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Gp130 cytokines in multiple sclerosis: a role in autoimmunity and remyelination

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“Challenges are what make life interesting and overcoming them is what makes life meaningful.“

–Joshua J. Marine

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α-CD3	anti-CD3 antibody
APC	antigen-presenting cell
AVV	adeno-associated virus
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
Breg	regulatory B cell
CCL21	chemokine (C-C motif) ligand 21
CFA	complete Freud's adjuvant
CIS	clinically isolated syndrome
CLC	cardiothrophin-like cytokine
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CP-MS	chronic progressive multiple sclerosis
CSF	cerebrospinal fluid
CT-1	cardiotrophin-1
CXCL1	chemokine (C-X-C motif) ligand 1
DAB	3,3'diaminobezidine
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DMA	disease modifying agent
DRG	dorsal root ganglia
EAE	experimental autoimmune encephalomyelitis
EDSS	expanded disability status scale
eGFP	enhanced green fluorescent protein
FCS	fetal calf serum
FDA	food and drug administration
FGF-2	fibroblast growth factor 2
FOXP3i1	CpG nucleotides located in the first intron of FOXP3

GFAP	glial fibrillary acidic protein
gp130	glycoprotein 130
HC	healthy control
HIV-1	human-immunodeficiency virus 1
HLA	human leukocyte antigen
ICAM-1	intracellular adhesion molecule 1
IFN-γ	interferon gamma
IGF-1	insulin-like growth factor 1
IL	interleukin
JAK	janus kinases
KO	knock-out
LFB	luxol fast blue
LIF	leukemia inhibitory factor
LIFRβ	leukemia inhibitory factor receptor beta
LV	lentiviral vector
LV-eGFP	lentiviral vector encoding enhanced green fluorescent protein
LV-LIF	lentiviral vector encoding leukemia inhibitory factor
LV-OSM	lentiviral vector encoding oncostatin M
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein 1
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MOG_{35-55}	myelin oligodendrocyte protein 35-55 peptide
MS	multiple sclerosis
MTT	3-(4,5-Dimethylthiazol- 2-yl)-2,5 diphenyltetrazolium bromide
NAWM	normal appearing white matter
NGF	nerve growth factor
NK cell	natural killer cell

NT-3	neurotrophin 3
OPC	oligodendrocyte precursor cell
OSM	oncostatin M
OSMRβ	oncostatin M receptor beta
PBMC	peripheral blood mononuclear cell
PDGFa	platelet-derived growth factor subunit a
PI3K	phophatidylinositide 3-kinases
PLP	proteolipid protein
PMA	phorbol-12-myristate-13-acetate
PNS	peripheral nervous system
PP-MS	primary progressive multiple sclerosis
pSTAT3	phosphorylated signal transducer and activator of transcription 3
RR-MS	relapsing remitting multiple sclerosis
SOCS	suppressor of cytokine signaling
SP-MS	secondary progressive multiple sclerosis
STAT	signal tranducer and activator of transcription
TEM	transmission electron microscopy
TGF-β	transforming growth factor beta
Th	T helper
TIMP-1	tissue inhibitor of metalloproteinase 1
TMEV	Theiler's murine encephalomyelitis virus
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
WT	wild type

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INTRODUCTION AND AIMS

Parts of this chapter are based on:

Immunomodulatory properties of the IL-6 cytokine family in multiple sclerosis

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Submitted

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1.1 Multiple sclerosis

Multiple sclerosis (MS) was first described in 1868 by the French neurologist Jean-Martin Charcot. In patients with intermittent episodes of neurologic dysfunction, he found an accumulation of inflammatory cells in a perivascular distribution in the brain and spinal cord and called it “sclérose en plaques” [1]. Elvin Kabat provided further evidence for the inflammatory nature of the disease by revealing increased oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) of MS patients in 1948 [2]. The generally accepted hypothesis that MS is an autoimmune disease came from experiments of Thomas Rivers, who repeatedly injected rabbit brain extracts into primates leading to an inflammatory reaction accompanied by demyelination in the central nervous system (CNS) [3]. Over the last decades, extensive research resulted in a better understanding of the etiology and pathogenesis of MS.

1.1.1 Clinical aspects and diagnosis

Nowadays, MS is described as a chronic disabling disease of the CNS, characterized by multifocal inflammatory infiltrates, demyelination, and axonal degeneration. The insulating myelin sheath surrounding the axons is destructed, resulting in disturbed neuronal signaling and leading to a variety of symptoms including muscles weakness, fatigue, spasticity and tremor, visual and sensory disturbances, bowel and bladder dysfunction, and cognitive impairment [4].

MS is the most common cause of non-traumatic neurological disability in young adults, which mostly becomes clinically apparent in early adulthood (20-40 years). The estimated prevalence rate of MS is 83 per 100 000 in Europe, with women more frequently affected than men (female:male ratio of 2) [5, 6]. According to clinical observations, MS patients are divided into relapsing remitting (RR), secondary progressive (SP) and primary progressive (PP) [7-9]. The majority of the

patients (80-90%) are diagnosed with RR-MS, which is characterized by relapses of clinical symptoms that can last days, weeks or months, followed by spontaneous recovery or remission. The relapse rate varies between patients, with an average of one or two episodes per year. After 8 to 20 years, the majority of the RR-MS patients evolves in SP-MS, in which there is continuous neurological decline without recovery. In addition, 10-20% of MS patients are diagnosed as PP-MS. In these patients there are no relapses and the disease is progressive from onset [7-9]. The RR phase is thought to be caused by a strong inflammatory response, which is less apparent in the progressive phase in which neurodegeneration becomes increasingly evident [7].

The first episode of neurological symptoms suggestive of MS is called clinical isolated syndrome (CIS). This term refers to an 'attack' that has one or more symptoms resembling MS that lasts for at least 24 hours. However, not all CIS patients will develop clinical definite MS [10].

