are mainly inactive lesions with no inflammatory cellular infiltrate (24).

1.1.3 Multiple sclerosis - cortical lesions of PP-MS and SP-MS

As noted above, besides white matter lesions, there are also cortical lesions which were first described systematically by Brownell & Hughes in 1962, who quantified cortical lesions in an autopsy study (26). Even though cortical lesions were known, MS was considered to be mainly a white matter disease, due to difficulties in recognizing cortical lesions in imaging, were they were under detected (27), but also in classical histological staining techniques without immunohistochemistry (28). However, with time it was shown that cortical lesions are a frequent finding in MS patients (27,29). Then it was discovered by Bo et al. that the cortical demyelinating lesions of patients with progressive forms of MS, show no signs of an increased inflammatory cellular infiltrate compared to healthy controls or non-demyelinated areas in the cortex of the same individual (30). They also classified cortical lesions into four different histological categories (31). Furthermore, it was a change in perspective, when it was shown by Kutzelnigg & Lassmann et al., that these cortical demyelinating lesions of the grey matter and the diffuse white matter injury occur significantly more frequently in the progressive forms, PP-MS and SP-MS, of the disease (24). Whereas, these findings are not common or absent in RR-MS (24), making it a characteristic feature of progressive MS forms, where the cortex can be extensively affected by cortical lesions (32). In the progressive forms, these cortical lesions present themselves mainly as subpial band-like demyelinated areas with various depth and affect the cortex focally or as a widespread demyelination (32).
In summary, according to the literature provided, the inflammatory cellular infiltrate in the white matter lesions of PP-MS and SP-MS, is compared to the RR-MS disease

course, reduced or absent (24). In addition, there is also no increased inflammatory cellular infiltrate in the cortical lesions of progressive MS forms (30). This indicates that these cortical demyelinating lesions occur and might develop independently from a peripheral inflammatory cellular infiltrate in the cortical parenchyma.

1.2 Experimental models of multiple sclerosis

Basic laboratory research is the foundation to investigate pathologies and to find new therapeutic options for diseases. Therefore, proper animal experimental models are necessary to represent the main features of the investigated disease and to carry out experiments. For the investigation of MS, there are different animal models known, in which a demyelinating process is evoked by an experimental autoimmune encephalitis (EAE), a viral infection or through administration of toxins (33). There are various EAE models established, where an inflammatory process is induced by immunization against components of the myelin sheath, using different techniques (34). Due to the fact that MS is a complex disease with different phenotypes (3) and histopathological findings (24), none of these models is able to represent the whole spectrum of the disease. Most variants of EAE models utilize autoimmunization of animals against a component of myelin (myelin basic protein, proteolipid protein or myelin-oligodendrocyte glycoprotein (MOG) antigenic epitopes) together with completes Freund's adjuvant (34,35). These models successfully mimic the white matter plaques in the cerebellum and spinal cord with T-cell infiltrates (36,37). Depending on the used species (rat or mouse), strain of the animal, the used variant of the EAE model as well as the used antigenic epitope, a variety of disease pathologies can be reproducibly generated, including the relapsing-remitting phenotype (34). In these commonly used animal models, however, cortical structures and subsequently the grey matter stays spared and are mostly not affected (36–38), therefore other models were developed to recapitulate cortical lesions of MS (38,39).

On of these, is a widespread cerebral demyelination model of Dark Agouti rats developed by Prof. Hochmeister and colleagues which successfully reproduces cerebral cortical pathology observed in progressive types of the disease (40,41). In this model, pro-inflammatory cytokines were injected through an implanted catheter into the cortex of rats, which were subclinically immunized against a recombinant myelin oligodendrocyte glycoprotein ($rMOG_{1-125}$) (40,41). Inflammatory response

triggered by the atraumatic infusion of cytokines through the catheter results in a cortical demyelination of both cerebral hemispheres with little to no inflammatory cellular infiltration (40,41), recapitulating the key features of histopathological findings of progressive MS forms (24,30,32). Consequently, this model seems suitable for the investigation of progressive forms of MS.

1.3 Disease modifying therapies of multiple sclerosis

According to the guidelines of the German neurological association for the diagnosis and treatment of MS, there are several disease modifying treatment options for RR-MS approved (table 4), which are categorized into three different categories, depending on their clinical efficacy in a relative reduction of inflammatory activity (42).

***Table 4: Categories of substances for the treatment of RR-MS** (42)*

class I substances	dimethylfumarat, diroximelfumarat, glatirameroide, teriflunomide, beta-interferone
class II substances	cladribin, S1P-receptor modulators (fingolimod, ozanimod, ponesimod)
class III substances	alemtuzumab, CD20-antibodies (ocrelizumab, ofatumumab, rituximab, ublituximab), natalizumab

Inflammatory activity is defined through relapse rate, MRI activity and disease progression through relapses (3,42). Aims of the treatment are a decrease in disease progression, relapses, subclinical disease activity measured with MRI and an improvement in the quality of life (42).

According to current evidence, for the treatment of PP-MS, the cluster of differentiation (CD) 20 antibodies Ocrelizumab and Rituximab show some efficacy (42–45). For the treatment of SP-MS, a distinction between an active or inactive form, as described above (3,42), is necessary (42). According to a guideline for the treatment of active SP-MS siponimod (42,46), beta-interferone (1b) (42), and mitoxantrone (42,47) are evidence based and approved, however, the usage of

mitoxantrone is not recommended anymore (42). Furthermore, no effect on risk of disability progression through treatment with beta-interferone was found in a meta-analysis, there was only a reduction in relapse risk detected (48). For the treatment of inactive SP-MS, there are currently no treatment options which are founded on evidence (42).

There are some medications that show efficacy in RR-MS and in PP-MS or SP-MS (42). The CD20-antibodies rituximab and ocrelizumab show efficacy in RR-MS and in PP-MS (42–45,49,50). Ocrelizumab leads to a declined disease progression confirmed through clinical and MRI measurements compared to Interferon beta 1a in the treatment of RR-MS (49) and in the treatment of PP-MS compared to a placebo (44). An extension of this study confirmed the efficacy of ocrelizumab over a longer (6 years) period, where a beneficial influence of earlier vs. later ocrelizumab intervention was shown (43). Furthermore, it was shown that Rituximab has a beneficial influence on relapse rate compared to class I substance dimethyl fumarate in the treatment of RR-MS (50). In the treatment of PP-MS, there was a beneficial influence of Rituximab on disease progression in patients younger than 51 years and patients with gadolinium enhancing MRI lesions (45). A cumulative effect was found when these two features occurred combined, so especially in younger patients with inflammatory lesions (45). There is also evidence, that suggests, that rituximab could decline disease progression in SP-MS (51). Furthermore, in RR-MS and SP-MS, sphingosine-1-phosphate (S1P) receptor modulators and beta-interferone are influencing the disease in a beneficial way (42,46,48,52). A large network meta-analysis showed, that treatment with the S1P-recepotor modulator fingolimod, had a beneficial effect on disability aggravation and leads to a reduced relapse rate over a 24-month period, however, interferon beta-1a or interferon beta-1b, also reduced the relapse rate, but less, in the same time period in RR-MS compared to placebo (52). In the treatment of SP-MS, beta-interferone showed no beneficial influence on overall disability progression, but they decreased relapse rate (48). The S1P-receptor modulator siponimod showed in a clinical study a beneficial influence on SP-MS disability progression, other clinical and imaging parameters (46). Taking all this literature into account, despite there are several treatment options for MS, therapeutic interventions especially in the progressive types are limited. Therefore, it is of utmost importance to explore further

therapeutic options for MS, especially for the progressive forms. In this study, the effect of the disease modifying substance siponimod, which shows efficacy in SP-MS (46) is investigated.

1.4 Siponimod

Siponimod is a S1P receptor modulator, that is approved for the treatment of SP-MS, with disease activity, which must be confirmed through clinical relapses or signs of inflammatory activity in imaging (53). S1P-receptors are cell surface receptors which are G protein linked, and they have five different subtypes (S1P1-5) (54). Siponimod is able to specifically bind to the S1P receptor subtypes one (S1P1) and five (S1P5) (55). These receptors are expressed in various cells, tissues and organs throughout the body, and they are involved in a variety of different biological functions (54). They are among others expressed in the nervous system, the heart, the lungs, the kidneys, the liver, by immune cells and primary and secondary lymphatic organs like the thymus and the lymphoid system (54,55).

S1P1 plays an important role in the immune system by influencing the traffic of lymphocytes (56). It was shown in an animal model that S1P1 signaling drives the egress from B and T cells out of the peripheral lymphatic organs like the lymph nodes to the peripheral tissues and the wandering from mature thymocytes out of the thymus (56). Loss of S1P1 signaling, resulted in a significant reduction in the egress from these cells out of the lymphatic organs into the blood and the lymph (56). These findings indicate that S1P1 signaling seems necessary for the egress of these immune cells out of the lymphatic organs to the periphery (56). Furthermore, these results suggest that an antagonist of S1P1 signaling has immunosuppressive effects by preventing the egress of immune cells (56). This mechanism described is the main known mechanism of action of siponimod, which results in a reduction of T cells infiltrating into the CNS, by working as a functional antagonist on the S1P1 on lymphocytes (53).

In a clinical study, it was shown that treatment with siponimod attenuates the risk for a 6-month confirmed disability progression, delays disability progression, improves clinical features of SP-MS like the cognitive processing speed, reduces brain atrophy measured with an MRI and inflammatory disease activity, in a 5-year time period (46). In addition, it was observed in experimental studies that siponimod also has direct effects on the resident glial cells in the CNS and can influence their

gene expression (57–60). Furthermore, there is evidence that it has a beneficial impact on EAE disease severity thought to be independent of its peripheral effects (61).

1.5 Hypothesis

Taken together, the known primary mechanism of action of the S1P receptor modulator siponimod, in the treatment of progressive MS is, that it binds to S1P1 on lymphocytes and works as an antagonist on this receptor and subsequently reduces the egress from T cells out of the lymph nodes into the peripheral tissues like the CNS (53). But, as noted above, there is also evidence that it has direct effects on the glial cells in the CNS (57–60). Furthermore, it was proven in clinical studies, that siponimod has a beneficial effect on SP-MS, influencing disease progression, functional and morphological features (46). Accordingly, preliminary experiments by the working group, by which this work has been supervised, showed that siponimod was protective against inflammation induced demyelination of cortex in the aforementioned rat model, despite the substantially high anti-MOG titres. Since these lesions in the animal model are also free from T-cell infiltrates (40), it raises the question how does Siponimod exert its beneficial effects leading to significant myelin protection.

Therefore, we hypothesize that siponimod might act in this way by influencing the gene expression of the resident glial cells in the CNS and promoting an anti-inflammatory phenotype by upregulating the expression of anti-inflammatory genes and by downregulating the expression of pro-inflammatory genes. The aim of this study is to reveal a previously unknown mechanism of action of Siponimod. To test this hypothesis, primary mixed glial cell (MGC) cultures are cultivated and treated in different constellations with the drug siponimod and pro-inflammatory cytokines. After cultivation and treatment of the MGC cultures, the mRNA expression of certain inflammation related genes will be quantitatively evaluated with a quantitative real time-polymerase chain reaction (qRT-PCR), to analyse the gene expression followed by a statistical analysis.

1.6 Mixed glial cell culture

The neuronal tissue in the CNS consists of neurons and glial cells which are astrocytes, oligodendrocytes, ependymal cells and microglial cells which play a role in many different functions of the CNS (62–67). To investigate the effect of siponimod on these cells, a MGC culture consisting of astrocytes, oligodendrocytes and microglial cells is cultivated. There are methods for separate glial cell cultures known, which enable one to investigate specifically one distinct glial cell type (68). But it has been shown, that glial cells behave differently in an inflammatory environment when they are separated or when they can interact with each other (69,70). Therefore, also methods were established to cultivate a MGC culture containing astrocytes, oligodendrocytes and microglial cells (71). This indicates, that MGC cultures seem suitable to represent the interactions between glial cells (69,70) similar as they occur in a brain of a living organism. In order to reflect these glial cell interactions, a MGC culture is used in this study to perform the planned experiment and to test the hypothesis.

1.7 Investigated genes

In this experimental study, the mRNA expression of following genes, listed in the tables 5 and 6, was quantified and subsequently analysed to test the hypothesis, if siponimod reduces the expression of pro-inflammatory genes and increases expression of anti-inflammatory genes in glial cells and thus promotes an anti-inflammatory phenotype. The used genes as markers are presented in table 5 and table 6.

Table 5: Used genes as markers for M1 microglia, M2 microglia and reactive astrocytes

M1 microglia	M2 microglia	Reactive astrocytes
inducible NO-synthase (iNOS)	arginase 1 (Arg1)	upregulated complement component C3 (C3)
-	insulin like growth factor 1 (IGF-1)	Downregulated potassium inwardly rectifying channel subfamily J member 10 (KCNJ10)

Table 6: Used genes as markers for protective astrocytes, a pro-inflammatory and an anti-inflammatory reaction

Protective astrocytes	Pro-inflammatory	Anti-inflammatory
brain derived neurotrophic factor (BDNF)	interleukin 6 (IL6)	interleukin 10 (IL10)
toll like receptor 3 (TLR3)	prostaglandinsynthase 2 (PTGS2)	tumor necrosis factor receptor associated factor 5 (TRAF5)
ciliary neurotrophic factor (CNTF)	-	-

1.8 Glial cells

1.8.1 Microglial cells

Microglial cells are part of the glial cell system of the CNS (62), more specifically they are an ontogenetically unique group of the mononuclear phagocyte system (72), and they make up for approximately 25 percent of the non-neuronal cells in the human cortex of the brain (73). Microglial cells are involved in a variety of functions. Among other functions, they are part of immune reactions by recognizing pathogens through different pattern-recognition receptors or by phagocytosis of redundant cell parts (64). Furthermore, microglial cells are expressing various receptors for neurotransmitters, by which their behaviour is modulated (65).

Concerning pathological states, there is a paradigm regarding microglial cells which is that there are two types of microglial cells: M1 and M2 microglial cells (64,74–76). M1 microglial cells represent the pro-inflammatory phenotype and they are associated with pro-inflammatory cytokines and neurotoxic factors, whereas M2 microglial cells represent the anti-inflammatory phenotype which is associated with neuroprotective factors (64,74–76). Regarding MS, there is evidence in experimental studies that M2 microglial cells are associated with remyelination (77). In contrast, inducible NO-synthase (iNOS) expression is associated with active lesions of progressive forms of MS (78) and iNOS is used as a marker of M1 microglia (75). This evidence supports the M1/M2 paradigm regarding MS. In this study, as a marker for the pro-inflammatory M1 microglial cells the mRNA

expression of iNOS is used. Whereas as a marker for the anti-inflammatory M2 microglial cells the mRNA expression of arginase 1 (Arg1) and insulin like growth factor 1 (IGF-1) is used.

1.8.2 Astrocytes

Astrocytes are part of the glial cell system in the CNS (62), and they make up for approximately 35 per cent out of the non-neuronal cells in the human cortex (73). Among other things, astrocytes take part in the homeostasis of ions, keeping balance of the water and the extracellular volume in the brain and they are playing a role in the regulation of neurotransmitters (66). Furthermore, astrocytes communicate and interact with neurons, and they are involved in the controlling of the development of synapses (67). In summary, in the physiological healthy brain astrocytes are involved in many different processes which are essential for its proper function.

Concerning pathological circumstances, astrocyte reactivity or astrogliosis is a response of astrocytes which is associated with events that harm the CNS like ischemia, trauma or neuroinflammation (79). According to evidence, there are two types of reactive astrocytes, which differ in the molecular phenotype, depending on the primary inflammatory stimulus: lipopolysaccharide (LPS) or ischemia (80). The astrocytes stimulated with LPS show a molecular phenotype of genes expressed which suggests it is harmful, whereas the reactive astrocytes induced by ischemia show a molecular phenotype which suggests it is beneficial (80). In another study conducted, it was proven that these reactive astrocytes, called “A1” reactive astrocytes, induced by an inflammatory stimulus are neurotoxic and that they are associated with neurodegenerative disorders like Alzheimer disease or MS (69). The suggested protective astrocytes were labelled as “A2” reactive astrocytes (69). In addition, it was shown in an experimental mouse model, that a reduction in the A2 astrocyte molecular phenotype was associated with a more severe neurological damage and microglial activation, compared to a state with an A2 astrocyte molecular phenotype, which was associated with a neuroprotective effect after a global ischemia of the brain (81). These findings support the hypothesis that A2 reactive astrocytes are neuroprotective.

There is also data regarding reactive astrocytes with an emphasis on the morphological features. According to literature and concerning these features, there

are also two subtypes. First, the proliferative border-forming reactive astrocytes, which are forming borders around areas with damaged cells, and second, the non-proliferative reactive astrocytes that are able to keep its structure but react to the injury (82), for example in altering its gene expression (80,82). According to this literature, it must be considered that astrocyte reactivity has not a dichotomic outcome which is either beneficial or harmful to the organism, instead it is multilayered and has to be seen in a disease and context dependent manner (82). Since in this study only data about the mRNA expression was evaluated and no morphological features were assessed, the A1 and A2 reactive astrocyte concept is used, but the results will be interpreted in a disease and context dependent manner. In this study, an upregulated mRNA expression of complement component C3 (C3) and a downregulated mRNA expression of potassium inwardly rectifying channel subfamily J member 10 (KCNJ10) is used as a marker for A1 reactive astrocytes. The mRNA expression of brain derived neurotrophic factor (BDNF), toll like receptor 3 (TLR3) and ciliary neurotrophic factor (CNTF) is used as a marker for A2 protective astrocytes.