As no single diagnostic test or clinical feature is able to diagnose MS, the McDonald Criteria are used [11, 12]. The criteria include clinical and paraclinical diagnostic methods to establish disease dissemination in both space and time, meaning occurrence of at least two different episodes of disease and evidence of lesions/inflammation in at least two different regions in the CNS. Radiological and laboratory investigations include magnetic resonance imaging and analysis of the CSF for the presence of oligoclonal bands [11, 12].

1.1.2 Genetic predisposition and environmental factors

The exact etiology of MS is unknown, however it is thought that MS is triggered by environmental factors in genetically predisposed individuals. The prevalence of MS varies considerably around the world, but it follows a geographic pattern with higher frequencies with increasing distance from the equator [13]. Furthermore, the risk of developing MS diminishes with migration from high-risk to low-risk

regions in childhood [14]. These differences are thought to be associated with sunlight and vitamin D levels, as well as nutrition [15, 16]. Other environmental factors which have been associated with MS susceptibility are infections with Epstein-Barr virus, herpesvirus 6 and varicella-zoster virus [17].

Besides environmental factors, genetic predisposition also determines the risk of developing MS. The familial recurrence rate of MS is approximately 20% and there is a reduced risk with further degree of relation to a MS patient [13, 18]. Moreover, higher clinical concordance rates were found in monozygotic twins compared to dizygotic (25% vs 5%) [19, 20]. Associations between alleles of major histocompatibility complex (MHC) and MS were already identified in the early 1970s. Genes in the human leukocyte antigen (HLA)-DR2 haplotype, HLA-DRB1*1501, HLA-DRB5*0101 and HLA-DQB1*0602 encoding respectively HLA-DR2b, HLA-DR2a and HLA-DQ6, are the main genetic risk factors for developing MS [21]. Besides the HLA region, genome wide association studies have identified genes mainly involved in the immune response such as interleukin 2 receptor α (IL-2Rα), IL-7Rα, MHCI, CD40, CD125, CD58 and signal transducer and activator of transcription 3 (STAT3) to be associated with MS [22, 23].

1.1.3 Pathogenesis

MS is classically considered as an autoimmune disorder in which myelin specific T cells initiate an inflammatory and demyelinating pathology. T cells become activated in the periphery and home to the CNS were they cross the blood-brain barrier (BBB) by binding receptors on endothelial cells. In the CNS they are reactivated by antigen-presenting cells (APCs), which triggers the production of soluble mediators and initiates recruitment of other inflammatory cells. The following aberrant inflammatory reaction leads to demyelinated lesions throughout the CNS (Fig. 1.1) [4, 24]. However, based on pathological observations others believe that MS is a primary degenerative disorder, in which

the inflammatory response is secondary as a result of cytodegeneration and the release of highly antigenic components [9, 25]. Regardless of the initial trigger, the inflammatory autoimmune response is crucially involved in the demyelinating pathology. In the following paragraphs the role of the immune and CNS cells in the pathogenesis of MS is discussed.

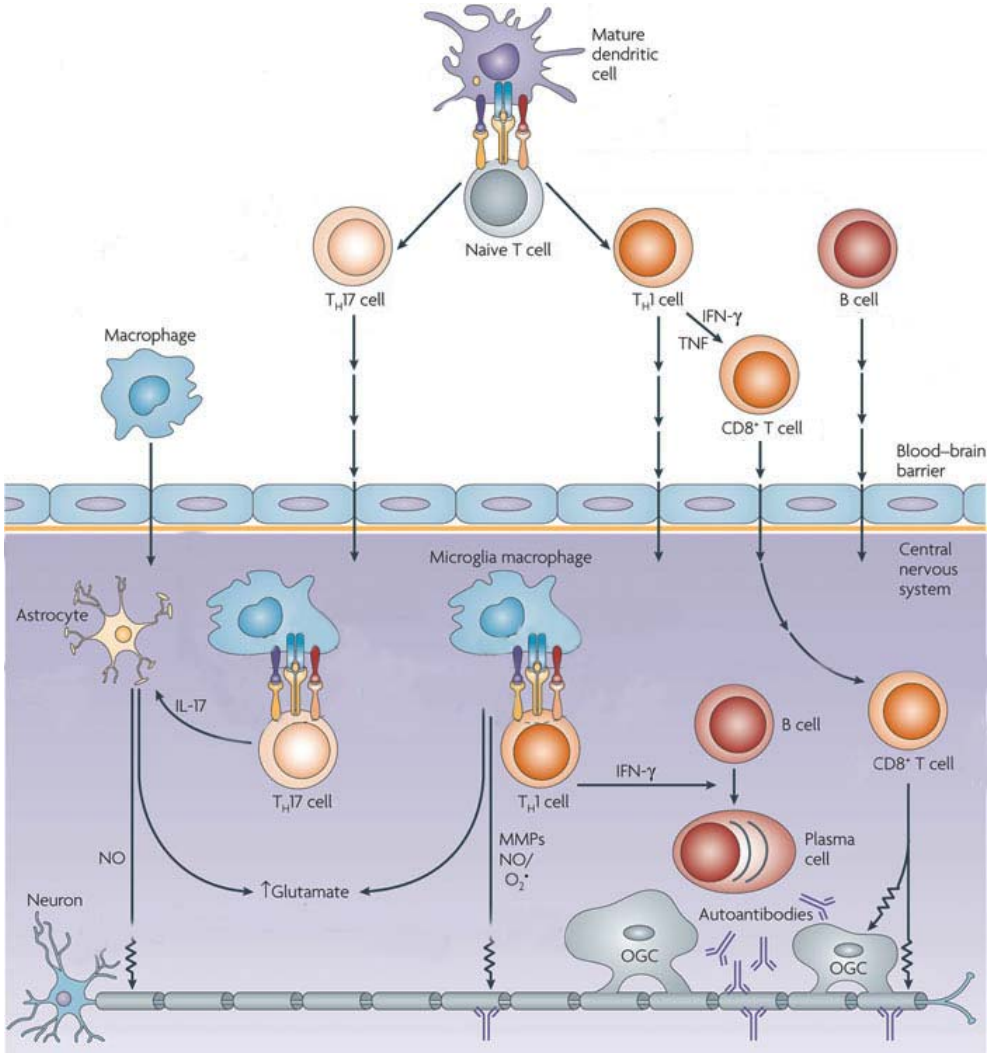

Fig. 1.1. A simplified schematic representation of the pathogenesis of MS. T cells are activated in the periphery by APCs which enables them to cross the BBB. In the CNS, they

are reactivated and produce cytokines and chemokines thereby attracting T cells, B cells and macrophages. The infiltration of these immune cells together with the activation of astrocytes and microglia leads to the production cytokines, reactive oxygen species, glutamate, autoantibodies and direct cytotoxicity resulting in damage to the myelin, axons and the BBB. T_H cell, T helper cell, OGC, oligodendrocyte; IFN-γ, interferon-γ; NO, nitric oxide; IL-17, interleukin-17; TNF-α, tumor necrosis factor α; MMPs, matrix metalloproteinases. Reprinted with permission from the Nature Publishing Group [26].

$CD4^+$ T helper cells

$CD4^+$ T cells are classically considered as the main effector cells in the pathogenesis of MS due to the strong association of certain MHC class II molecules and MS, in addition to studies on experimental autoimmune encephalomyelitis (EAE), the most widely used animal model of MS. Depending on the inflammatory environment, naïve $CD4^+$ T helper (Th) cells differentiate into several subsets after activation. Th1 and Th17 cells have proinflammatory properties and produce interferon-γ (IFN-γ) and IL-17 respectively. Th2 cells, in contrast, have anti-inflammatory effects in MS and are characterized by the production of IL-4 [27]. Before Th17 cells were identified, Th1 cells which require IL-12 for differentiation were thought to be the primary effector cells in MS and EAE. This was based on observations that Th1 cytokines are present in MS lesions and that MS is exacerbated by IFN-γ administration [28, 29]. However, mice deficient in IL-12 and IFN-γ are still susceptible to EAE [30, 31]. IL-23 deficient mice, in contrast, are completely resistant to EAE. IL-23 is a crucial factor for maintenance and expansion of Th17 cells [32]. Differentiation towards Th17 cells is regulated by transforming growth factor-β (TGF-β) and IL-6 [33, 34]. Although IL-23 deficient mice are completely resistant to EAE, mice lacking IL-17A and IL-17F still develop EAE [35]. Thus, while it is still a subject of debate whether Th1 or Th17 cells are the main effector cells in MS, these studies demonstrate that both cell types contribute to the pathogenesis.

CD8⁺ cytotoxic T cells

Far less research has focused on the role of $CD8^+$ T cells in MS. However, these cells are more prominent in MS lesions compared to $CD4^+$ T cells and clonal expansions are more frequently detected in $CD8^+$ T cells isolated from lesions than in $CD4^+$ T cells [36-38]. Effector mechanisms of these cells are cell contact-mediated lysis and production of soluble mediators. Production of IL-17, IFN-γ and TNF-α by $CD8^+$ T cells of MS patients is demonstrated [39, 40]. In EAE, myelin-specific $CD8^+$ T cells are highly pathogenic [41, 42]. Transfer of these cells in naive mice induces a demyelinating disease of which certain pathological features are similar to MS patients, but differ from $CD4^+$ T cell-mediated EAE as they are more associated with upper motor neuron impairment such as ataxia, spastic reflexes and loss of coordinated movement [42]. Although both T cell subsets contribute to MS pathogenesis, $CD4^+$ T cells are thought to be critically involved in initiating the disease, while $CD8^+$ T cells are a predominant cell type causing CNS damage during relapses and possibly in chronic disease stages [43].

Regulatory T cells

As a result of regulatory defects an aberrant autoimmune response can induce damage to the CNS. The function of regulatory T cells (Tregs) is to maintain tolerance to self-antigens and prevent autoimmunity [44, 45]. They inhibit activation or suppress effector activity of autoreactive T cells directly by contact or indirectly by the synthesis of immunosuppressive cytokines. $CD4^+$ Tregs are divided into natural occurring Tregs, which are produced in the thymus, and inducible Tregs, which develop from naïve $CD4^+$ T cells during immune responses [44]. In MS patients, loss of suppressive function of circulating $CD4^+$ Tregs is demonstrated [46-48]. Furthermore, RR-MS patients have an impaired migration of Tregs into the CNS [49]. In EAE, adoptive transfer of $CD4^+$ Tregs amiliorates disease [50, 51]. Besides the impaired supressive capacity, $CD4^+$ effector T cells of

active RR-MS patients are shown to be resistant to supression by Tregs, a proces in which IL-6 was found to be involved [52].

In accordance with $CD4^+$ Tregs, $CD8^+$ Tregs also display reduced supressive function in MS patients [53]. Moreover, the immunomodulatory therapy glatiramer acetate exerts it benificial effect at least partly via the induction of $CD8^+$ Tregs. Therefore, therapeutic stategies that enhance both $CD4^+$ and $CD8^+$ Tregs could be benificial for MS patients.

B cells

B cell depletion therapy in MS patients clearly revealed their importance in the disease pathogenesis [54]. B cells can contribute to the disease by the production of antibodies, as revealed by the presence of oligoclonal bands in the CSF of MS patients [55]. Moreover, the formation of tertiary lymphoid follicles containing B cells in the meninges of patients further supports their role in antibody production and antigen presentation in the CNS [56]. The presence of these follicles in SP-MS patients is associated with more severe pathology and earlier onset of disease [57]. In similarity with Tregs, regulatory B cells (Bregs) are identified in MS and found to be reduced in numbers [58]. In EAE, IL-10 producing Bregs suppress disease [59]. These studies point towards an essential role of B cells in the pathogenesis of MS.