1.8.3 Oligodendrocytes

Oligodendrocytes play also a vital role in the CNS as a part of the macroglia by forming the myelin sheath around the axons (62). They account for approximately 40 per cent of the non-neuronal cells in the human cortex and therefore they are the most frequent glial cell type (73). They play a crucial role in many different functions in the CNS. By forming the myelin sheath around the axolemma between the nodes of Ranvier, they increase the speed of signal transmission by enabling the saltatory conduction, which is a fundamental part for the function of the nervous system of vertebrae (62,83). Furthermore, there is evidence, that in the physiological state oligodendrocytes are required maintain proper function and survival of axons and neurons by supporting them with energy metabolites (63).

With regards to MS, oligodendrocytes play also a key role due to the fact myelin sheaths, which are formed by oligodendrocytes (62), are destroyed in demyelinated lesions, what is a key feature of the disease (24). In summary, oligodendrocytes play among other things an essential role in the physiology of axons and neurons, stimulus transmission and in the pathology of MS. The effect of siponimod on

oligodendrocytes is also investigated in this study, due to the presence of these cells in the MGC culture, however, no specific markers for oligodendrocytes were used.

2 Materials and methods

2.1 Isolation of primary glial cells

Neonatal rats (P5-P6) were euthanized with cryoanaesthesia and decapitation. The head was placed on a petri dish, located on ice for cooling and was brought to a biological safety cabinet with vacuuming system (Herasafe biological safety cabinet, Kendro, Langenselbold, Germany) for the following steps of cell culture preparation. Then, the skin of the skull was cut on top in the midline with scissors, folded to the side with two fingers, and then the skull was cut on top in the midline and in the transversal plane, just beneath the ears, on both sides. The two skull parts were removed with a tweezer from the cerebrum. The cerebellum was removed from the brain with scissors. Using a small spatula, the two hemispheres of the cerebrum were removed carefully and were placed on the teflon plate of the tissue chopper (McILWain Tissue chopper Modell TC7, The Mickle Laboratoy Engineering Co.Ltd., Surrey, England). The brain tissue was chopped two times in a 90-degree angle at 100µm steps. The tissue was brought with the small spatula into 1 mL of warm accutase in a 15mL centrifuge tube, and the tube was transferred into a waterbath (GFL water bath, LAUDA DR. R. WOBSER GMBH & CO. KG, Lauda-Königshofen, Germany) with a temperature of 37° Celsius for 20 min. The tube was shaken from time to time. Immediately after 20 minutes, 5 mL of a solution containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, MA, USA) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco) were added into the tube. This suspension was transferred into a 50mL centrifuge tube using a cell strainer (70 µm). The tube was centrifuged (Heraeus Multifuge 1 L-R, Kendro, Germany) for 5 mins at 1000 rpm, afterwards the supernatant was discarded, by using the vacuum system of the cell culture bench. The cells were re-suspended with 5 mL of the MGC culture medium, composed of 38,75 mL DMEM, 10 mL FBS (20 %), 500 µL Sodium pyruvate (1 mM) (Gibco), 500 µL GlutaMAX (2 mM) (Gibco), Penicillin-Streptomycin 250 µL (10.000 U/mL) (Gibco) for 50 mL of the medium, and seeded in a Poly-D-Lysine (Gibco) coated T-25 cell culture flask (25 cm2 cell culture flask cellstar, greiner bio-one, Frickenhausen, Germany).

2.2 Mixed glial cell culture and cultivation

The cells were cultured, at 37°C with 5% CO_2, in a humidified cell incubator (Binder, Tuttlingen, Germany) for ten days in the MGC medium. The MGC medium was refreshed every 2-3 days. The cultures were checked, using a reflected light microscope (Olympus IX 51), before changing the media.

2.2.1 Treatment of the cells

The cells were treated on the 10th day in the following constellations:

1) siponimod + cytokines (interferon-gamma (IFNγ + tumor necrosis factor alpha (TNFα))
2) cytokines + dimethylsulfoxide (DMSO)
3) siponimod
4) DMSO
5) naive – without treatment

In the first group, the MGC culture was treated with final concentrations of 2 µM siponimod (MedChemExpress, USA, Catalog# HY12355/CS-3876) (10mM in DMSO) (DMSO 100%, Sigma-Aldrich, MO, USA), 12 ng/mL IFNy (Recombinant Rat IFN-gamma, PEPROTECH, Thermo Fisher Scientific, MA, USA, Catalog# 400-20) and 2 ng/mL (TNFalpha (Recombinant Rat TNF-alpha, R&D Systems, MN, USA, Catalog# 510-RT-050/CF) both solved in Dulbecco's Phosphate Buffered Salin (-) (D-PBS (-)) (Gibco).

The second group was treated with the same concentration of cytokines, but siponimod was replaced with 1 µl DMSO as vehicle control.

The MGC culture in the third group was treated with 2 µM siponimod alone, whereas the fourth group was treated with solely 1 µL of DMSO. The fifth group (naive controls) was left untreated. All experimental groups were incubated for a further 20 hours after the treatment, before harvesting the cells for RNA isolation.

2.2.2 Cell harvesting

The cells were harvested on the 11th day of cultivation, after 20 hours of treatment, as follows: medium was aspirated, the culture was washed once with 5 mL of D-PBS (-). The cells were detached from the bottom of the flask, using a cell scrapper, and were resuspended with 2 mL of a solution containing DMEM + 10 % FBS. The suspension was transferred into a 2 mL Eppendorf tube (safe lock tube, Eppendorf,

Germany) and was centrifuged (Heraeus Multifuge 1 L-R, Kendro, Germany) for 5 min at 2000 rpm. The supernatant was discarded, the remaining cell pellet was snap frozen in liquid nitrogen and stored at -80 °C in a freezer until further use.

2.3 RNA Extraction

The RNA extraction was performed using the Qiagen RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). First, 750 µL of the RLT Buffer, containing 1% beta-mercaptoethanol (AppliChem GmbH, Darmstadt, Germany), was transferred into each tube to the cell pellet. By pipetting in and out several times, the cell pellet was disrupted into small particles, until there was macroscopically an even distribution of the cell pellet in the liquid. Then this suspension was transferred to a QIAShredder column, in a 2 mL tube, and was centrifuged (Heraues Biofgue pico, Kendro, Germany) for 13000 rpm for 2 minutes. The flow-through was used for further steps. One volume of 70% ethanol was given into the lysate and was mixed five to seven times with the pipette, to get a homogenous liquid. Then 700 µL of this solution were transferred to a RNeasy mini spin column, in a 2 mL collection tube and was centrifuged for 15 seconds at 13000rpm. The flow through was discarded. This step was done a second time with the same sample to use all the lysate. After that, 700 µL of the RW1 Buffer was added to the mini spin column and it was centrifuged for 15 seconds at 13000 rpm. The flow through was discarded. Then 500 µL of the Buffer RPE were transferred into the mini spin column and the column was centrifuged for 15 seconds at 13000 rpm. This step was done another time. Then the mini spin column was placed into a 1.5 mL collection tube of the kit, and 50 µL of RNase free water was added directly on the membrane of the mini spin column and was subsequently centrifuged for one minute at 13000 rpm. The eluate was transferred back to the spin column, directly on the membrane, and was centrifuged for another time for one minute at 13000 rpm. After that, the RNA was extracted, and the concentration of the RNA was measured at a photometer (Nanodrop 2000c, spectrophotometer, Thermo Fisher Scientific, MA, USA). Then it was stored at –80°C for the further steps of the experiment.

2.3.1 RNA concentration measurement

First, the photometer was cleaned, by pipetting 3 µL of distilled and deionized water (dd H2O) on the location for the sample on the lower arm of the photometer. The upper arm was closed and reopened, the remaining liquid was removed with a paper towel, and dust was removed with a butane duster. Then, 1 µL of the RNAse free water of the Qiagen RNeasy Mini Kit was measured as a blank sample. Afterwards, 1 µL of each sample was measured. Finally, the RNAse free water was measured again as a sample, to check if the blank sample is still negative.

2.3.2 RNA gel

The quality of the extracted RNA was checked with RNA gel electrophoresis. For the gel, 1.2 g of Agarose powder (Biozym LE Agarose, Biozym Scientific GmbH, Hess, Germany), weight with a scale (GP3202-0CE, Sartorius, Göttingen, Germany), and 10 mL of 10x Formaldehyde Agarose gel buffer (consisting of: 200mM 3-[N-morpholino] propanesulfonic acied (MOPS) free acid, 50mM sodium acetate, 10mM EDTA, pH to 7.0 with NaOH) was mixed and filled up to 100 mL with diethylpyrocarbonat treated-H_2O (DEPC-H_2O), under the laminar airflow, in a 250 mL Erlenmeyer flask. Afterwards, it was heated up in a microwave with 200 Watt until the solution was clear and it was boiling visibly. The solution was shaken from time to time during the heating process to solve the agarose gel and to avoid boiling delay. Then, it was cooled down under water to approximately 65 degrees. Next, 1.8 mL of 37 % Formaldehyde (Carl Roth, Karlsruhe, Germany) and 1 µL of GelRed Nuclei Acid Gel Stain (Biotium, CA, USA) was added to the solution under the laminar airflow. Then, the gel was poured into the mold of the gel electrophoresis system (Thistle scientific, Rugby, UK) and air bubbles in the gel were removed with the tip of a pipette. After the gel was solid, it was equilibrated in the running buffer for 30 minutes. The running buffer was mixed under the laminar airflow. It contained 880 mL DEPC-H_2O, 20 mL of 37% formaldehyde, and 100 mL of 10x formaldehyde agarose gel buffer. For the gel electrophoresis 15 µL volumes, containing 300 ng of RNA, were used.

Therefore, the samples were thawed and for the first batch a volume containing 500 ng of RNA, according to the RNA concentration of the sample, was transferred to a 0.2 mL polymerase chain reaction (PCR) tube and adjusted to 25 µL with nuclease free water (New England Biolabs, MA, USA). Then, a volume of 15 µL of this diluted

sample, containing 300 ng of RNA, was transferred to another 0.2 mL PCR tube. For the second and third batch, a volume containing 1000 ng of RNA, according to the RNA concentration of the sample, was transferred to a 0.2 mL PCR tube and adjusted to 15 μL with nuclease free water. Then, a volume of 4.5 μL of this diluted sample, containing also 300 ng of RNA, plus 10.5 μL nuclease free water (to get also a volume of 15 μL), was transferred to a 0.2 mL PCR tube. See tables 9-13 in the appendix for the exact volumes used.

The remaining samples were used for complementary DNA (cDNA) synthesis, after the quality of the RNA was checked with the gel electrophoresis. For the third cDNA batch, instead of the gel, a single stranded DNS measurement, with a photometer, was performed. Then, 5 μL of an RNA Loading Dye (Thermo Fisher Scientific, MA, USA) was transferred to each sample that was used for gel electrophoresis. After this step, the samples were vortexed for a few seconds and centrifuged (centrifuge/vortex combi – spin FVL-240) for 30 seconds to spin all the liquid to the bottom of the tubes. Then, the samples were incubated at 65°C for 8 minutes (lid temperature was 72°C), and then cooled on ice for 2-3 minutes, before loading onto the gel. The electrophoresis was done at 85 volts for 35 min. The samples on the gel were imaged using ChemiDoc XR+ Gel Documentation System (BioRad, Germany).

2.4 cDNA synthesis

The cDNA was synthesized using LunaScript RT Super Mix Kit (New England Biolabs). The 0.2 mL PCR tubes, containing the samples with the RNA for cDNA synthesis, were put on ice and 4 μL of the LunaScript RT Super Mix was transferred to the same 0.2 mL PCR tubes and it was filled up to 20 μL with nuclease free water. Reverse transcriptase (RT) negative (RT-) controls were prepared using RNA pool stocks. These were prepared by mixing equal mass of RNA, 300 ng in the first batch, and 700 ng in the second and third batch, taken from multiple samples. Then, the same volume of nuclease free water as the RNA sample was added in each pooled sample. Afterwards, 12 μL of this pooled RNA sample was transferred to a 0.2 mL PCR tube, 4 μL of LunaScript No-RT Control Mix was added, and it was filled up to 20 μL with nuclease free water. See tables 9-13 in the appendix for the exact volumes used.

In addition, one no template control was prepared by transferring 4 µL of the LunaScript RT Super Mix to a 0.2 mL PCR tube and filling it up to 20 µL with nuclease free water. Then the samples were vortexed to get a homogenous solution and centrifuged for a few seconds to ensure that all the liquid was at the bottom of the tube. Then, a thermocycler (PeqStar 96X PCR-Cycler, VWR Peqlab by Avantor, PA, USA) was used to synthesize the cDNA by using the following protocol:
Lock cycler with 100 N - Heat lid to 45°C - Primer annealing temperature by 25°C for 2 min. - Heat Lid to 65°C - cDNA synthesis at 55°C for 10 minutes - Heat Lid to 110°C - Heat inactivation by 95°C for 1 minute - Deactivate Lid Heating - cool down to 10°C.
Afterwards, the cDNA samples were diluted according to the used RNA concentration, before the cDNA synthesis, to get a concentration of 2.5 ng/µL. Then, they were transferred to 1.5 mL DNA LoBind tubes (Eppendorf) and stored at -20°C until further use.

2.5 Quantitative real time PCR

2.5.1 Primers

The primers (Eurogentec, Belgium) that were used for the experiments are depicted in table 7:

Table 7: Used primers for the gene expression experiment in the MGC cultures

Gene	Forward primer	Reverse primer	Amplicon length (bp)
Arg1	5'-GCCCTCTGTCTTTTAGGGCT-3'	5'-GCTTTCCTTAATGCTGCGGG-3'	200
BDNF	5'-CATCTGTTGGGGAGACGAGA-3'	5'-ACGTTTGCTTCTTTCATGGGC-3'	158
C3	5'-TGGAGCTAATGGCCAAAGGG-3'	5'-TTGGGTCACCTTTCACCACC-3'	95
CCNA2	5'-AGTGCCGCTGTCTCTTTACC-3'	5'-ACATTCACTGGCTTTTCGTCT-3'	131
CNTF	5'-TTCGCAGAGCAAACACCTCT-3'	5'-AGGCCCTGATGTTTTACATAAGATT-3'	125
PTGS2	5'-TGTTCGCATTCTTTGCCCAG-3'	5'-AGGATACACCTCTCCACCGAT-3'	200
G6PD	5'-AACCGTCTGTTCTACCTGGC-3'	5'-CGGTAGATCTGGTCCTCACG-3'	191
GAPDH	5'-TTGTGCAGTGCCAGCCTC-3'	5'-AGGTCAATGAAGGGGTCGTT-3'	145
HPRT1	5'-GTTGGACTTGACATGTGCGG-3'	5'-GTCCTGTCCATAATCAGTCCATGA-3'	149
IGF1	5'-AAGCGATGGGGAAAATCAGC-3'	5'-CACGAACTGAAGAGCGTCCA-3'	200
IL6	5'-CACTTCACAAGTCGGAGGCT-3'	5'-TCTGACAGTGCATCATCGCT-3'	114
IL10	5'-CCTCTGGATACAGCTGCGAC-3'	5'-GTAGATGCCGGGTGGTTCAA-3'	200
iNOS	5'-ACACAGTGTCGCTGGTTTGA-3'	5'-AACTCTGCTGTTCTCCGTGG-3'	135
TFRC	5'-CTTTCAGAAGGCGTGTGGAG-3'	5'-ACCGGGTGTATGACAATGGC-3'	148
Tlr3	5'-AAAACTGCCCGAGTCACAGT-3'	5'-TGCATCATAGTCTACTCCTTGCT-3'	101
TRAF5	5'-CTCAGGGAGGGACTCGAACC-3'	5'-CCGCTTGCTCCTCTGAATGA-3'	138
KCNJ10	5'-TCCCACTACCCCAGGATTCA-3'	5'-TCCATTCTCACGTTGCTCCG-3'	175

cyclin A2 (CCNA2), glucose-6-phopshate dehydrogenase (G6PD), glycerin-aldehyde-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT1), tranferrin receptor coding gene (TFRC)

2.5.2 Primer annealing temperature

Optimal primer annealing temperature was determined by doing a gradient PCR. Therefore, a pool of samples of the first batch of cDNA, was created by transferring 8 µL of each sample into a 1.5 mL LoBind DNA-tube to get a total volume of 200 µL. Then, it was diluted by factor 1:4 with nuclease free water to get a concentration of 2.5 ng/µL and a volume of 800 µL. Another gradient PCR was performed with the samples of the third batch to evaluate the optimal annealing temperature for the housekeeping genes. Therefore, 1 µl of each sample was transferred to a 1.5 mL LoBind DNA tube to get a total volume of 25 µL. Then it was diluted by the factor 1:14 to get also a concentration of 2.5 ng/µL cDNA and a volume of 350 µL. The gradient PCR was performed using a white 96-well PCR plate (Hard-Shell 96-Well PCR Plate, thin wall, white, BioRad, Germany). Therefore, 4 µL of the pooled sample and 6 µL of the ReactionMix were transferred into the wells of the plate. Then, the plate was sealed with foil (Light cycler 480 Sealing Foil, Roche Diagnostics, IN, USA) and centrifuged (Heraeus Multifuge 3 L-R, Kendro, Germany) for one minute with 900 rpm.

The ReactionMix contained 5 µL of the SsoAdvanced Universal SYBR Green Supermix (BioRad, Germany) and 0.5 µL of the investigated forward and 0.5 µL of the reverse primer. Final concentration of each primer in the reaction mix was 50 nM.

Afterwards, a qRT-PCR (CFX Opus 96 Real-Time PCR System, BioRad, Germany) was performed, and the temperature gradient was set with seven steps from 50 to 70°C. The used protocol of the gradient PCR is displayed in figure 1.

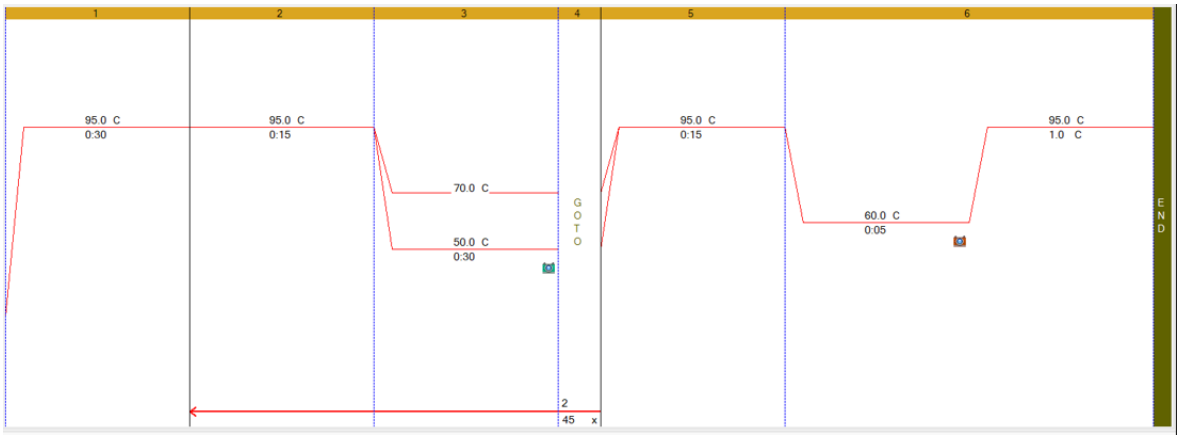

Figure 1: The protocol used for the temperature gradient PCR

Afterwards, the optimal annealing temperature was evaluated considering the CT value and by optical analysis of the melt curve by using the CFX maestro software (BioRad, Germany). Table 8 shows the evaluated temperature for the genes.

Table 8: Temperatures evaluated for the genes with gradient PCR

58 °C	**54°C**
BDNF	KCNJ10
Arg1	TRAF5
C3	IL6
CNTF	TLR3
IL10	IGF1
PTGS2	G6PD
INOS	-
GAPDH	-
HPRT1	-
TFRC	-
CCNA2	-

2.5.3 qRT-PCR

The samples were thawed and spun down (Heraues Biofuge pico, Kendro, Germany) shortly before handling. The ReactionMix was prepared and stored on ice and protected from sunlight afterwards. Then, 6µL of the ReactionMix was transferred into each well with a stepper (Multipipette plus, Eppendorf, Germany) and afterwards 4 µL of the cDNA sample (equivalent to 10ng RNA) was added using micropipettes. The qRT-PCR was performed in triplicates for each sample. For the genes KCNJ10, Arg1, tumor necrosis factor receptor associated factor 5 (TRAF5), C3, interleukin 6 (IL6), CNTF, TLR3, interleukin 10 (IL10), IGF-1, prostaglandinsynthase 2 (PTGS2) glucose-6-phopshate dehydrogenase (G6PD) and iNOS a 384 white PCR-plate (Hard-Shell 384-Well PCR Plate, thin wall, white, BioRad, Germany) was used. For the genes glycerin-aldehyde-phosphate dehydrogenase (GAPDH), cyclin A2 (CCNA2), hypoxanthine-guanine-phosphoribosyltransferase (HPRT1), tranferrin receptor coding gene (TFRC) and BDNF a 96 white PCR-plate (Hard-Shell 96-Well PCR Plate, thin wall, white,

BioRad, Germany) was used. The used protocol for the qRT-PCR is depicted in figure 2.

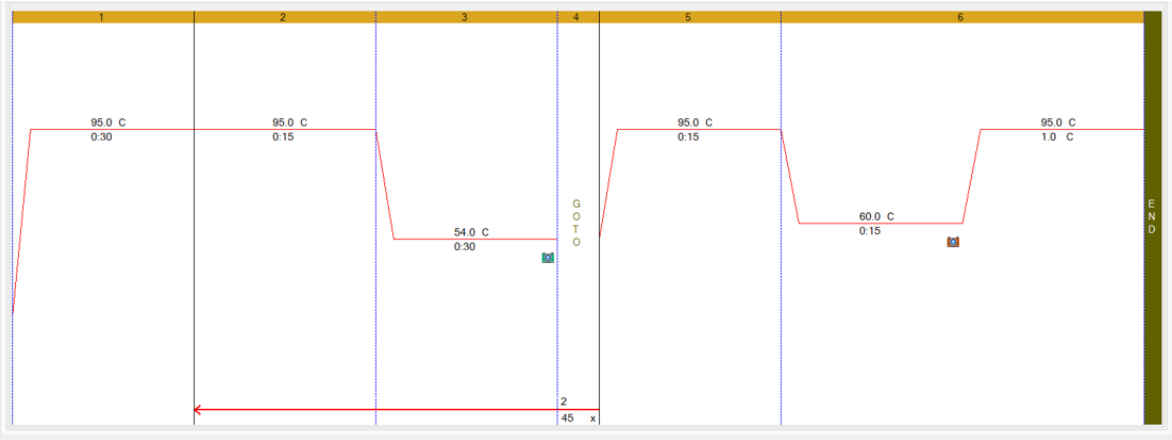

Figure 2: The protocol used for the qRT-PCR. *It was performed at 54° Celsius or 58° Celsius according to the evaluated temperature for each gene in the gradient PCR.*

In addition, for each gene a triplicate of the RT-1, RT-2, RT-3 samples, and a triplicate of negative controls with nuclease free water were included on the reaction plate. After pipetting, the plate was sealed with the foil and centrifuged (Heraeus Multifuge 3 L-R, Kendro, Germany) for 1 min with 900 rpm. Then, the qRT-PCR was run on a CFX384 Touch Real-Time PCR Detection System (Biorad, Germany) or on a CFX Opus 96 PCR-system (BioRad, Germany).

2.6 Data analysis

Mean cycle time (CT)-values for each gene and sample were calculated from qRT-PCR in triplicates. Five different candidate genes evaluated for internal reference were tested (G6PD, GAPDH, CCNA2, HPRT1, and TFRC) based on literature (84–86) and experience from previous work of the laboratory host group by which the work has been supervised. GAPDH and CCNA2 showed the most stable Ct-values across the experimental groups. Thus, both were taken as the internal reference gene index. See the appendix and figure 14 in the appendix for the results of the reference gene index. The geometric mean of their mean CT-values for each sample was used for normalization. Relative gene expression was calculated with the $2^{-\Delta\Delta CT}$ method without correction for primer efficiency with the formula below:

$$\Delta CT = CT\ target\ gene - CT\ reference\ gene\ index$$

$$\Delta\Delta CT = \Delta CT\ treated\ group - \Delta CT\ control\ group$$

$$Fold\ change = 2^{-\Delta\Delta CT}$$

Log_2 (fold change) was then used for statistical analyses.

2.7 Statistical analysis

Statistical analysis of the results was done using IBM SPSS (version 29, USA). Normality of the data distribution within a group was assessed by using the Shapiro Wilk test, Kolmogorov-Smirnov test, and visual inspection of the Q-Q plot. Variance homogeneity was tested with Levene's test. Statistical significance of the observed differences for data with parametric distribution was tested with one-way analyses of variance (ANOVA) followed by a post hoc analysis with Tukey's honestly significant difference (HSD) test. In case of a variance heterogeneity, Welch´s-ANOVA followed by Games-Howell test was used. When at least one of the compared groups had non-normal data distribution, the differences were tested with Kruskal-Walli's test followed by Dunn's pairwise comparison with Bonferroni correction for multiple comparisons. All comparisons with a $p \leq 0.05$ deemed statistically significant.

3 Results

3.1 M1 microglial cells

3.1.1 Inducible NO-synthase – iNOS

iNOS was used as a marker for M1 microglial cells. It works as an enzyme catalysing the reaction from L - Arginine to NO, it influences inflammatory processes, and it is involved in the non-adaptive immune reaction against pathogens (87). It is used as a marker for microglial cells (88) specifically for the proinflammatory M1 subtype (75). iNOS mRNA expression is elevated under inflammatory conditions in microglia (89) and its elevation is further associated with reactive astrocytes and neuroinflammation (90). iNOS protein expression is elevated in oligodendrocytes after treatment with pro-inflammatory cytokines as well (91).

There was a statistically significant difference between groups revealed by one-way ANOVA ($F(4,18)=145.65$, $p<0.001$) in iNOS expression. Tukey's post hoc test showed that the mRNA expression of iNOS was significantly higher in the group treated with cytokines (9.14±0.65) than in the naive group (0.00±0.35, $p<0.001$), the group treated with DMSO (-3.32±1.19, $p<0.001$) and the group treated with siponimod (-4.10±1.23 $p<0.001$). In contrast to the hypothesis, there was no statistically significant reduction in the group treated with siponimod and cytokines (8.11±1.56, $p=0.644$) compared to sole cytokine treatment. Consecutive there was a statistically significant elevation in the expression in the group treated with siponimod and cytokines compared to the naive group ($p<0.001$), the group treated with DMSO ($p<0.001$) solely and the group treated with siponimod ($p<0.001$).

The expression of iNOS was according to the hypothesis statistically significant reduced in the group treated with siponimod ($p<0.001$) and similar decreases were also observed in the group treated with DMSO ($p=0.002$) compared to the naive group. The differences between the group treated with DMSO and the group treated with siponimod were, however, not statistically significant ($p=0.828$). Figure 3 shows the explained results of iNOS expression in the used MGC cultures in response to cytokine and/or siponimod treatment.

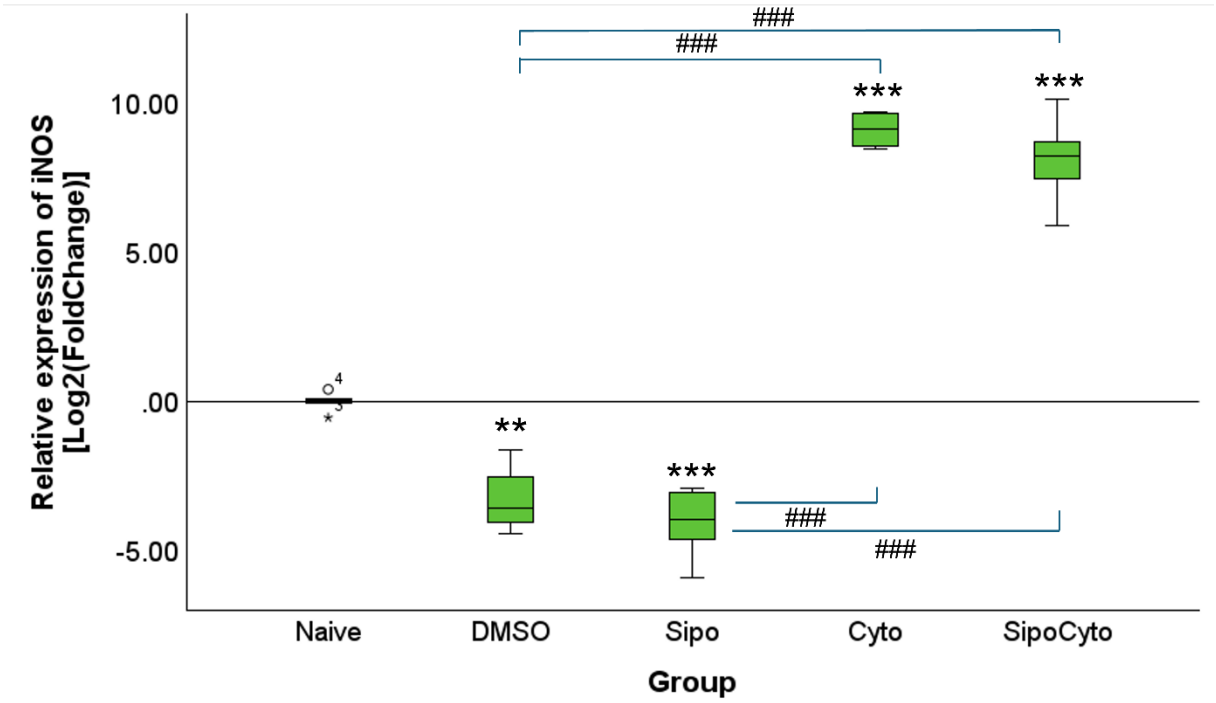

Figure 3: mRNA expression of iNOS in MGC cultures in response to cytokine and/or siponimod treatment. *Gene expression was analysed by qRT-PCR following a 20-hour treatment. Cytokine treatment induced significant increases in iNOS expression in MGC cultures. A concomitant siponimod treatment, however, did not show a significant influence on the iNOS expression. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.2 M2 microglial cells

3.2.1 Arginase 1 – Arg1

Arg1 is an enzyme which catalyses the reaction from arginine to urea and ornithine (92). It uses the same substrate (arginine) as iNOS uses for NO production. Arg1 is used as a marker of microglial cells (88) specifically for the alternatively activated M2 subtype, which is promoting an anti-inflammatory phenotype (75). There is evidence that Arg1 might exert its neuroprotective effects in the CNS by counteracting the harmful effects of iNOS (93). Astrocytes also express Arg1 (94).

There was a statistically significant difference between groups as determined by one-way ANOVA (F (4,18=77.47, p=<0.001). Tukey's post hoc test revealed that the mRNA expression of Arg1 was significantly reduced in the group treated with cytokines (-2.00±0.40) compared to the naive group (0.00±0.23, p<0.001), to the group treated with DMSO solely (0.84±0.34, p<0.001) and to the group treated with siponimod (0.15±0.28, p<0.001). Treatment with siponimod and cytokines (-2.67±0.52) led also to a statistically significant decrease in Arg1 expression compared to the naive group (p<0.001), to the group treated with DMSO (p<0.001) and as well compared to the group treated with siponimod (p<0.001). Although lower expression levels were observed in the group with concomitant treatment of siponimod with cytokines compared to sole cytokine treatment, the differences were not statistically significant (p=0.980).

Further, a statistically significant elevated expression in the group treated with DMSO compared to the naive group (p=0.023) was found. There was no statistically significant difference between siponimod treatment, and the naive group (p=0.968) and the group treated with DMSO (p=0.760). Figure 4 presents the described influence of cytokine and/or siponimod treatment on relative Arg1 expression in the used MGC cultures.

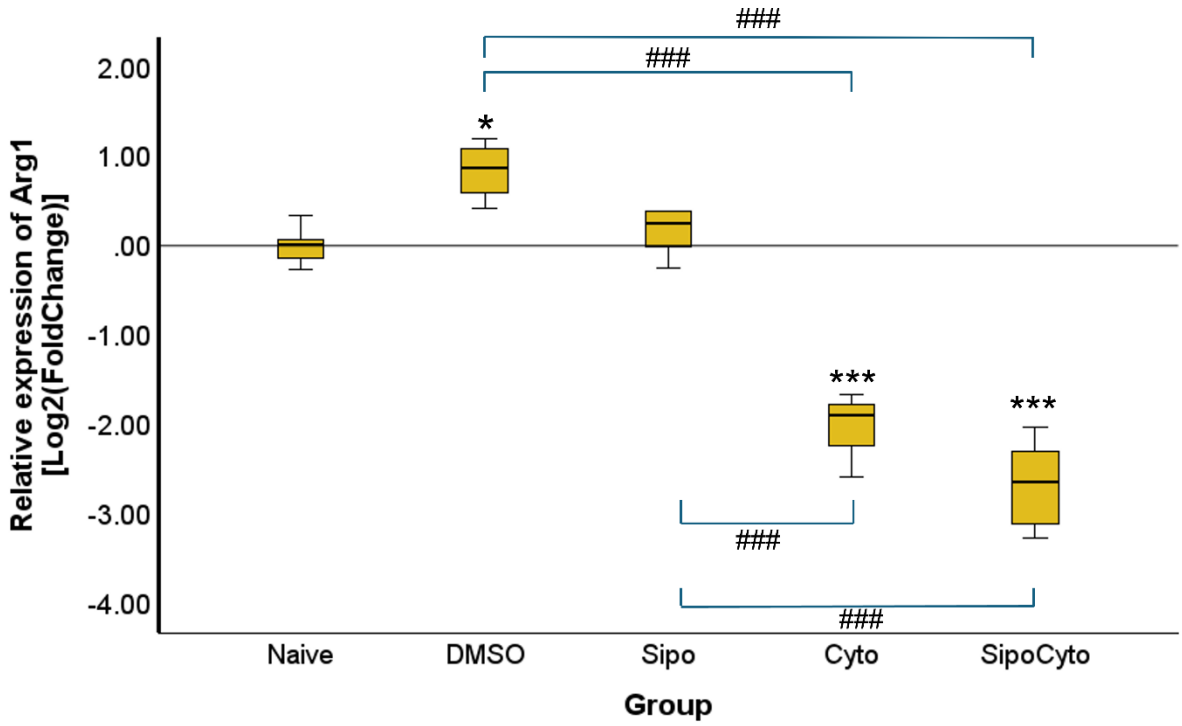

Figure 4: Influence of cytokine and/or siponimod treatment on relative Arg1 expression in MGC cultures. *The expression of Arg1 was evaluated after a 20-hour treatment by qRT-PCR. Although treatment with siponimod and cytokines led to a significant decrease in the expression of Arg1 in the mixed glial cells, there was no significant difference compared to the sole cytokine treated group, which also reduced the expression significantly. However, sole DMSO treatment induced the expression of Arg1 significantly. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.2.2 Insulin like growth factor 1 – IGF-1

IGF-1 is expressed by M2 microglial cells which favour an anti-inflammatory phenotype (64,75). There is evidence in an experimental study that astrocytes and oligodendrocytes, but less than astrocytes, also express IGF-1 protein under pathological conditions (95). It was shown in another experimental study, that the mRNA expression of IGF-1 is downregulated under inflammatory conditions in microglia (96). Further, there is data suggesting that IGF-1 has physiologically neuroprotective and neurotrophic effects in the CNS, because it promotes rat derived hypothalamic neuron cell survival and differentiation as well as glial cell proliferation (97).