Monocytes/macrophages/microglia

Perivascular cuffs of MS patients mainly consist of monocytes, which after extravasation differentiate into macrophages [60]. In lesions of MS patients, activated macrophages and microglia are abundantly present and strongly outnumber lymphocytes [60-62]. Eliminating infiltrating macrophages or preventing microglial activation represses EAE symptoms [63, 64], indicating their essential role in the disease pathogenesis. Upon activation macrophages/

microglia secrete proinflammatory cytokines, nitric oxide, reactive oxygen species and glutamate which induce tissue damage [65-68]. Moreover, their antigen presenting capacities provide a crucial role in modulating autoreactive T cells [62]. However, macrophages/microglia also exert beneficial effects, such as production of trophic factors and phagocytosis of myelin debris essential for axonal sprouting and remyelination [69, 70]. This dual role of macrophages/ microglia can be explained by the fact that different phenotypical subpopulations exist which are dependent on the inflammatory environment. The two originally identified subsets are classically activated (M1) macrophages, which have proinflammatory and cytotoxic properties and alternatively activated (M2) macrophages, which have anti-inflammatory and wound-healing functions [71, 72]. Additional subdivisions in M2 macrophages, namely M2a, M2b and M2c, were made based on different induction stimuli. In a toxin-induced demyelination model, a shift from M1 to M2 microglia and macrophages is found at the initiation of remyelination [73]. During remyelination M2 cells drive oligodendrocyte differentiation [73]. Thus, macrophages/microglia have both detrimental and beneficial effects in the MS pathogenesis, and promoting their beneficial functions may result in CNS regeneration in MS patients.

Dendritic cells, natural killer cells, neutrophils and mast cells

Besides the above mentioned immune cells, dendritic cells (DCs), natural killer (NK) cells, neutrophils and mast cells are also thought to be involved in the MS pathogenesis [74]. DCs are professional APCs which may contribute to activation and expansion of autoreactive T cells [75]. NK cells can be cytolytic for oligodendrocytes and neurons, but they also have immunoregulatory activity [76]. The role of neutrophils and mast cells in MS is not fully revealed, although most studies suggest a contributing role to inflammation and CNS damage [74, 77].

Astrocytes

Astrocytes are the most numerous cell type in the CNS [78, 79]. They have a wide variety of functions including providing trophic support, maintaining metabolic homeostasis, formation of the BBB and regulating synaptogenesis [78, 80]. The role of astrocytes in MS has long been underestimated [81]. They contribute to the inflammatory response and CNS degeneration by antigen presentation, production of proinflammatory cytokines and chemokines and formation of a glial scar [80, 81]. Nevertheless, astrocytes also play an essential role in CNS repair by the production of trophic factors, anti-inflammatory cytokines and chemokines that modulate axonal regeneration and oligodendrocyte differentiation and myelination [78, 82, 83].

Neurodegeneration

As stated above, it is proposed that MS is a primary neurodegenerative disorder. Pathological studies, especially in the initial stages of the disease, have shown that the earliest myelin abnormalities begin at the inner myelin sheaths, while outer myelin wraps are often intact [84]. Moreover, in cortical MS lesions substantial neurite injury is apparent with far less inflammatory infiltration compared to white matter lesions [85, 86].

Classically, oligodendrocyte death and demyelination are thought to be caused by proinflammatory cytokines, oxidative stress, glutamate excitotoxicity, autoantibody- and complement-mediated damage, and cytotoxic immune cells. Demyelination results in an increased axonal vulnerability to toxic mediators. Already in early stages of disease axonal swelling and transection, neuronal cell atrophy and neuronal cell death are detected [87-89]. Moreover, cumulative axonal loss is considered to be the cause of progressive and irreversible neurological disability in MS [90].

Remyelination does occur in MS lesions and gives rise to so-called “shadow” plaques [91]. The newly formed myelin sheaths are generally thinner and shorter than the original sheaths, but they are functional and protect axons from injury [92-95]. In a subset of individuals remyelination is extensive, however it often fails during the course of the disease [96-98]. Remyelination is mediated by oligodendrocyte precursor cells (OPCs) which are widespread throughout the adult CNS [99]. In response to mediators such as insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor a (PDGFa), OPCs proliferate and migrate to the lesion site, where they differentiate into oligodendrocytes and enwrap the naked axons with new myelin sheaths. An environment permissive for OPC differentiation is of high therapeutic value for MS patients and therefore great efforts are being made to understand which factors promote and inhibit remyelination [100].

1.1.4 Animal models

While no single animal model mimics all clinical features and pathological aspects of MS, several animal models are good representations of pathological features such as inflammation, demyelination and remyelination. A short overview is provided here.

Experimental autoimmune encephalomyelitis

EAE is the most extensively used animal model for MS, which has been developed in multiple species including mice, rats, rabbits, guinea pigs, marmosets and rhesus monkeys [101, 102]. EAE is induced by active immunization with whole CNS homogenate or myelin proteins such as myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) or myelin basic protein (MBP). Additionally, it can be induced by adoptive transfer of myelin-reactive T cells into naïve recipient animals [102]. The pathological and clinical features depend on the antigen used,

the mode of induction and the genetic background of the animal. In addition, spontaneous EAE models in transgenic mice are developed [103-105].

The translation of results gathered from EAE studies to MS is a topic of debate. Promising therapeutics in EAE failed in MS patients, for example TNF neutralizing agents and the anti-IL-12/23p40 monoclonal antibody ustekinumab [106, 107]. Nevertheless, the EAE model has helped to clarify immunopathogenic mechanisms and led to the successful development of immunotherapies such as glatiramer acetate, mitoxantrone, fingolimod and natalizumab [108].

Viral-induced models

Theiler's murine encephalomyelitis virus (TMEV) is the most commonly used viral-induced animal model of MS and results in a chronic progressive disease [109] [110]. TMEV induces a biphasic disease in susceptible mouse strains. One week after infection, meningoencephalomyelitis develops characterized by infection and apoptosis of neurons in the gray matter of the brain. Approximately one month after infection, glial cells and macrophages are infected which causes chronic demyelination and axonal degeneration, especially in the white matter of the spinal cord [109].