There was a statistically significant difference between groups revealed by one-way ANOVA ($F(4,18)=17.39$, $p<0.001$). Tukey's post hoc test showed that the mRNA expression of IGF-1 was significantly lower in all the groups, the one treated with

DMSO solely (-1.52±0.48, p=0.008), the one treated with siponimod (-2.36±0.61, p<0.001) the one treated with cytokines (-2.29±0.81, p<0.001) and the one treated with siponimod and cytokines (-2.72±0.31, p<0.001) compared to the naive group (0.00±0.61). There was a statistically significant reduction in IGF-1 in the group treated with siponimod and cytokines compared to DMSO (p=0.043) treatment. No additional differences between the other groups were found. The mentioned relative quantification of IGF-1 gene expression of mixed glial cells after cytokine and/or siponimod treatment is depicted in figure 5.

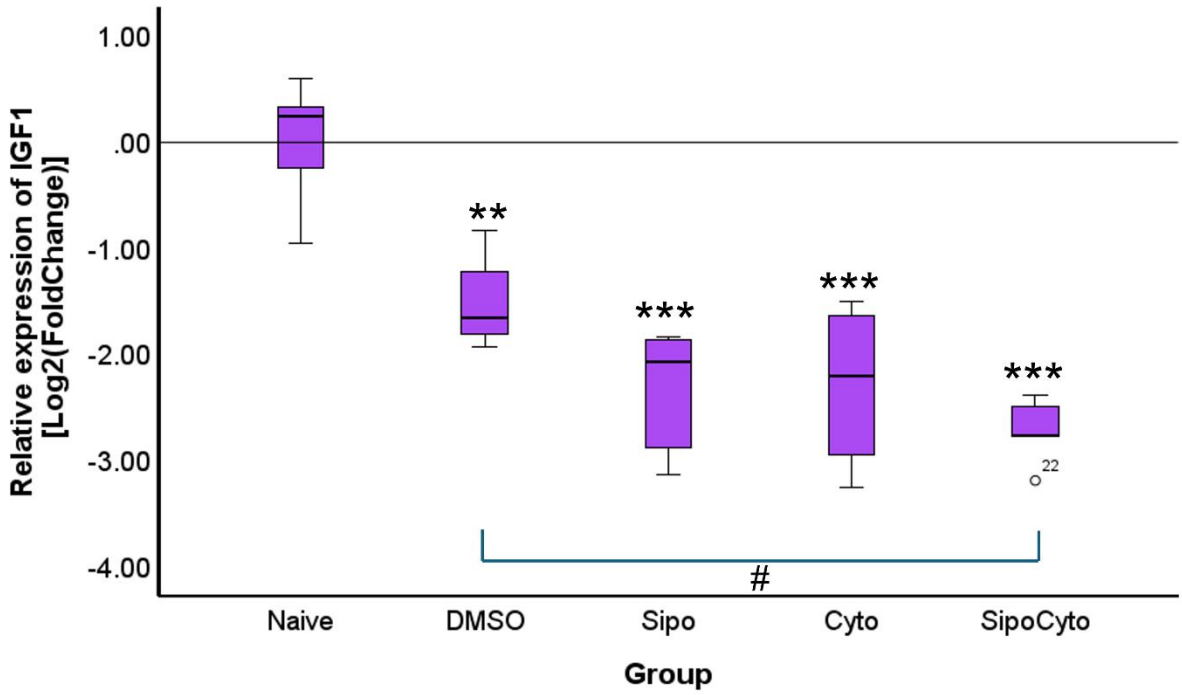

Figure 5: Relative quantification of IGF-1 gene expression of mixed glial cells after cytokine and/or siponimod treatment. *The gene expression of IGF1 was evaluated after 20 hours of treatment with siponimod and cytokines by a qRT-PCR. Sole Siponimod, sole cytokine and concomitant treatment decreased the expression of IGF-1 significantly in the mixed glial cells. Furthermore, DMSO also reduced the expression of IGF-1 significantly. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.3 Reactive astrocytes

3.3.1 Complement component 3 - C3

C3 is part of the complementary system, and it was shown that its mRNA expression is elevated in microglial cells, which is the main source, but also in astrocytes, in an EAE model (98). In this inflammatory model, C3 promotes neurodegeneration in the gyrus dentatus (98). An upregulation of C3 levels is associated with A1 reactive astrocytes, which are neurotoxic and promote cell death of neurons and oligodendrocytes (69). Furthermore, A1 astrocytes are associated with neurodegenerative diseases like MS, and they are triggered by activated microglial cells (69). It has been shown that oligodendrocytes also express C3 (99). It was shown that astrocytes potentiate their expression of C3 in the presence of microglia, and they promote microglia to express C3 as well, creating an upward spiral (70). Taken together, C3 is expressed by all glial cell types in the CNS, and it is associated with neurodegenerative diseases like MS. Furthermore, astrocytes have a higher expression of C3 in the presence of microglia and vice versa, and elevation of C3 favours a proinflammatory and neurotoxic phenotype in the context of MS.

Our results showed a statistically significant difference between groups revealed by one-way ANOVA ($F(4,18)=386.72$, $p<0.001$) in C3 expression. Tukey's post hoc test showed that the mRNA expression of C3 was as expected significantly higher in the group treated with cytokines (2.34±0.56) than in the naive group (0.00±0.47, $p<0.001$), the group treated with DMSO (-6.32±0.49, $p<0.001$) and the group treated with siponimod (-7.530±0.50, $p<0.001$). A statistically significant reduction in the group treated with siponimod and cytokines (2.41±0.58) compared to the sole cytokine treatment ($p=1.00$) was not detected. Subsequent, there was a statistically significant elevation in the group treated with siponimod and cytokines compared to the naive group ($p<0.001$), the group treated with DMSO ($p<0.001$) solely and the group treated with siponimod ($p< 0.001$).

However, there was a statistically significant reduction of C3 in the group treated with DMSO ($p<0.001$) and the group treated with siponimod ($p<0.001$) compared to the naive group. In line with the study hypothesis, a statistically significant reduction of C3 in the group treated with siponimod compared to the group treated with DMSO ($p=0.020$) was revealed. Figure 6 shows the explained quantification of the relative

gene expression of C3 in the MGC cultures after concomitant or sole cytokine or siponimod treatment.

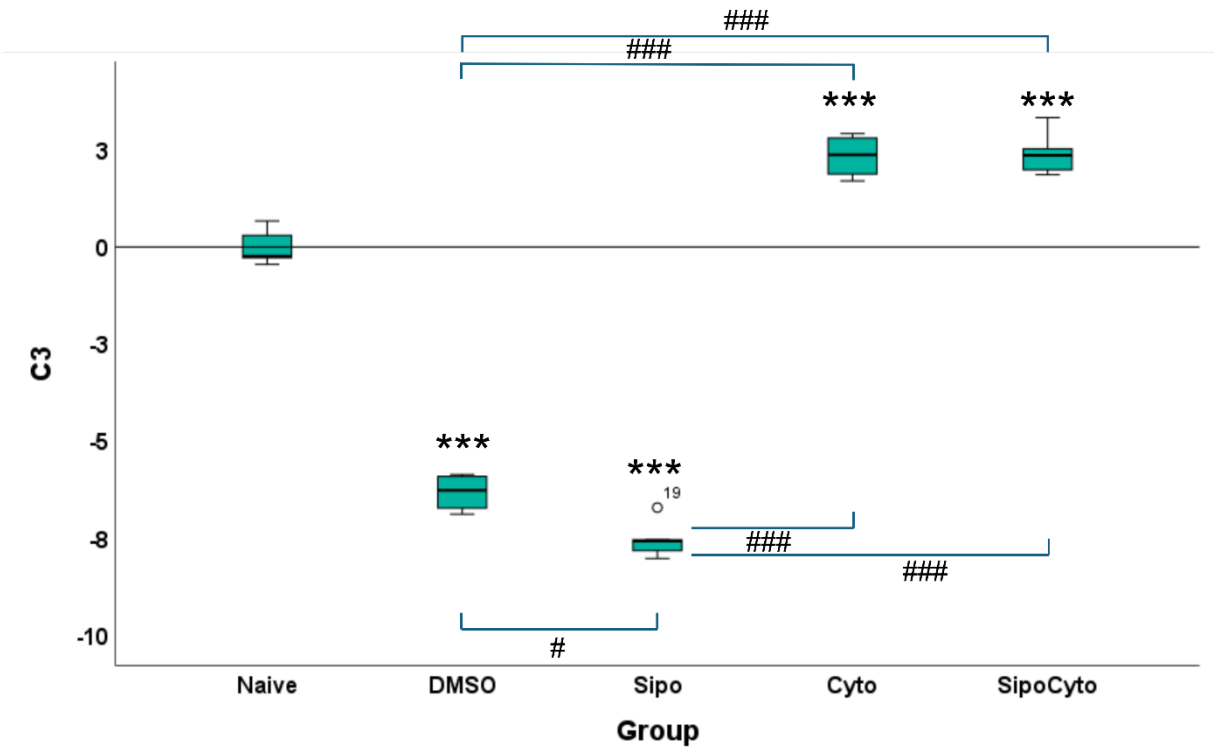

Figure 6: Relative gene expression of C3 in mixed glial cells after concomitant or sole cytokine or siponimod treatment. *The expression of C3 was quantified by a qRT-PCR after a 20-hour treatment with siponimod and cytokines. Treatment with siponimod and cytokines or sole cytokine treatment resulted in a significant elevation of C3 expression. However, there was no significant influence of siponimod upon concomitant cytokine treatment. Sole siponimod and sole DMSO treatment resulted in a significant reduction of C3 gene expression. Moreover, sole siponimod treatment significantly reduced C3 gene expression even more, compared to DMSO. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.3.2 KCNJ10

KCNJ10 codes for an inwardly rectifiying potassium channel (Kir) called Kir4.1 (former KAB2), which is expressed by glial cells in the CNS (100) and therefore contributes to the homeostasis of potassium in the brain (101). There is data that stimulation with the inflammatory cytokine interleukin-1β leads to a decreased expression of KCNJ10, whereas treatment with TNFα did not alter the expression significantly in an astrocyte culture (102). It has been shown that KCNJ10 knockout mice develop a demyelinating phenotype in the CNS, suggested due to the lack of Kir4.1 expression in oligodendrocytes (103). Moreover, there is data that in MS patients there are autoantibodies against Kir4.1 indicating a possible role in pathogenesis (104). Injection of IgG Kir4.1 antibodies in an experimental model, led to a decreased expression of Kir4.1 and to complement activation in the CNS (104). KCNJ10 is expressed in astrocytes (101) and oligodendrocytes (103) but there was no evidence found that it is expressed in microglial cells.

In summary taking all this data into account, it has been shown that the reduction of KCNJ10 plays a possible role in the pathogenesis of MS and promotes a demyelinating phenotype. This indicates a pro-inflammatory influence of a reduced KCNJ10 expression in the CNS in the context of MS. Thus, we analysed the expression of KCNJ10 in our study to understand its potential role with respect to siponimod.

There was a statistically significant difference between the groups shown by Welch-ANOVA ($F(4,8.27)=61.85$, $p=<0.001$). Games-Howell post hoc test revealed that the mRNA expression of KCNJ10 was significantly lower in the group treated solely with siponimod (-6.19±0.78) compared to the naive group (0.00±0.42, $p<0.001$), the group treated with cytokines and DMSO (0.36±0.64, $p<0.001$) and the group treated with siponimod and cytokines (0.54±0.56, $p<0.001$). However, no significant difference was detected in the group treated with DMSO (-4.61±2.51, $p=0.751$) compared to siponimod treatment. No further statistically significant difference between the groups was found. Figure 7 presents the described results of the mRNA expression of KCNJ10 in the used MGC culture after treatment with cytokines and/or siponimod.

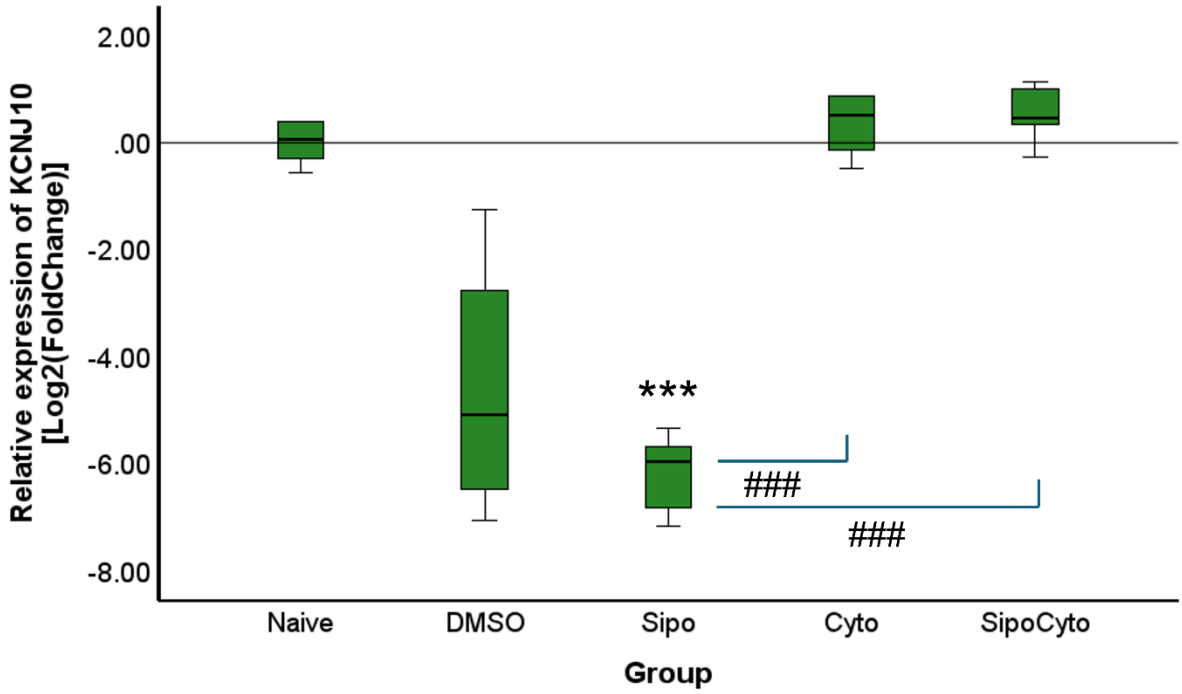

Figure 7: mRNA expression of KCNJ10 in a MGC culture after treatment with cytokines and/or siponimod. *The expression of KCNJ10 was evaluated by treatment of mixed glial cells for 20 hours followed by a qRT-PCR. Treatment with siponimod led to a significant reduced expression of KCNJ10 compared to sole cytokine challenge and concomitant cytokine and siponimod treatment. A reduction in the expression was also observed in the DMSO vehicle control group although it was not significant. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with Welch-ANOVA followed by Games-Howell test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.4 Protective astrocytes

3.4.1 Brain derived neurotrophic factor - BDNF

BDNF is a neurotrophin which takes part in different processes, like enhancing neuron survival (105) or promoting oligodendrocyte precursor cell (OPC) DNA synthesis or proliferation, in a pro-proliferative environment (106). Further, there is evidence that increased BDNF expression of astrocytes is associated with an increased expression of proteins of the myelin sheath in demyelinated lesions in an animal model (107). In addition, it was shown that blood BDNF levels are reduced in MS patients compared to healthy individuals (108). Stimulation with TNFα increases BDNF protein and mRNA expression in astrocytes (109) and BDNF is expressed in astrocytes (107), microglial cells (110) and oligodendrocytes (111). Thus, we analysed whether its mRNA expression is influenced in MGC cultures upon siponimod treatment, with or without inflammatory insult.

There was a statistically significant difference between groups revealed by one-way ANOVA ($F(4,18)=9.009$, $p<0.001$). Tukey's post hoc test showed that the mRNA expression of BDNF was significantly higher in the group treated with cytokines (2.14±0.48, $p<0.001$) and the group treated with siponimod and cytokines (1.77±0.348, $p=0.002$) compared to the naive group (0.00±0.51). Further, there was a statistically significant elevation in the expression of the group treated with cytokines compared to the group treated solely with siponimod (0.68±0.58, $p=0.017$). There was no other significant difference detected, including the group treated with DMSO (1.15±0.95). The mentioned influence of cytokines and/or siponimod on relative BDNF expression in the used MGC culture is presented in figure 8.

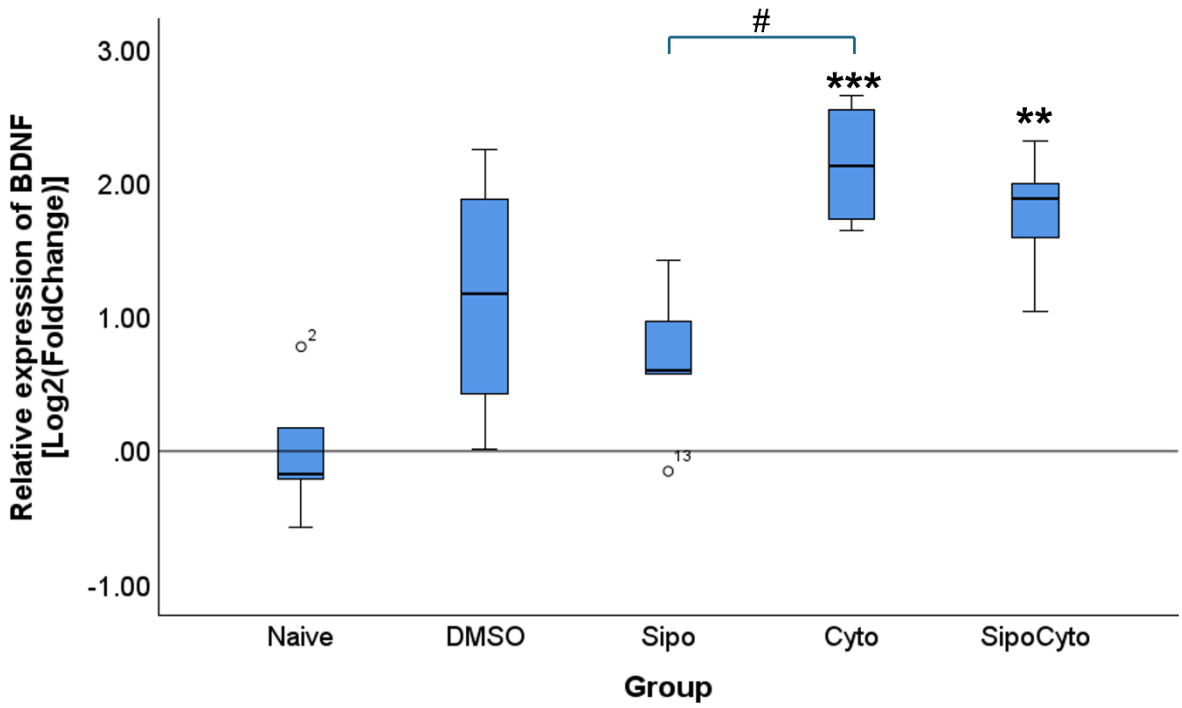

Figure 8: Influence of cytokines and/or siponimod on relative BDNF expression in MGC cultures. *The relative expression of BDNF was analysed using a qRT-PCR after 20 hours of treatment with siponimod and/or cytokines. The relative expression of BDNF was significantly elevated in the group challenged with cytokines and upon concomitant cytokine and siponimod treatment. However, no significant influence on BDNF expression of the siponimod treatment under cytokine challenge was revealed. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.4.2 Toll-like-receptor 3 - TLR3

TLR3 is part of the innate immune system, and its function is to recognize body foreign structures and subsequently to induce an immune response (112). TLR 3 signalling has a dichotomic influence, it can protect from viral induced diseases, but it can also have harmful effects depending on the virus and the location of infection (113). For example, there is evidence that suggests a promoting role of TLR3 signalling in developing a Tick-borne encephalitis (114) and it was shown in a Theiler's murine encephalomyelitis model depending on the timing of the TLR3 stimulation, prior or after virus infection, that it can have a detrimental or beneficial influence (115). Nevertheless, it was shown that TLR 3 signalling reduces oxidative cell damage of astrocytes (116) and that it has protective functions in astrocytes during viral (herpes simplex virus 2) infections (117). Further, there is data that TLR 3 signalling on astrocytes promotes the production of anti-inflammatory cytokines (118). TLR 3 is expressed by astrocytes, microglial cells (119) and by oligodendrocytes (120). Thus, it is of interest how mixed culture of primary glial cells alter their TLR3 expression in response to inflammatory insult and treatment with siponimod.

There was a statistically significant difference in the mRNA expression of TLR3 between the groups revealed by Kruskal-Wallis H test ($\chi^2(2)=16.21$, $p=0.003$). Post hoc Dunn's pairwise comparison with a Bonferroni correction showed that the expression of TLR 3 was reduced in the group treated solely with siponimod (-1.64±0.20) or DMSO (-1.225±0.275) compared to the naive cultures (0.002±0.158), yet the differences were statistically not significant. Interestingly, however, treatment with cytokines (0.010±0.312) or siponimod and cytokines (0.22±0.35) did not induce a detectable change in TLR3 expression compared to the naive cultures. The only statistically significant difference was detected between the group treated with siponimod only and that with siponimod and cytokines ($p=0.018$). Figure 9 shows the described results of the relative mRNA expression of TLR3 in mixed glial cells after treatment with cytokines and/or siponimod.

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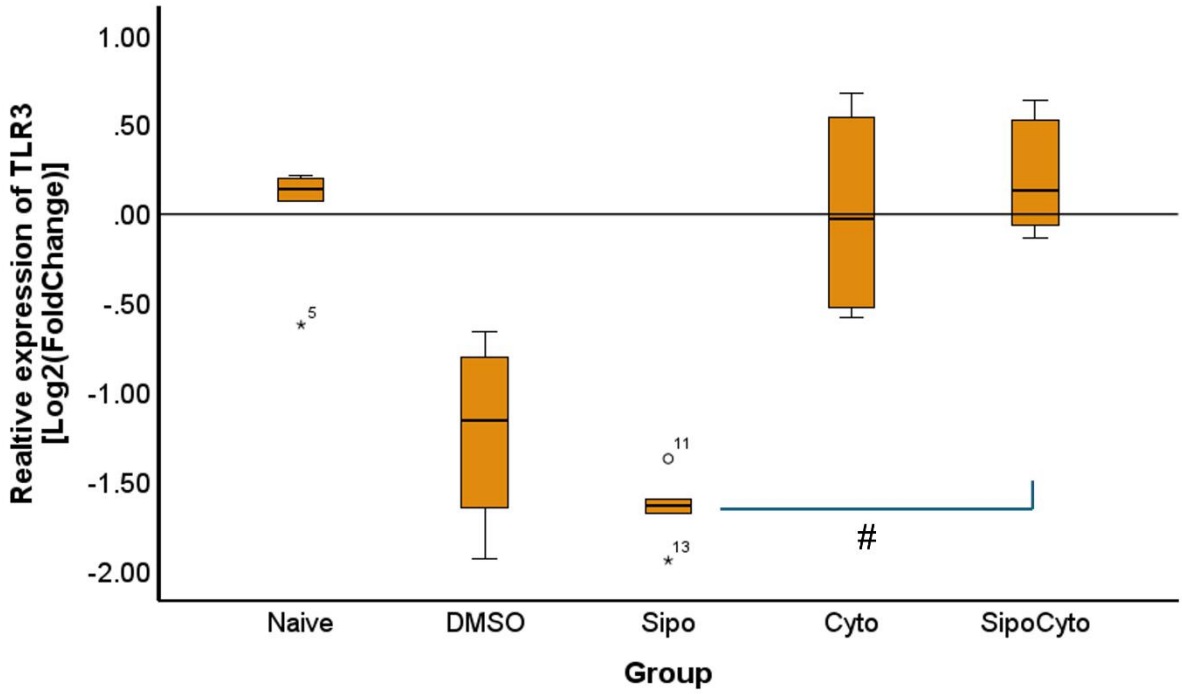

Figure 9: Quantification of relative mRNA expression of TLR3 in mixed glial cells after treatment with cytokines and/or siponimod. *The expression of TLR3 was quantified by using a qRT-PCR after a 20-hour treatment with cytokines and/or siponimod. The only significant alteration of mRNA expression found, regarding TLR3, was that treatment with siponimod reduced the expression compared to treatment with siponimod and cytokines. Sole treatment with DMSO reduced the expression but not significantly, whereas cytokines and siponimod and cytokines had no effect. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with Kruskal-Wallis H test followed by a Dunn's pairwise comparison with a Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.4.3 Ciliary neurotrophic factor - CNTF

There is evidence suggestive, that in an experimental model astrocytes expressing CNTF prevent the death of axotomized neurons (121) and that CNTF expressed by astrocytes enhances axon regeneration (122). It was also shown in vitro that CNTF enhances the survival of OPC's and their differentiation into oligodendrocytes, and their survival as well (123). Further, it was shown in a MOG-induced EAE model, that lack of CNTF increases the severity of the disease course and leads to less oligodendrocyte survival and OPC proliferation (124) indicating an anti-inflammatory effect of CNTF. Furthermore, it was shown that an inflammatory stimulus with IFNγ increases the mRNA expression of CNTF in an astrocyte culture (125). CNTF is expressed in the CNS by astrocytes (122,126) but no evidence was found that oligodendrocytes and microglial cells express CNTF. These findings suggest that elevated CNTF expression could potentially indicate a protective response by astrocytes, thus we investigated its expression in MGC cultures in response to treatment with cytokines and/or siponimod.

There was a statistically significant difference between groups as determined by one-way ANOVA ($F(4,18=22.77$, $p=<0.001$). Tukey's post hoc test revealed that the mRNA expression of CNTF was, as expected, significantly elevated in the group treated with cytokines (1.44±0.51) compared to the naive group (0.00±0.58, $p=0.020$), the group treated with DMSO (-0.33±0.71, p=0.006) and the group treated with siponimod (-1.68±0.27, $p<0.001$). Siponimod treatment concomitant to the inflammatory insult with cytokines did not induce a change in CNTF expression (1.60±0.86) compared to the cytokine treated group ($p=0.996$), and it was significantly higher compared with the naive group ($p=0.006$), DMSO ($p=0.002$) and siponimod ($p<0.001$), groups. Interestingly, treatment with siponimod alone induced a significant reduction of CNTF expression compared to the naive controls ($p=0.04$) and those treated with DMSO ($p=0.031$). The described effects of cytokines and/or siponimod on the relative gene expression of CNTF in the used MGC cultures are shown in figure 10.

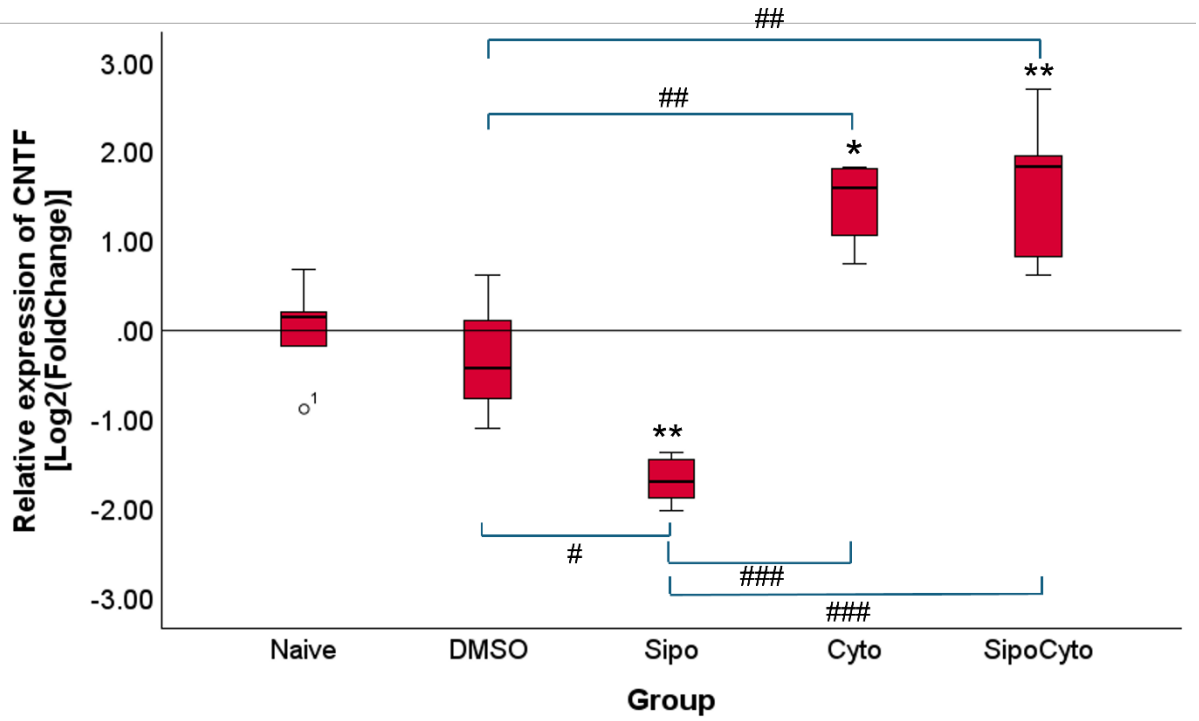

Figure 10: Effects of cytokines and/or siponimod on the relative gene expression of CNTF in MGC cultures. *The mRNA expression was quantified using a qRT-PCR after a 20-hour stimulation with cytokines and/or siponimod. A cytokine stimulus led to a significant increase in the mRNA expression of CNTF in MGC cultures, regardless of a concomitant treatment with siponimod. However, sole siponimod treatment led to a significant reduction in the expression of CNTF in comparison to all other groups. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.5 Pro-inflammatory markers

3.5.1 Interleukin 6 - IL6

IL6 was used as a marker for a pro-inflammatory reaction. IL6 is a cytokine with pleiotropic effects (127,128). These pleiotropic effects are thought to be caused by two different signalling pathways the so called “trans-signalling” and the “classical signalling” pathway (127,128). There is evidence that suggests that the classic signalling pathway might promote anti-inflammatory, whereas the trans-signalling pathway might promote pro-inflammatory functions (127). However, it was shown, that IL 6 is elevated intrathecally in an inflammatory autoimmune disease, in progressive MS, and it shows a positive connection with disease severity (129). Further, it shows elevated levels in an infectious disease in patients plasma compared to healthy individuals (130), indicating it is a secure biomarker for inflammatory reactions. There is also suggestive evidence from an experimental study that IL 6 plays a proinflammatory role in the CNS (131). IL 6 is expressed by astrocytes, microglial cells (60,132) and in oligodendrocytes (133).

We investigated the influence of siponimod on IL6 expression in MGC cultures. Highest expression was observed in the cultures that were challenged with cytokine treatment (1.63±1.20), whilst the lowest levels were observed upon concomitant treatment with siponimod and cytokines (-.47±2.25). The differences, however, were not statistically significant as determined by Welch-ANOVA ($F(4,7.53)=2.57$, $p=0.12$). Figure 11 shows the mentioned response of the used mixed glial cells in relative IL6 mrNA expression after challenge with cytokines and/or siponimod.