Toxin-induced demyelination models

To study demyelination and remyelination, lysolecithin and cuprizone are two widely used toxins [111]. Lysolecithin (lysophosphatidylcholine) is a membrane-dissolving agent which is particular harmful for myelin and results in oligodendrocyte death [112]. This substance is stereotactically injected into white matter tracts of the CNS, mostly into the dorsal and ventral funiculi of thoracic and lumbar spinal cord or in the corpus callosum [111, 113]. Hours after injection demyelination is apparent which lasts for approximately 7 days, followed by

remyelination [114]. Microglia and infiltrated macrophages are observed in the lesions with minimal T cell responses [111].

Cuprizone (bis-cyclohexanone-oxaldihydrazone) is a copper chelating agent, which induces degeneration of oligodendrocytes [115]. The pathological features of cuprizone-induced demyelinating lesions are similar to pattern III lesions in MS patients, including indistinct lesion borders and abundant accumulation of microglia, without a marked lymphocytic response [116, 117]. Mice are fed with 0.2% of cuprizone for 4-6 weeks or 12 weeks to induce acute or chronic demyelination respectively in specific brain regions such as the corpus callosum, cerebellar peduncles, internal capsule and anterior commissure. This demyelination is followed by spontaneous remyelination after omitting cuprizone from the diet [115]. Acute demyelination is followed by very rapid spontaneous remyelination in the corpus callosum. Chronic demyelination, in contrast, results in pronounced depletion of oligodendrocytes and their progenitors [118], leading to limited spontaneous remyelination. The underlying mechanism of cuprizone-induced oligodendrocyte death is poorly understood. Early findings suggested direct toxicity to oligodendrocytes, as copper deficiency leads to dysfunction of several mitochondrial enzymes such as cytochrome oxidase, monoamide oxidase and succinyl dehydrogenase, essential for the mitochondrial respiratory chain [119]. However, *in vitro* cuprizone treatment of primary oligodendrocytes causes metabolic stress, but does not induce cell death, unless inflammatory cytokines are added [120, 121]. Moreover, mice lacking proinflammatory mediators such as IFN-γ or neuronal NO synthase are relatively resistant to cuprizone-induced demyelination [122, 123]. These data suggest that inflammatory mediators produced by activated microglia and astrocytes are essential to induce oligodendrocyte depletion after cuprizone treatment. In conclusion, lysolecithin and cuprizone-induced demyelination are highly reproducible models, which

provide insights in to determinants of oligodendrocyte death and allow to study mechanisms of demyelination and remyelination.

1.1.5 Therapeutic strategies

To date, there is no cure for MS. However, during the last decade therapeutic strategies have radically changed. At this moment, ten disease modifying agents (DMAs) are approved by the Food and Drug Administration (FDA) for the treatment of MS.

First-line therapeutics

Twenty years ago the first immunomodulatory therapy, IFN-β (Avonex®, Betaseron®, Rebif®, Extavia®), was approved by the FDA, followed by glatiramer acetate (Copaxone®) a few years later. The mechanism by which both therapies work is not fully understood, but several modes of actions have been proposed. IFN-β reduces cell-trafficking across the BBB, stimulates the production of trophic factors such as nerve growth factor (NGF) and anti-inflammatory cytokines, and reduces proinflammatory cytokine production [124, 125]. Glatiramer acetate stimulates polarization of Th2 cells, Tregs and M2 macrophages, and increases secretion of neurotropic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) [125, 126]. Both DMAs reduce disease activity by approximately 30% in RR-MS and 40% in CIS, however there is a strong variation between patients [127]. Approximately one third of the patients develops neutralizing antibodies against IFN-β which reduce efficacy [125].

Second-line therapeutics

When first-line therapies fail or in case of aggressive disease, there are several second-line therapeutics. Natalizumab (Tysabri®) is a humanized monoclonal antibody against the α-4 subunit of very late antigen, an adhesion molecule that

enables activated leukocytes to cross the BBB. This therapy has an impressive effect on the relapse rate and disease progression. However, the risk of developing progressive multifocal leukoencephalopathy, due to reactivation of a latent infection with JC virus, is a major disadvantage [128]. Therefore, it is only used in highly active RR-MS patients not responding to first-line therapies [129].

Fingolimod (FTY720, Gilenya®) was the first available oral drug, which binds to spingosine-1-phosphate receptor leading to internalization of the receptor [26, 130]. As a result, lymphocytes can no longer egress from the secondary lymphoid organs. Moreover, in organotypic cerebellar slice cultures fingolimod enhances remyelination [131, 132]. Reported adverse effects of fingolimod are cardiovascular events (bradycardia and atrioventricular block), macular edema and increased risk of mild infections such as bronchitis and herpesvirus [26, 130].

Mitoxantrone (Novantrone®) is a cytotoxic drug that causes DNA strand breaks by intercalation and interferes with DNA repair by inhibiting topoisomerase II. Thereby, it diminishes lymphocyte numbers and reduces relapses and disease progression [133, 134]. Severe adverse effects of mitoxantrone treatment are cardiotoxicity and leukaemia. Mitoxatrone is the only FDA approved treatment for SP-MS and PP-MS [133].

Teriflunomide (Aubagio®) is an inhibitor of dihydroorotate dehydrogenase, an essential enzyme involved in pyrimidine synthesis [135, 136]. De novo pyrimidine synthesis is required for highly proliferating cells including activated lymphocytes. Therefore, teriflunomide treatment results in reduced proliferation of T and B cells [135]. Teriflunomide can cause renal failure, peripheral neuropathy, and should not be taken during pregnancy due to birth defects [136].

Dimethyl fumarate or BG12 (Tecfidera®) is thought to protect CNS cells against oxidative stress via activation the nuclear (erythroid-derived 2) relate factor pathway [137, 138]. In addition, immunomodulatory properties such as shifting DC differentiation and reducing proinflammatory cytokine production have also

been reported [137, 139]. Main adverse effects of dimethyl fumarate are gastrointestinal tract irritations and flushing [137].