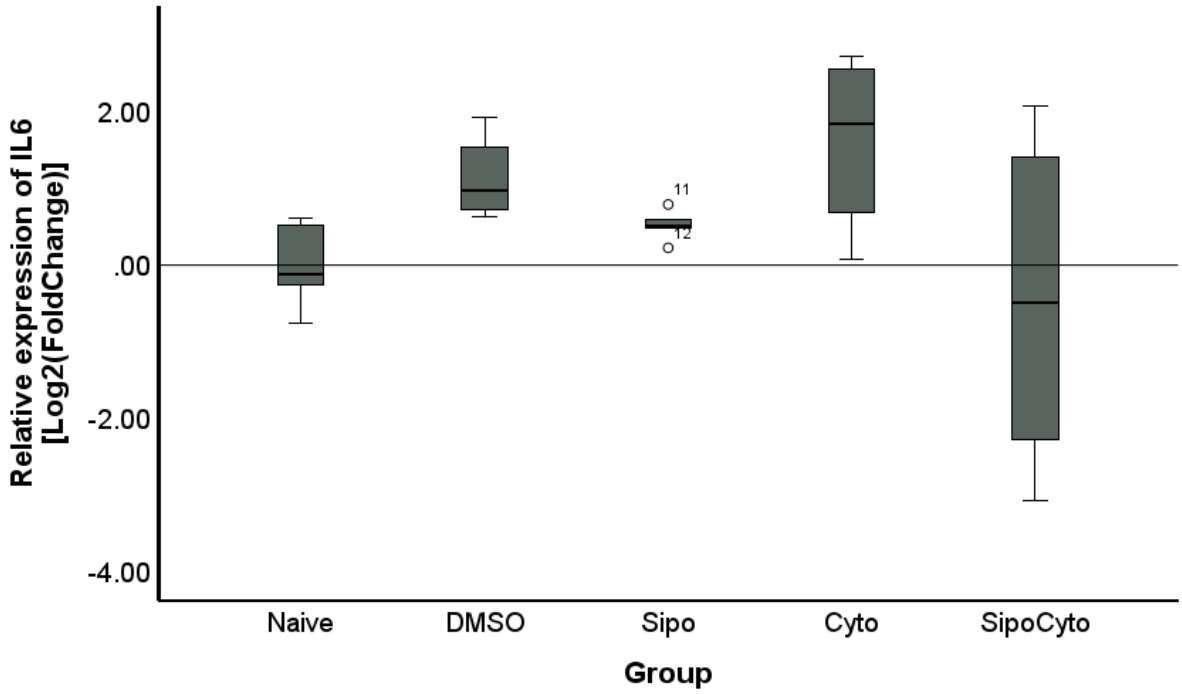

Figure 11: Response of mixed glial cells in IL6 expression after challenge with cytokines and/or siponimod. *The mRNA expression in response to a 20-hour treatment was quantified by using a qRT-PCR. No significant changes were observed regarding the expression of IL6 in this experimental setting across all groups. However, the highest expression was detected in response to sole cytokine challenge, whereas the lowest expression was detected in the group with cytokine and concomitant siponimod treatment. Nevertheless, these results are not statistically significant. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with Welch-ANOVA. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.5.2 Prostaglandinsynthase 2 - PTGS2

PTGS2, also called cyclooxygenase-2 (COX2), was used as a marker for a pro-inflammatory reaction. COX2 is an enzyme which catalyses the reaction from arachidonic acid to prostaglandins which have a broad variety of functions in the organism (134). It has been shown that PTGS2/COX2 mRNA expression is upregulated after an inflammatory stimulus in microglial cells and promotes IL6 expression (135). Elevated expression of COX2, compared to control subjects, is associated with neuroinflammation and demyelination and was found in chronic active lesions of MS patients (136). Further, elevated COX2 expression is associated with cell death in oligodendrocytes in lesions of MS patients and treatment with a COX2 inhibitor reduced demyelination significantly in an animal model of MS (137). PTGS2 is expressed in microglial cells (136), astrocytes (138) and oligodendrocytes (137). Although, it is well known for its proinflammatory role, there is also evidence for some anti-inflammatory functions (139). However, with respect to the literature provided, in the context of MS it seems that there is a pro-inflammatory influence of PTGS2. Therefore, we investigated how its expression changes in response to siponimod treatment in MGC cultures.

There was a statistically significant difference between groups as determined by one-way ANOVA ($F(4,18)=20.12$, $p=<0.001$). Tukey's post hoc test revealed that the mRNA expression of PTGS2 was in line with the hypothesis and underwent a significant reduction in the group treated with siponimod and cytokines (-1.977±0.492) compared to all the other groups, the naive group (0.00±0.25, $p<0.001$), the group treated with DMSO solely (0.54± 0.68, $p<0.001$), the group treated with siponimod (-0.54±0.20, $p<0.001$) and the group treated with cytokines (-0.9±0.57, $p=0.018$). It should be noted that cytokine treatment alone did result in decreases in PTGS2 expression compared to the untreated cultures, although statistically not significant ($p=0.62$). Nevertheless, these were significantly lower than that of those treated with DMSO ($p=0.003$). Similarly, reductions observed with siponimod alone were not statistically siginificant compared to the naive cultures ($p=0.363$), although the differences to the DMSO group ($p=0.017$) were significant. The explained relative expression of PTGS2 in the used MGC cultures after treatment with cytokines and/or siponimod is depicted in figure 12.

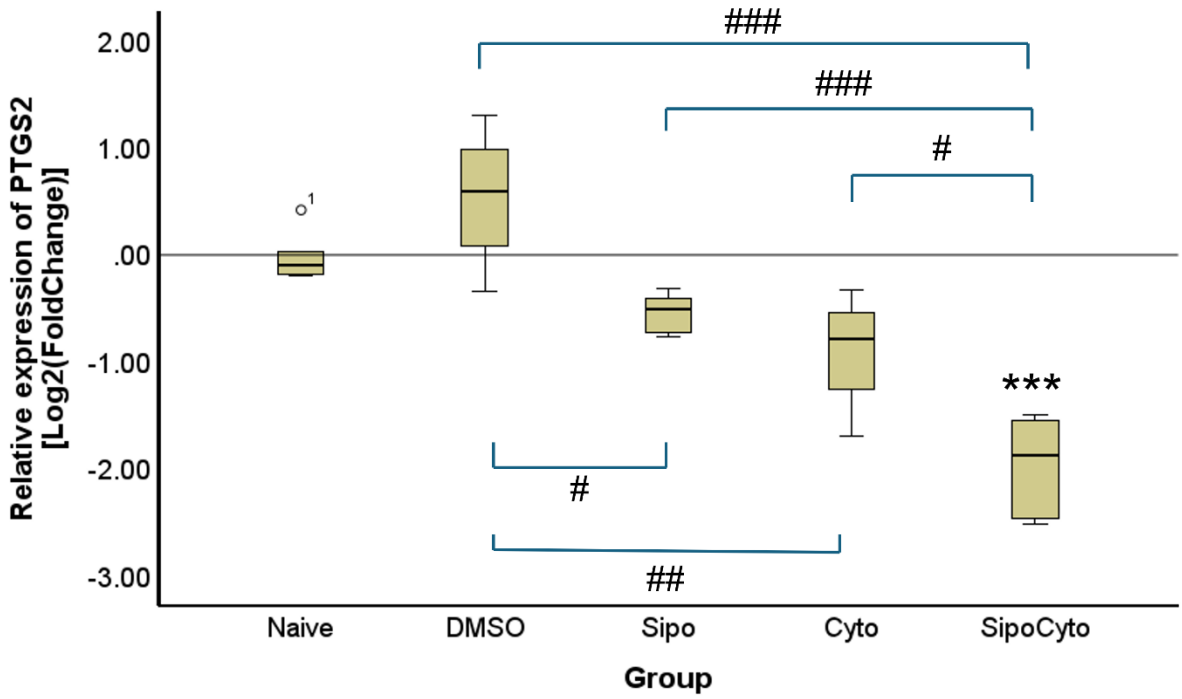

Figure 12: Relative expression of PTGS2 in MGC cultures after treatment with cytokines and/or siponimod. *A qRT-PCR was used for analysing the expression of PTGS2 after a 20-hour treatment. Only the vehicle control with DMSO increased the expression of PTGS2, however not significant compared to the controls but significant to the other groups. Siponimod, cytokine and concomitant treatment induced a decrease in the expression in MGC cultures, whereas only the treatment with siponimod and cytokines resulted in a significant reduction compared to the naive controls. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

3.6 Anti-inflammatory markers

3.6.1 Tumor necrosis factor receptor associated factor 5 - TRAF5

TRAF5 is part of the tumour necrosis factor receptor-associated factor (TRAF) group which are cytoplasmatic adapter proteins which were summarized into the mentioned group consisting of TRAF 1-6 (140). TRAF 5 is expressed by microglial cells, and its expression is elevated under inflammatory conditions (141) and by astrocytes where the expression is elevated after injury (142). There is evidence from an EAE model that mice deficient of TRAF5 showed an exaggerated disease course (131) indicating a possible anti-inflammatory role of TRAF5 in the CNS in the context of MS. Thus, we investigated the expression of TRAF5 mRNA in the MGC cultures to reveal a possible anti-inflammatory effect of siponimod.

In our experiments with cytokine and/or siponimod treatment of mixed cultures of primary glial cells there was a statistically significant difference between groups revealed by a one-way ANOVA ($F(4,18)= 6.348$, $p<0.002$). Tukey's post hoc test showed that the mRNA expression of TRAF5 was unexpectedly elevated to a statistically significant level in the group treated with DMSO (0.63±0.10) compared to the naive group (0.00±0.25, $p=0.013$) and the group treated with siponimod (-0.20±0.30, $p=0.001$). The lower levels observed in the siponimod only group or the higher levels observed in those treated with both siponimod and cytokines (0.22±0.29), however, were statistically not significant compared to the untreated naive cultures. The group treated with cytokines (0.09±0.24) showed no significant alteration in the expression. The described results of cytokine and/or siponimod treatment on TRAF5 expression in the used MGC cultures is presented in figure 13.

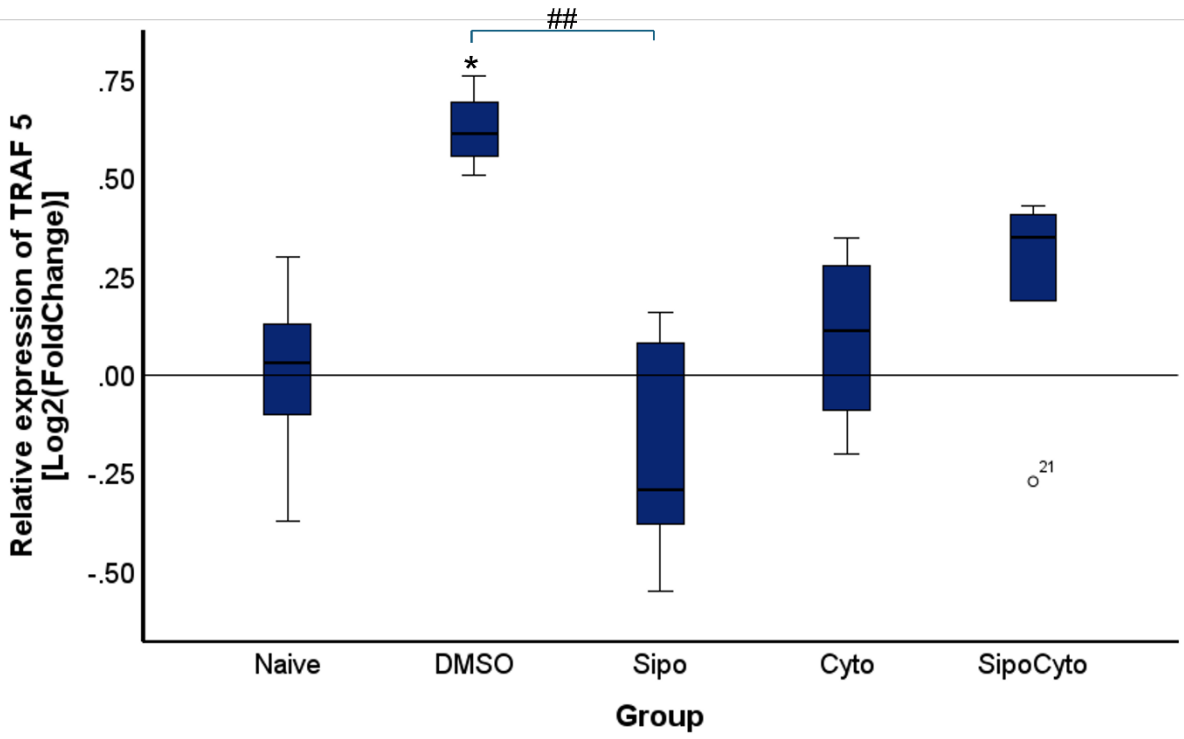

Figure 13: Influence of cytokine and/or siponimod treatment on TRAF5 expression in cultures of mixed glial cells. *TRAF5 mRNA expression was quantified by using a qRT-PCR after a 20-hour treatment. Only the vehicle control with DMSO led to a significant alteration, an increase in the expression of TRAF5, compared to sole siponimod treatment. Siponimod with concomitant cytokine treatment resulted in an elevated expression, however, these influences are not significant to the controls. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with one-way ANOVA followed by Tukey's HSD test. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*

4 Discussion

4.1 M1 microglial cells

The mRNA expression of iNOS was evaluated to examine how siponimod acts on the microglial cells and their behaviour in the different experimental constellations on the M1/M2 microglial cell phenotype spectrum in the MGC cultures (64,74–76). iNOS served as a marker for the M1 microglial cells (75,88), which are considered to promote a pro-inflammatory phenotype (64,74–76). Therefore, a decrease in the expression of iNOS would indicate a reduction of M1 phenotype microglial cells and thus an anti-inflammatory reaction.

In both groups treated with cytokines, as expected, a statistically significant increase in the gene expression compared to the naive group was shown. There was a decrease in expression through siponimod treatment upon concomitant cytokine challenge relative to the sole cytokine treatment observed, although the reduction was not statistically significant. However, in another study conducted, it was shown that siponimod is able to reduce the protein expression of iNOS and promote an anti-inflammatory phenotype in microglial cells under inflammatory conditions (58), whereas in this study, no such effect was proven. Causative for this deviating observed effect, could be a longer exposure of the microglial cells to siponimod (72h vs. 20h), a much higher used concentration of siponimod (50 µM or 10 µM vs. 2 µM) and the usage of a pure mature microglia culture in the mentioned study (58). Furthermore, it was shown that siponimod reduces the mRNA expression, of other pro-inflammatory genes, but a higher concentration of siponimod (50 µM for 24-hours) led to this effect (58). In the treatment of SP-MS, a dose of 2 mg once daily is given which leads to a mean maximal plasma concentration of 30.4 ng/mL, which equals 0,059 µM (516.6 g/mol, molar mass of siponimod), when given over 10 days and reaching a steady-state concentration (53). In another conducted study, the best efficacy of siponimod in a mouse EAE model was reached by a level of 3 – 3,5 µM of siponimod in a brain homogenate, which equals 0.400-0.500 µM in blood (143). Furthermore, a mean brain to blood drug exposure ratio around 6 in rats was revealed (143) which would equal 0.33 µM plasma concentration of siponimod in our experiment. Therefore, the dose used in this experiment is still higher, but much closer to the plasma level which is reached in vivo in humans. Nevertheless, with respect to the mentioned studies where a higher dose was used (58,143), this

suggests that this study was not able to reproduce a significant reduction of iNOS mRNA expression, in an in-vitro setting with a concomitant inflammatory stimulus, due to a lower used dose. This implicates, that in future studies, which investigate the effect of siponimod on gene expression in glial cells, the usage of a higher concentration and a longer treatment duration of siponimod should be considered. However, this would result in a larger deviation from the steady-state dose reached in humans.

However, there was a statistically significant reduction in the expression of iNOS in the group with siponimod treatment compared to the naive group. This would be in accordance with the hypothesis, but the expression was also statistically indifferently decreased in the group with the vehicle control DMSO, compared to siponimod treatment. Since siponimod is solved in DMSO and they are statistically indifferently decreased, it cannot be differentiated if the decreased expression is caused by siponimod or the DMSO treatment. Therefore, it was not proven that this reduction of a pro-inflammatory gene, is caused by siponimod and there might be an influence of DMSO as well. In other studies, there is evidence, that DMSO can have an influence on iNOS expression (144,145). It was shown that it can reduce iNOS mRNA expression in microglia (145) and the protein expression in bone marrow derived macrophages (144), but this effect was only found at higher concentrations, like 0,2% (145) or 0,08 % (144) than at this study (0,02%) was used. In contrast, in the before mentioned study, a DMSO concentration of 0.1 % was used (58). However, there was no naive culture to control for a decreasing influence of DMSO or siponimod treatment on gene expression (58). Therefore, more detailed studies which investigate the effect of DMSO on glial cells, the influence on their gene expression and the underlying mechanisms should be conducted.

Taking these results into a context, it has been shown that NO, which is produced by iNOS (87), can harm DNA and subsequently cells (146), exert damage to neurons (147) and oligodendrocytes through formation of reactive molecules, such as peroxynitrite (148). Furthermore, it was shown that iNOS expression is associated with active lesions of progressive forms of MS (78). In addition, it has been shown that cerebral demyelinated cortical MS lesions are associated with an increased activity of myelin-peroxidase (MPO), compared to controls and non-demyelinated cortical grey matter of the same individuals (149). The observed

elevated MPO activity could be attributed to CD68 positive microglial cells (149). CD68 is a marker of M1 microglial cells (75,88). This data provided here suggests, that M1 microglial cells could be causally involved in the demyelination of cerebral cortical MS lesions. Subsequently, influencing microglial cells, to reduce their M1 phenotype cells could provide a mechanism for the observed protection from demyelination in the unpublished experiment by Ücal & Haindl et al..

In this unpublished experiment by Ücal & Haindl et al., a protective effect of siponimod against cytokine induced demyelination in the cortex of rats and a significantly reduced number of activated microglial cells through siponimod treatment was revealed. This beneficial effect observed in vivo, could not be reproduced regarding iNOS expression, in this in vitro setting. One of the reasons causative for these deviating findings, could be the different timing of the cytokine and siponimod treatment. In the in vivo experiment, siponimod was given for four weeks before the inflammatory stimulus, whereas in this experiment a simultaneously cytokine injection was performed. It might be speculated, that the cytokine stimulus influenced the gene expression before siponimod was able to exert its beneficial effect on the mRNA expression. Therefore, a prior treatment of siponimod, using this dose, to the inflammatory stimulus seems necessary in order to study the influence of siponimod on gene expression in glial cells and could lead to greater influence on it. Subsequently, despite siponimod treatment reduced iNOS expression, and there is evidence that it influences inflammation related genes in an anti-inflammatory way (58), in this experimental setting, it could not be proven that siponimod promotes an anti-inflammatory phenotype by influencing microglial cells and their iNOS expression. Therefore, these results cannot explain the observed protection from demyelination in the performed preliminary experiment by Ücal & Haindl et al..

4.2 M2 microglial cells

The effects of siponimod on the microglial cells in the MGC cultures and their behaviour on the M1/M2 microglial phenotype spectrum in the different experimental constellations were investigated by analysing the mRNA expression of Arg-1 and Igf-1, which served as markers of M2 microglial cells (75,88). M2 microglial cells are considered to promote an anti-inflammatory phenotype (64,74–76). Therefore, an

increase in the expression of these genes, would indicate an anti-inflammatory reaction and an increase in M2 phenotype microglial cells.

As expected, a statistically significant downregulation in the expression of Arg-1 was observed in both groups treated with cytokines compared to the naive group. However, no ameliorating influence of siponimod on the reduction in mRNA expression, relative to the cytokine treated group, was found. In another conducted study, it has been shown, that treatment with 2mg/kg bodyweight (BW) or 6 mg/kg BW siponimod is able to induce Arg-1 expression in glial cells in vivo and reduces the clinical score of mice with an induced experimental autoimmune encephalomyelitis (57). Interestingly, the greatest reduction in the clinical score was shown with a prophylactic treatment on the day of immunization compared to later treatment (day 14 or day 30) (57). However, also therapeutic treatment on day 14 after immunization showed a significant reduction in the EAE score, but less than prophylactic treatment (57). Even in this group, with treatment on day 14, a statistically significant elevated gene expression of Arg-1 was detected by doing a quantitative PCR of samples of the optic nerves of these mice (57). Nevertheless, the greatest influence of the treatment on the EAE score was shown, using a prophylactic treatment (57) indicating the best efficacy when a preventive treatment is given. Also, in the unpublished in vivo experiment by Ücal & Haindl et al., a preventive treatment approach before the cytokine injection was used (3mg/kg BW, started 4 weeks before cytokine injection). Thus, it might be speculated, that this experimental setting could not reproduce a beneficial effect on gene expression in glial cells, because a concomitant cytokine and siponimod stimulus was performed. Whereas a treatment prior to the inflammatory stimulus could be necessary, using this dosage, to influence gene expression. Moreover, the results in this study could be influenced by the fact that Arg-1 and iNOS are using the same substrate and working antagonistic against each other (87,92,93) and no decrease in iNOS expression in this study was found.

The sole siponimod treatment (2 µM) alone, did not alter the expression of Arg-1. Interestingly, in a BV2 cell line culture, a pure microglia culture compared to our used MGC culture, a sole siponimod dose of 5 µM and 7.5 µM led to an increase in Arg1 expression, but a dosage of 2.5 µM did not lead to a statistically significant increase (57). This suggests that a higher dose as well, could be necessary to

observe an influence on gene expression in glial cells in vitro. Interestingly, a statistically significant elevated expression of Arg-1 in the group treated with DMSO was seen. In another study, an effect of DMSO on the mRNA expression of Arg-1 was observed in microglial cells, but a higher concentration (0,2%) (145) than in this study (0,02%) was used. Thus, more detailed studies are needed which investigate the influence of DMSO, especially at low concentrations, on gene expression in glial cells for a safe usage in further gene expression experiments.

With respect to IGF-1, surprisingly, there was a statistically significant decrease in all the investigated groups in the expression of IGF-1 compared to the naive controls. As expected, the expression of IGF-1 was decreased in the groups treated with cytokines compared to the naive group. However, there was no elevating effect of siponimod on mRNA expression with concomitant cytokine challenge compared to the sole cytokine treatment. This result is in line with another study, where no effect of siponimod treatment for 24 hours with a dosage of 100 nM on the gene expression of IGF-1 was found (59). This was confirmed with real time PCR under inflammatory conditions (treatment with LPS (1µg/mL)) and physiological conditions (59). In the noted study, a pure microglial cell culture, compared to a MGC culture in this study, was used (59). However, in the mentioned study, an influence on the gene expression of other genes was discovered (59). This shows that siponimod is able to influence gene expression, but with a concomitant inflammatory stimulus no statistically significant beneficial effect compared to the untreated controls was observed (59). This suggests, that in an in-vitro setting, when a concomitant inflammatory stimulus is given, a higher dose of siponimod might be necessary to significantly influence gene expression of glial cells beneficially. On the other hand, it could be deduced that siponimod does not influence IGF-1 expression. Therefore, a possible influence of siponimod on IGF-1 expression in glial cells should be clarified in future studies.

The DMSO group and the siponimod treated group, showed also a reduced mRNA expression. Analogous with the results obtained from iNOS expression, it cannot be differentiated if the effect is caused by siponimod or by DMSO treatment. In the aforementioned study, siponimod had no statistically significant effect on the gene expression of IGF-1 in microglial cells under physiological condtions (59), which would rather indicate an influence of DMSO. However, no studies were found on the

effect of DMSO on the expression of IGF-1 for comparison. As noted above, DMSO can have an influence on gene expression (144) of microglial cells (145), but this effect was found at higher used concentrations (144,145). Therefore, as mentioned before, more detailed investigations on the effect of DMSO on glial cells, the influence on their gene expression and the underlying mechanisms should be conducted.

In the M1/M2 microglial cell paradigm, M2 microglial cells are considered to promote an anti-inflammatory phenotype (64,74–76). With respect to MS, it was shown in an animal model where white matter demyelinating lesions were induced, that M2 microglial cells are associated with remyelination (77). A change from a M1 to a M2 microglial phenotype was observed at the time when remyelination started (77). Furthermore, it was shown that M2 microglial cells are needed for the differentiation of oligodendrocytes in those lesions (77), which form the myelin sheaths around the axons (62). This data demonstrates, that M2 microglial cells might play a beneficial role regarding remyelination in MS.

In conclusion, however, the results in this study regarding Arg-1 and IGF-1 expression, do not show an enhanced M2 microglial cell type caused by siponimod treatment under inflammatory or non-inflammatory conditions and therefore do not provide an explanation for the observed protection from demyelination in the unpublished in vivo experiment by Ücal & Haindl et al..

4.3 Reactive astrocytes

The effect of the siponimod treatment on astrocyte reactivity and their behaviour in the different experimental constellations was evaluated by analysing the mRNA expression of C3 (69) and KCNJ10 (101,103,104). An upregulation of C3 and a downregulation of KCNJ10 was used in this study to indicate an increase in an A1 reactive astrocyte phenotype, which is considered to be pro-inflammatory and neurotoxic (69).

First, regarding the expression of C3, both groups treated with cytokines showed as expected a statistically significant upregulation of the mRNA expression, but a concomitant treatment with siponimod did not attenuate this inflammatory upregulation of C3. Interestingly, there was a statistically significant reduction in the expression of C3 in the group with DMSO and the group with siponimod treatment compared to the naive group. Siponimod decreased the expression statistically

significant even more than DMSO, which indicates an anti-inflammatory effect of siponimod under non-inflammatory conditions on astrocytes. These results are in accordance with another study, where an anti-inflammatory influence of siponimod treatment (1µM) on the gene expression of other pro-inflammatory genes of astrocytes (60) was confirmed. This effect was detected with a quantitative PCR, under inflammatory conditions after treatment with LPS (1µg/mL) in a murine primary pure astrocyte culture (60). Causative for the deviating finding in the group with the inflammatory stimulus, could be a different response of the used cell cultures or the timing of the treatment, since in the mentioned study the cell culture was treated 1-hour before the inflammatory stimulus (60), compared to the concomitant treatment in this experiment. It was shown that treatment of mouse derived astrocytes with 1 µM siponimod for 1-hour leads to an internalization of the S1P1 receptor (132). These considerations are supported with the results obtained in the unpublished in vivo experiment of the working group, where a prophylactic treatment of siponimod was used to prevent a widespread demyelination in the cortex of rat. As mentioned before, this indicates that a prior treatment before the inflammatory stimulus could be needed to influence inflammation related gene expression of glial cells in a beneficial way. Another reason could be, that a higher dosage and longer treatment duration seems necessary to alter gene expression of glial cells in vitro, when a concomitant treatment and inflammatory stimulus is given, as seen in this study (58).

Despite there were several studies conducted analysing the effects of siponimod on gene expression, no investigation regarding the expression of C3 was found (57–60,150,151). In conclusion, this study shows for the first time that siponimod promotes an anti-inflammatory phenotype by reducing the expression of C3 in mixed glial cells and therefore reduce an A1 reactive astrocyte phenotype under non-inflammatory conditions.

However, in this study it was shown that DMSO had a decreasing influence on the expression of C3, despite it was used at a very low concentration (0,02%). No literature was found investigating C3 expression and DMSO treatment, but as already mentioned it was shown that DMSO can have an impact on gene expression (144,145), whereas this effect of DMSO was found at higher concentrations (144,145) than used in this study. Therefore, the influence of DMSO on gene

expression of glial cells and the underlying mechanisms should be evaluated in more detailed studies.

With respect to the expression of KCNJ10, it was shown that the cytokine treatment had no influence on the gene expression in both groups compared to the control group. In addition, no effect of siponimod with concomitant cytokine treatment could be found relative to the sole cytokine treatment. There was a reduction in the mRNA expression of KCNJ10 in the group with sole siponimod treatment and the group with DMSO treatment, compared to all other groups. However, no statistically significant alteration between the sole DMSO and siponimod treatment was found, thus, it cannot be differentiated if the decreased expression of KCNJ10 is caused by siponimod or DMSO. As with C3, no literature was found exploring the effect of DMSO on KCNJ10 expression, but as noted above, it was shown that DMSO can influence gene expression (144,145). No studies for comparison were found investigating siponimod treatment and the expression of KCNJ10, although the influence of siponimod on gene expression was investigated in several other studies (57–60,150,151). In conclusion, it could not be proven that siponimod had a beneficial effect under inflammatory or non-inflammatory conditions on KCNJ10 expression. In line with other genes in this study, it seems that DMSO, despite it was used at very low concentrations, had an impact on mRNA expression, which should be investigated in more detail in future experimental studies.

Taking these results into a context, it was shown that reactive astrocytes with upregulated C3 expression, are associated with neurodegenerative diseases like Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis and MS (69). Furthermore, it was shown that these A1 reactive astrocytes are toxic and lead to the death of neurons and mature oligodendrocytes (69). This indicates, that C3 expressing reactive astrocytes could contribute to the demyelination in cortical lesions by promoting the cell death of the myelin sheath forming oligodendrocytes. Since in this study a decreasing impact of siponimod treatment on the mRNA expression of C3 in the MGC cultures and therefore a reduction in reactive astrocytes was found, this could be part of an explanation of the observed prevention from demyelination in the in vivo experiment by Ücal & Haindl et al.. However, no effect of siponimod was found under inflammatory conditions. This could be caused by the fact, that reactive astrocytes are induced by activated

microglial cells (69,70), and vice versa (70). This pro-inflammatory upward spiral of astrocytes and microglia could have surpassed the observed beneficial effect of siponimod. As already mentioned before, a higher effective concentration (58) or a treatment prior to the inflammatory stimulus (57,60), as it was also used in the preliminary in vivo experiment conducted by the working group, of siponimod could be needed to have a beneficial effect on glial cells and their gene expression under inflammatory conditions.

4.4 Protective astrocytes

To evaluate the effect of siponimod on astrocytes and their reactivity, the mRNA expression of BDNF, TLR3 and CNTF was quantified in this study. These genes were used as markers for protective astrocytes, which are considered to be anti-inflammatory and neuroprotective (69,80,81), therefore, in this study an upregulation of these genes was used to indicate an increase in protective astrocytes and a promotion of an anti-inflammatory phenotype.

First, concerning the expression of BDNF, there was in both groups treated with cytokines a statistically significant elevation compared to the control group. However, there was no influence of siponimod on the expression with concomitant cytokine treatment, relative to the sole cytokine treatment. These findings are in line with the results of an aforementioned study, which also found no alteration in BDNF expression, and other neurotrophins, under inflammatory conditions in astrocytes through siponimod, despite an influence on the expression of other genes was revealed (60). In conclusion, in this study no influence of siponimod on astrocytes concerning the expression of BDNF under non-inflammatory and inflammatory conditions was shown. Supporting these results, it was shown in an experimental study, that a clinical improvement in an EAE model that was caused by siponimod treatment, was independent of BDNF expression in microglia and immune cells (152).

Second, it was shown that cytokine treatment had no influence on TLR3 expression compared to the control group. There was also no alteration, between the cytokine treated group and the group with siponimod and concomitant cytokine treatment. Thus, no effect of siponimod under inflammatory conditions in TLR3 expression was found. Surprisingly, the sole siponimod treatment showed a statistically significant decrease in the expression compared to the group with siponimod and concomitant

cytokine treatment. Despite several other studies were conducted investigating the influence of siponimod on gene expression, no results were found for comparison regarding the effects on the mRNA expression of TLR3 (57–60,150,151). Since there was no difference between the vehicle control group and the group with sole siponimod treatment, which is solved in DMSO, it cannot be deduced that this reduction is caused by siponimod and there might be an influence of DMSO on TLR3 expression. As noted above, it was shown that DMSO can have influence on gene expression at higher concentrations (144,145), but no studies were found investigating the effect of DMSO specifically on TLR 3 expression. However, there is evidence that DMSO has an influence on astrocytes, similar to ischemia or excitotoxicity, by increasing glial fibrillary acidic protein expression and cell proliferation, but this effect was found at higher concentrations (0,5 - 1%) in a 24-hour or 48-hour treatment period (153). In summary, it was not proven that there is an influence of siponimod on astrocytes by influencing the expression of TLR3. Whereas it was shown that there might be an influence of DMSO, despite it was used at a very low concentration (0,02 %). As it was shown that DMSO can influence astrocytes (153), and there seems to be a possible influence in this study as well, further investigations in more detail are needed to evaluate the influence of DMSO on glial cells and the involved mechanisms, especially at low concentrations.

Third, with respect to the expression of CNTF, a statistically significant elevation in both groups treated with cytokines compared to the naive and vehicle control group was found. There was no influence on the expression of DMSO compared to the naive group. However, no influence of siponimod treatment between both groups with cytokine treatment in the expression was found, indicating no influence of siponimod on CNTF expression under inflammatory conditions. Unexpectedly, rather contradictory to the hypothesis, it was observed that sole siponimod treatment led to a decrease in the expression of CNTF compared to the control groups. Despite several other studies conducted (57–60,150,151), there were none found investigating the expression of CNTF. However, another study showed no influence of siponimod on other neurotrophic factors (60). It was shown that CNTF might have a beneficial role concerning MS, since it was shown that it is associated with enhancing remyelination after injury in an animal model (123). Moreover, CNTF expression or administration has a beneficial influence on the disease course of

mice were an EAE was evoked (124,154), indicating an anti-inflammatory and neuroprotective role. In summary, these results are rather contradictory to the hypothesis, since a decrease in CNTF mRNA expression, indicating a decrease in a protective astrocyte phenotype in the MGC cultures, was observed upon sole siponimod treatment.

In summary, in this study no anti-inflammatory influence of siponimod, regarding the gene expression of the investigated protective astrocyte marker genes, was shown. Therefore, these results cannot explain the observed protection from demyelination in the unpublished in vivo experiment performed by Ücal & Haindl et al.. On the one hand, there is evidence that siponimod can have a beneficial influence on gene expression of glial cells (57,58,60). As explained before, it can be speculated, that a prior treatment of siponimod to an inflammatory stimulus, or a higher concentration and longer treatment duration in vitro, with a concomitant cytokine stimulus, could be needed to influence the mRNA expression of glial cells of these genes in a beneficial way. On the other hand, since there is supporting evidence that siponimod does not influence BDNF expression (60), it could be deduced that siponimod exerts its beneficial effects independently of the investigated genes, except CNTF. With respect to the controversial results of CNTF, further investigation is needed to analyse the effect of siponimod on its expression by glial cells.

4.5 Other markers

4.5.1 Pro-inflammatory genes: IL6 and PTGS2

The anti-inflammatory properties of siponimod, were analysed by quantifying the mRNA expression of the pro-inflammatory markers IL6 (130,131) and PTGS2 (135–137). Therefore, in this study a reduction in these markers, would indicate an increase of an anti-inflammatory phenotype.

In this study, in line with the hypothesis, a statistically significant reduction in the expression of PTGS2 in the group with siponimod and concomitant cytokine treatment was observed compared to all other groups. This indicates, that siponimod had a beneficial influence regarding the expression of the pro-inflammatory gene PTGS2 when a concomitant inflammatory stimulus is given. These results are in line with other aforementioned studies, where there is evidence that siponimod influences the gene expression of other inflammation related genes

in glial cells (57,58,60). However, no literature regarding PTGS2 expression was found for comparison. Interestingly, DMSO showed a slight increase in gene expression, with a statistical significance in comparison to all the groups except the naive group. Vice versa, the groups with the sole siponimod and sole cytokine treatment, showed a statistically significant reduction in mRNA expression, but just in comparison to the DMSO group and not to the naive group. Therefore, no influence of the sole siponimod treatment was proven under non-inflammatory conditions. Surprisingly, the sole cytokine treated group showed no enhanced expression of PTGS2. In other studies conducted, it has been shown that PTGS2 expression is elevated after an inflammatory stimulus with TNFα (5 ng/mL for 4-hours) in primary astrocytes (155), LPS (0.5-2µg/mL for 24-hours) in BV2 microglial cells (135) or TNFα + IFNy (50 ng/mL+20 ng/mL for 24-hours) in primary rat derived microglial cells (89). These results were not reproduced in this study, potentially caused by a lower used dosage of cytokines.