Upcoming therapeutics

Multiple upcoming drugs entered or completed phase II and III clinical trials, including laquinimod, daclizumab, alemtuzumab and rituximab. Laquinimod modulates the function of several APCs, thereby downmodulating proinflammatory T cell responses. Moreover, experimental data indicate that laquinimod directly affects CNS cells to reduce demyelination and axonal damage [140]. Daclizumab is humanized monoclonal antibody which targets the IL-2Rα (CD25) and is designed to inhibit T cell activation. In addition, several other immunomodulating effects are revealed, such as the expansion of immunoregulatory NK cells and modulation of antigen presentation by DCs [141]. Alemtuzumab is humanized monoclonal antibody that binds CD52, thereby depleting lymphocytes. Development of secondary autoimmunity represents the major side effect of this therapy [142]. Finally, rituximab is a chimeric monoclonal antibody directed against CD20, which results in depletion of B cells [143].

As mentioned above mitoxatrone is the only FDA-approved therapy for SP-MS and PP-MS. All approved therapies modulate the inflammatory response, thereby limiting the initial relapsing-remitting phase. They delay the time to disabling stages, but they fail to prevent permanent neurological disability. Therefore, there is a high need for novel therapies that not only modulate the immune response, but are neuroprotective and even promote CNS regeneration. Members of the glycoprotein 130 (gp130) cytokine family, including leukemia inhibitory factor (LIF) and oncostatin M (OSM), have the potential to exert these beneficial effects.

1.2 Gp130 cytokine family

The gp130 cytokine family includes IL-6, IL-11, LIF, OSM, ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC) and neuropoietin (NP) [144, 145]. All members of this family share the common signal transducer receptor protein gp130 (Fig. 1.2). This receptor subunit combines with another signal transducing β-receptor (LIFRβ, OSMRβ) or with a non-signaling α-receptor (IL-6Rα, IL-11Rα, CNTFRα). IL-6, IL-11, CNTF and CLC first bind to their respective α-receptor subunit, which then recruits the signal transducing β-receptor subunits. LIF and OSM directly bind the signaling receptors, LIFRβ or OSMRβ, without the need of an α-receptor subunit. Only IL-6 and IL-11 signal via gp130 homodimers, while other members signal via heterodimers of gp130-LIFRβ (LIF, CNTF, CT-1, CLC, OSM) or gp130-OSMRβ (OSM) [144, 145]. Mouse OSM can only signal via the gp130-OSMRβ complex, while human and rat OSM can also activate the gp130-LIFRβ complex [146, 147].

Expression of gp130 is widespread, whereas the expression of the other subunits is limited and tightly regulated, restricting the number of cells that respond to this cytokine family [144]. However, soluble forms of the α-receptors, such as IL-6Rα and IL-11Rα, do exist. As a result, cells which do not express these α-receptors can respond to IL-6 or IL-11, after binding of the soluble receptor-ligand complex to membrane-bound gp130 [148, 149].

Binding of the cytokines to the receptor subunits results in phosphorylation and activation of multiple Janus kinases (JAKs), including JAK1, JAK2 and Tyk2. This in turn leads to recruitment and activation of STATs, including STAT1, STAT3 and STAT5. The recruited STATs dimerize and translocate to the nucleus where they regulate gene expression [145, 150]. Suppressor of cytokine signaling 1 (SOCS1) and SOCS3, which can be induced by several members of the gp130 cytokine family, are crucial negative regulators of the JAK/STAT pathway [151]. Additionally

to the JAK/STAT pathway, gp130 signaling leads to recruitment of protein tyrosine phosphatase SHP2, which provides a docking site for the adaptor protein GrB2. This induces recruitment of Raf by activated Ras resulting in induction of the mitogen-activated protein kinase (MAPK) cascade. A last pathway that can be activated by the gp130 cytokine family is the phosphatidylinositide 3-kinase (PI3K) cascade which leads to Akt phosphorylation [144, 145, 150].

The intracellular signaling of the family members seems largely overlapping, however these cytokines have diverse and contrasting effects. These different biological responses may be the result of temporal and spatial receptor activation, higher affinity binding or activation of negative regulators.

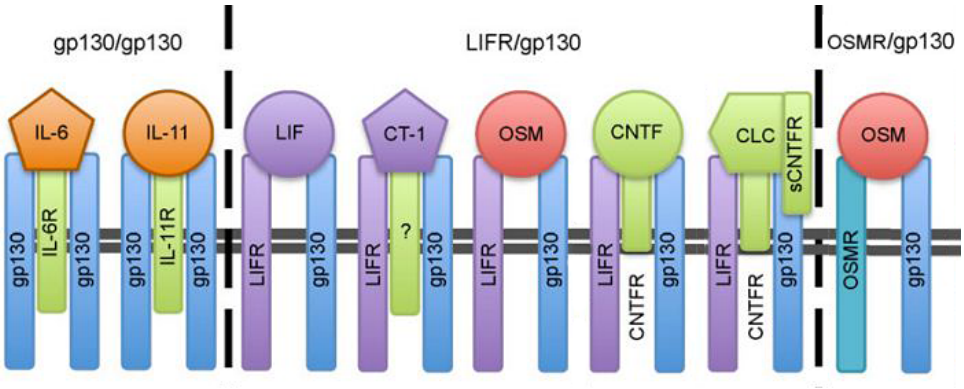

Fig. 1.2. Receptor complexes of the gp130 cytokine family. IL-6 and IL-11 first bind their α-receptor which then recruits a homodimer of gp130. LIF, CT-1, OSM, CNTF and CLC signal through a heterodimer of LIFRβ-gp130. OSM can also signal through its specific receptor, consisting of OSMRβ-gp130. IL, interleukin; LIF, leukemia inhibitory factor; CT-1, cardiotrophin-1; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; CLC, cardiotrophin-like cytokine; gp130; glycoprotein 130. Reprinted with permission from Elsevier [152].