In summary, it was shown that siponimod decreased the expression of PTGS2 upon concomitant cytokine treatment. It might be speculated, that in this gene the experimental setting was suitable to detect a decreasing influence of siponimod under inflammatory conditions, but not in other genes, because in this gene there was a synergistic action of cytokines and siponimod. It could be concluded, that an effect on gene expression of the sole siponimod treatment could have been revealed, when a higher dose had been given, as seen in a aforementioned study where a higher dose (5 µM and 7.5 µM) reduced Arg-1 expression in a BV2 cell culture (57). Nevertheless, the study shows for the first time an effect of siponimod on gene expression of PTGS2. Therefore, the results of this study suggest that siponimod promotes an anti-inflammatory phenotype in a MGC culture through acting directly on the glial cells by reducing their PTGS2 expression.

Bringing these results in context with MS, it was shown that elevated protein expression of PTGS2 (COX2) is found in chronic active lesions of MS patients, compared to healthy tissue and healthy control subjects and it is associated with demyelination (136). Furthermore, it was shown that PTGS2 (COX2) expression is associated with dying oligodendrocytes in lesions of MS patients (137). Moreover, in an experimental model of PTGS2(COX2)-knock out mice a survival benefit of oligodendrocytes to exitotoxic induced death compared to the wild-type could be

observed (137). This evidence provided here, suggests that a reduced expression of PTGS2 could promote the survival of oligodendrocytes. Therefore, the observed reduction of PTGS2 mRNA expression in this study through siponimod under inflammatory conditions in glial cells, might partially explain the observed protection from demyelination in the unpublished in-vivo experiment by Ücal & Haindl et al., by promoting an anti-inflammatory phenotype, the survival of oligodendrocytes and subsequently a reduced demyelination.

Regarding IL6 expression, it was shown that it is elevated intrathecally in the progressive forms of MS and that it has a positive correlation with disease severity (129). Furthermore, there is data that IL6 signalling promotes a pro-inflammatory phenotype and leads to a more severe disease course in an EAE model (131). These findings emphasise its pro-inflammatory role in regards of MS. However, in this study no influence of the cytokines nor of the treatment with siponimod, nor of DMSO on IL6 expression was observed. Whereas in other experimental studies, there was a decreasing effect of siponimod treatment on IL6 mRNA expression in a pure murine astrocyte culture after treatment with LPS (1µg/mL) (60) and protein levels in a murine astrocyte or microglia culture under inflammatory conditions (LPS 100ng/mL) (132). However, in both studies a preventive treatment of siponimod (1µM) 1-hour before the inflammatory stimulus was used (60,132). In microglial cells a reducing effect of siponimod on the gene coding for the IL6 receptor was found, but as mentioned before, a much higher concentration was used than in this study (50 µM vs. 2 µM) (58). Similar to other genes in this study, it could be speculated that these results could not be reproduced because of a different timing of the treatment and the inflammatory stimulus (concomitant vs. preventive). Furthermore, in other studies conducted, it was shown that exposure to TNFα and IFNy showed an enhanced mRNA expression of IL6 in human derived astrocytes (24h/TNFα-10 and 30 ng/mL) (156), rat derived astrocytes (24h/IFNy) (157) and protein levels in rat derived hippocampal slice cultures accompanied by a proliferation of microglia (72h/IFNy-1ng/mL) (158). These results could not be reproduced in this study. In this experiment the cytokines showed a reliable ability to influence gene expression, thus it could be speculated that regarding the results of this gene, there was some external influence like an insufficiency of the primer, since the results are not influenced in any group at all or an insufficient dose was used. However, this

speculation cannot be proven. Besides speculation and concerning the facts, no effect of the siponimod treatment could be observed in this gene. Therefore, no conclusion can be drawn from these results regarding the hypothesis and in this experimental setting the beneficial effect of siponimod on IL6 expression could not be reproduced. This implicates for future studies which evaluate the influence of siponimod on glial cells and their mRNA expression of IL6 and aim to reproduce the mentioned beneficial effects on IL6 expression, a prior treatment to the cytokine stimulus should be preferred.

4.5.2 Anti-inflammatory genes: IL10 and TRAF5

The effects of siponimod on glial cells and its ability to promote an anti-inflammatory phenotype was investigated by analysing the mRNA expression of TRAF 5 (131) and IL10 (159,160). Therefore, in this study an increase in these marker genes would indicate an anti-inflammatory reaction.

Regarding IL10, there is evidence that suggests it exerts anti-inflammatory effects in the CNS upon glial cells since it was shown that IL10 modulates inflammation in a MGC culture by decreasing the mRNA and protein expression of pro-inflammatory cytokines (159). Furthermore, there is evidence that intracerebroventricular treatment with viral vectors expressing IL10 are beneficial for the clinical course of an animal EAE model (160), which depicts its anti-inflammatory functions in an animal model of MS. Unfortunately, the results of the experiment were unusable because in the qRT-PCR incorrect amplification curves were generated and subsequently unusable results.

With respect to the results of TRAF5, it was shown that only DSMO treatment influenced the mRNA expression, which resulted in a statistically significant elevation of the expression compared to the naive group and the group treated with siponimod. However, no other statistically significant influences of the siponimod treatment nor the cytokines were found. In another study conducted, LPS (100ng/mL) and IFN-γ (20ng/mL) treatment for 24 h had an elevating effect on the protein expression of TRAF5 in a BV2 microglial cell culture (141). In this study, no significant increasing effect on gene expression after treatment with cytokines was observed. This difference might be caused by the fact, that the mRNA expression is influenced differently than the protein expression and, in this experiment, a different inflammatory stimulus was used (TNF-α + IFNy). Furthermore, in this study a MGC

culture was used which might respond differently than pure BV2 microglial cells. Siponimod, as noted above, can have direct influence on glial cells and their gene expression (58,60,132), but no literature was found concerning TRAF5 and siponimod treatment. In summary, no beneficial effect of siponimod was observed, but there was an anti-inflammatory influence of DMSO on TRAF5 expression in this study. As mentioned above, DMSO can have influence on gene expression (144,145) but no influence on TRAF5 was reported. Therefore, more detailed experimental studies on the effect of DMSO on glial cells, especially at low concentrations, the influence on their gene expression and the underlying mechanisms should be conducted.

4.6 Limitations of the study

Although we conducted this study to the best of our knowledge, there are some limitations. In this study the effect of siponimod on the gene expression of pro- and anti-inflammatory genes was investigated using a MGC culture. Therefore, no conclusions can be drawn on the influence of siponimod on distinct glial cell types but rather an overview on the influence off all glial cell types and the whole cell culture population, hindering direct comparison with isolated pure cultures. Moreover, some possible neuron-glia interactions, which could influence the results in an alive organism, could not be investigated and represented in this study. Concerning statistical issues, due to the relatively small sample size (n = 5), there is a reduced statistical power. Furthermore, in five out of eleven genes (iNOS, Arg1, IGF-1, C3, TRAF5), there was an influence of the solvent DMSO on the gene expression, despite it was used at a very low concentration (0,02%), indicating possible confounding effects by DMSO.

4.7 General conclusion and implications for further experiments

In this study, an influence of siponimod on the gene expression of glial cells in a MGC culture was revealed. There was an anti-inflammatory influence on the gene expression of C3, used as marker of reactive astrocytes, and on the pro-inflammatory gene PTGS2. Therefore, this confirms the hypothesis that siponimod has an anti-inflammatory influence on the gene expression of glial cells and can promote an anti-inflammatory phenotype. However, rather controversial to the hypothesis, siponimod downregulated the mRNA expression of the anti-

inflammatory gene CNTF. In conclusion, it was shown that siponimod can influence the gene expression of glial cells in an anti-inflammatory way, but there was also a pro-inflammatory influence. Therefore, further studies should be conducted to resolve these controversial results. With regards to the other investigated genes, no definite conclusions can be made from these results. Nevertheless, it was shown that siponimod has anti-inflammatory effects by acting on the resident glial cells of the CNS and their gene expression of C3 and PTGS2. These findings might partially explain the observed protection from demyelination and microglial activation through siponimod treatment in the unpublished in vivo experiment by the working group. In other conducted studies, an influence of siponimod on inflammation related gene expression was confirmed. With respect to these studies and the results in this study, in conclusion, a higher effective concentration, longer treatment duration and a siponimod treatment prior to the inflammatory stimulus, could have led to a greater influence on mRNA expression in the used MGC cultures. Furthermore, in the unpublished in-vivo experiment of the working group also a prophylactic treatment with siponimod before an inflammatory stimulus with cytokines was used, which resulted in a prevention of a widespread demyelination in the cortex of rat. This implicates as well, that a treatment prior to an inflammatory stimulus should be considered in future studies, to investigate the effect of siponimod on inflammation related gene expression in glial cells. This might lead to a greater influence, better represents the circumstances in the mentioned experimental in-vivo study and could possibly reveal more information about siponimods effect on inflammation related glial cell gene expression. Moreover, there was an influence of the solvent DMSO on gene expression, in five out of eleven genes (iNOS, Arg1, IGF-1, C3, TRAF5), revealed, despite it was used at a very low concentration (0,02%). This influenced the results and the significance of this study. Since DMSO is a commonly used solvent in experimental studies, it is necessary to perform further studies in more detail to determine in which concentrations DMSO can be used safely for investigations of gene expression of glial cells in vitro. Therefore, when conducting further studies, the effect of DMSO should be clarified, especially at low concentrations, or another vehicle should be used.

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Appendix

Exact used volumes for cDNA synthesis and for the RNA gel

Table 9: First batch of cDNA

Sample Nr.	Conc (ng/µL)	For 500ng (µl)	Adjust to 25 µL (πL)	To the gel-300ng (µL)	To the cDNA 200ng (µL)
9	410.6	1.22	23.78	15	10
10	341.4	1.46	23.54	15	10
11	206	2.43	22.57	15	10
12	205.3	2.44	22.56	15	10
13	257.9	1.94	23.06	15	10
14	333.7	1.50	23.50	15	10
15	312.2	1.60	23.40	15	10
16	313.3	1.60	23.40	15	10
17	277.4	1.80	23.20	15	10
18	134.1	3.73	21.27	15	10
40	162.1	3.08	21.92	15	10
41	340.8	1.47	23.53	15	10
42	201.5	2.48	22.52	15	10
43	154.5	3.24	21.76	15	10
44	151.8	3.29	21.71	15	10
45	184	2.72	22.28	15	10
46	154.3	3.24	21.76	15	10
47	184.6	2.71	22.29	15	10
48	136.9	3.65	21.35	15	10
49	125.1	4.00	21.00	15	10
61	220	2.27	22.73	15	10
62	306.6	1.63	23.37	15	10
63	192.9	2.59	22.41	15	10
64	256	1.95	23.05	15	10
65	236.2	2.12	22.88	15	10

Table 10 First batch RT- control

RT- control 1 (9-16;40-41)	RT- control 2 (17-18; 42-47)	RT- control 3 (47- 65)
18.74 µL + same volume of nuclease free water	23.21 µL + same volume of nuclease free water	18.21 µL + same volume of nuclease free water

Table 11 Second and third batch cDNA

Sample Nr.	Conc (ng/µL)	For 1000ng (µl)	Adjust to 15 µL (µL)	To the gel-300ng (µL) + 10,5 µL nuclease free water	To the cDNA 700ng (µL)
9	410.6	2.44	12.56	4.5	10.5
10	341.4	2.93	12.07	4.5	10.5
11	206	4.85	10.15	4.5	10.5
12	205.3	4.87	10.13	4.5	10.5
13	257.9	3.88	11.12	4.5	10.5
14	333.7	3.00	12.00	4.5	10.5
15	312.2	3.20	11.80	4.5	10.5
16	313.3	3.19	11.81	4.5	10.5
17	277.4	3.60	11.40	4.5	10.5
18	134.1	7.46	7.54	4.5	10.5
40	162.1	6.17	8.83	4.5	10.5
41	340.8	2.93	12.07	4.5	10.5
42	201.5	4.96	10.04	4.5	10.5
43	154.5	6.47	8.53	4.5	10.5
44	151.8	6.59	8.41	4.5	10.5
45	184	5.43	9.57	4.5	10.5
46	154.3	6.48	8.52	4.5	10.5
47	184.6	5.42	9.58	4.5	10.5
48	136.9	7.30	7.70	4.5	10.5
49	125.1	7.99	7.01	4.5	10.5
61	220	4.55	10.45	4.5	10.5
62	306.6	3.26	11.74	4.5	10.5
63	192.9	5.18	9.82	4.5	10.5
64	256	3.91	11.09	4.5	10.5
65	236.2	4.23	10.77	4.5	10.5

Table 12 Second batch RT- control

RT- control 1 (9-17)	RT- control 2 (18-46)	RT- control 3 (47- 65)
31.96 µL + same volume of nuclease free water	46.49 µL + same volume of nuclease free water	41.84 µL + same volume of nuclease free water

Table 13 Third batch RT- control

RT- control 1 (9-16)	RT- control 2 (17-45)	RT- control 3 (46- 65)
28.32 µL + same volume of nuclease free water	43.85 µL + same volume of nuclease free water	48.29 µL + same volume of nuclease free water

Reference gene index - CCNA2 and GAPDH

The mRNA expression of CCNA2 and GAPDH was used as described in the method section as a reference gene index. There was no statistically significant difference in the mRNA expression of CCNA2 and GAPDH used as a reference gene index between the groups revealed by a Kruskal- Wallis H test ($\chi^2(2) = 2.60$, $p = 0.626$). The effect of siponimod and/or cytokines on the expression of CCNA2 and GAPDH combined is presented in figure 14.

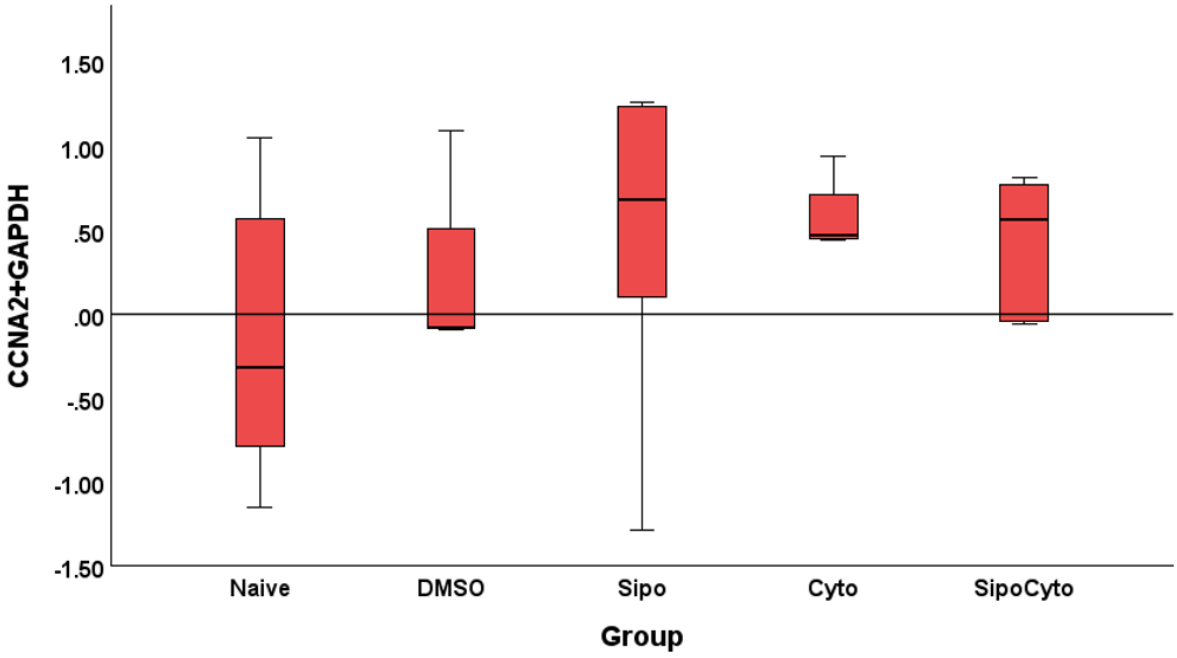

Figure 14: Influence of siponimod and/or cytokines on the expression of CCNA2 and GAPDH combined. *The expression of the reference gene index was analysed after a one-day treatment by using a qRT-PCR. No differences across all groups were found. Therefore, it is suitable for using CCNA2 and GAPDH combined as a reference gene index for relative quantification of the investigated genes. DMSO: dimethylsulfoxide treated (vehicle control for siponimod), Sipo: siponimod treated, Cyto: treated with cytokine and DMSO, SipoCyto: treated with siponimod and cytokines. Statistical significance was tested with Kruskal-Wallis H test followed by a Dunn's pairwise comparison with a Bonferroni correction. *p<0.05, **p<0.01, ***p<0.001 compared to the naive group; #<0.05, ##<0.01, ###p<0.001 compared to the indicated group.*