1.2.1 Leukemia inhibitory factor

LIF derives its name from the ability to inhibit differentiation of M1 leukemic myeloid cells into macrophages [153]. A wide range of functions are described for LIF. In early embryonic life, LIF is essential for blastocyst implantation and embryonic stem cell renewal [154-156]. LIF is also involved in tissue repair and heart and skeletal muscle regeneration [153, 156]. The neuroprotective and immunomodulating properties of LIF are discussed in detail in the following paragraphs.

Effects of LIF on cells of the nervous system

LIF production is increased after different types of damage to the nervous system, including spinal cord injury, brain trauma, Alzheimer's and Parkinson's disease, seizures and MS [157-160]. In MS patients, LIF is elevated in the serum, CSF and CNS lesions [161-163]. Upregulation of LIF seems to be an endogenous response to protect against damage.

Both in the peripheral nervous system (PNS) and CNS, LIF promotes survival of neurons. LIF treatment after transection of either sensory or motor neurons significantly reduces neuronal loss [164, 165]. Moreover, in a murine model of amyotrophic lateral sclerosis LIF rescues motor neurons [166, 167]. Besides promoting neuronal survival, LIF enhances regeneration of injured sensory neurons [168, 169]. Similar protective effects are demonstrated on CNS cells as LIF markedly stimulates neurite outgrowth of mature retinal ganglion cells *in vitro* [170]. After spinal cord injury LIF enhances regeneration and promotes recovery of locomotor function [171, 172]. Moreover, intracerebral injection of LIF attenuates neurological deficits after ischemic brain injury in rats [173]. In EAE, endogenous LIF reduces axonal loss and improves neurological outcome [174].

Besides direct neuroprotection, LIF also limits oligodendrocyte apoptosis and demyelination, thereby providing additional protection to axons and neurons. Our

group and others revealed that LIF protects mature rat oligodendrocytes *in vitro* against apoptosis induced by IFN-γ and TNF-α [162, 175, 176]. This anti-apoptotic response is induced by Akt-phosphorylation and enhances expression of the prosurvival molecule 14-3-3 [176]. *In vivo* LIF administration after spinal cord injury limits oligodendrocyte apoptosis and thereby demyelination [177, 178]. While a direct protective effect is found in the study of Azari and colleagues, Kerr and Patterson proposed that protection is mediated via an indirect effect on an ancillary cell type leading to augmented production of IGF-1, a prosurvival factor for oligodendrocytes. In EAE, several reports demonstrate a protective effect of LIF on oligodendrocytes. Systemic treatment abrogates oligodendrocyte apoptosis thereby preventing demyelination and reducing clinical severity [179]. This is in agreement with a study in our group which revealed that CNS-targeted expression of LIF ameliorates autoimmune-mediated demyelination [180]. Moreover, exogenous LIF administered during cuprizone-induced demyelination limits myelin loss [181]. The role of endogenous LIF is defined using neutralizing anti-LIF antibodies and LIF knock-out mice. Administration of the neutralizing antibodies after clinical onset of EAE augments the extent of demyelination and oligodendrocyte loss, thereby worsening EAE severity [182]. Furthermore, LIF knock-out mice exhibit exacerbated cuprizone-induced demyelination [181]. Thus, these studies demonstrate that LIF treatment as well as endogenous LIF protect against oligodendrocyte apoptosis and demyelination.

There is also evidence that LIF is involved in the remyelination process. Remyelination, which is mediated by OPCs, is crucial to restore axonal conduction and has neuroprotective properties. In cultures derived from rat cerebra, LIF enhances differentiation to immature oligodendrocytes [183]. Moreover, LIF has pro-myelinating effects on mixed cultures derived for embryonic mouse cerebra. In cultures of OPCs, astrocytes and dorsal root ganglia (DRG) neurons, LIF released by astrocytes in response to ATP promotes myelination [184]. In addition, TNF

receptor 2 activation of astrocytes leads to LIF secretion and promotes oligodendrocyte maturation in a co-culture model [185]. The myelin-promoting effects of LIF are also seen during development; in LIFR knock-out mice pronounced myelination defects are detected in the optic nerve at postnatal day 10 [186]. In adult mice, CNS-targeted LIF administration promotes proliferation of the OPC pool [187]. Moreover, LIF restores oligodendrocytes numbers and enhances hippocampal remyelination after cuprizone-induced demyelination [187]. In LIF deficient mice remyelination is impaired after cuprizone challenge [181]. Together, these data indicate that LIF plays a crucial role is oligodendrocyte survival, demyelination and remyelination.

Effects of LIF on immune cells

Besides these promising neuroprotective and repair promoting effects in preclinical models of MS, LIF may also directly modulate the autoimmune response. Both pro- and anti-inflammatory effects of LIF have been described. Denervated schwann cells attract macrophages by secreting LIF *in vitro* [188]. After injury to the sciatic nerve or cerebral cortex, LIF is involved in the activation and recruitment of macrophages [189]. Moreover, LIF overexpression in the spinal cord of mice results in high microglia/macrophage proliferation and even impairment of hind limb motor function [190]. However, in this model LIF overexpression probably reached toxic levels thereby causing motor dysfunction. Recruitment and activation of macrophages may not be solely detrimental, as they can produce trophic factors and clear myelin debris essential for remyelination. Our group revealed that LIF stimulates myelin uptake by macrophages *in vitro*, and reduces production of proinflammatory mediators, such as oxygen radicals and TNF-α [191]. Moreover, LIF promotes the generation of tumor-associated macrophages, which resemble M2 macrophages as they

produce high levels of IL-10 and TGF-β, low levels of IL-12, and have poor T-cell co-stimulatory properties [192].

Recent studies suggest that LIF could work opposing to its family member IL-6, which stimulates the development of Th17 cells and inhibits Treg generation [33, 193-197]. LIF suppresses IL-6 induced IL-17A protein release [193]. Moreover, it inhibits differentiation of Th17 cells in EAE and in human $CD4^+$ T cells [198]. This inhibition of differentiation results from an upregulation of ERK and SOCS3, leading to reduced STAT3 phosphorylation [198]. In studies on graft rejection, LIF is associated with transplantation tolerance and supports FOXP3 expression *in vitro* [193, 199]. Furthermore, in a non-human primate model LIF-coated nanoparticles induce expansion of $FOXP3^+$ T cells *in vitro* [193, 200]. These data are highly promising for the therapeutic potential of LIF in MS as Th17 and Treg responses play an essential role in the MS pathogenesis. Thus, additional murine *in vivo* and human *in vitro* studies are crucial to reveal whether LIF prevents Th17 induction and supports Treg functions in MS.

1.2.2 Oncostatin M

OSM is closely related to LIF, with a sequence identity of 27% [201]. The genes encoding for both proteins are located in close proximity on chromosome 22, suggesting that they are the result of gene duplication [202]. Similar biological effects can be explained by their shared receptor, namely LIFRβ-gp130. Human OSM can also signal via its specific receptor consisting of OSMRβ-gp130. In contrast, mouse OSM can only signal via OSMRβ-gp130, making it an excellent model to study OSMR signaling [146].

OSM was first purified from conditioned media of U937 monocytic cells and named based on the anti-proliferative effects on melanoma cell lines [203]. Subsequent studies have revealed a role for OSM in liver and heart regeneration, cancer, bone metabolism and cartilage remodeling [204-212]. Although,

compared to LIF, far less research has focused on the neuroprotective and immunomodulatory properties of OSM, the studies addressing these issues are discussed in detail in the following paragraphs.

Effects of OSM on cells of the nervous system

Comparable to LIF, OSM is upregulated in response to different types of CNS damage, such as nerve injury, epileptic seizures, spinal cord injury and MS [213-216]. In MS lesions, immunoreactivity is most prominent on microglia and hypertrophic astrocytes, and to a lesser extent on infiltrating leukocytes [215]. Similar to LIF this upregulation can be an endogenous response to protect against CNS damage, as OSM limits neuronal cell death caused by NMDA-mediated excitotoxicity *in vitro* and *in vivo* [217]. OSM, in contrast to LIF, protects neurons against excitotoxicity via neuronal adenosine A_1 receptor [218]. In a transgenic rat model of retinal degeneration, OSM treatment protects both rod and cone photoreceptors, additionally to promoting regeneration of cone outer segments in degenerating cones [219]. Moreover, we recently demonstrated that after spinal cord injury, OSM reduces lesion size and promotes neurite outgrowth and functional recovery [216]. These studies reveal neuroprotective and even regeneration-promoting properties of OSM.

In contrast to LIF, little is known about the effect of OSM on oligodendrocytes. A role is suggested in the ethidium bromide-induced demyelination model, where OSM modulates the expression of genes involved in OPC mobilization, oligodendrocyte differentiation and myelin production [220]. Further research is needed to determine the role of OSM in oligodendrocyte survival and differentiation and thereby on de- and remyelination.

Effects of OSM on immune cells

Depending on the microenvironment, OSM can have both pro- and anti-inflammatory effects. Proinflammatory actions are demonstrated in cultures of DCs, in which OSM stimulates their maturation and the production of the Th1-promoting cytokine IL-12 [221]. Moreover, OSM-treated DCs induce proliferation of T cells and enhance their IFN-γ production [221]. In two mouse models of arthritis, neutralizing OSM antibodies reduce cellular infiltration of the synovium, cartilage damage and clinical severity [222]. Adenovirus-mediated expression of OSM in the knee joint results in increased mononuclear cell infiltration, extracellular matrix deposition and synovial cell proliferation [223]. Moreover, pulmonary overexpression of OSM using adenovirus vectors causes marked eosinophil infiltration and extracellular matrix accumulation in the lungs [224].

Furthermore, the OSMR is highly expressed on endothelial cells [225]. These cells are crucial regulators of inflammation, as they line blood vessels and regulate immune cell infiltration after injury or infection. *In vitro* OSM treatment of human endothelial cells induces increased expression of chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2 and CXCL5 [226]. Furthermore, OSM augments chemokine (C-C motif) ligand 21 (CCL21) expression in endothelial cells lining the draining lymph nodes, thereby increasing DC trafficking to these lymph nodes [227]. OSM also upregulates expression of intracellular adhesion molecule-1 (ICAM-1), E-selectin, monocyte chemoattractant protein-1 (MCP-1) and IL-6 on human endothelial cells [215, 225, 226]. In cultures of rat brain capillary endothelial cells OSM increases permeability through structural disorganization of tight junctions [228]. Moreover, OSM stimulates neutrophil transmigration through primary human endothelial monolayers [226]. Our group demonstrated that local expression of OSM by means of lentiviral vectors (LVs) in the brain of healthy mice alters BBB permeability and induces tissue remodeling as revealed by marked vasodilatation, deposition of extracellular matrix proteins, reduced expression of the tight

junction protein ZO-1 and upregulation of matrix metalloproteinase 2 (MMP2). The increased BBB permeability is associated with a strong infiltration of lymphocytes and macrophages at the site of OSM expression (Slaets, in preparation). However, when EAE is induced in these mice, disease development is prevented. This results from an anti-inflammatory and repair-promoting M2 phenotype induced by OSM in macrophages, which redirects infiltrating Th1/Th17 cells. The induction of a M2 phenotype by OSM is confirmed in OSMR$\beta^{-/-}$ mice, in which adipose tissue macrophages are polarized to a M1 phenotype which augments adipose tissue inflammation [229]. Treatment of mice with OSM, in contrast, polarizes these macrophages to a M2 phenotype and increases insulin sensitivity [229]. Furthermore, hypoxic breast cancer cells release OSM, thereby recruiting macrophages and promoting their M2 polarization [230]. Taken together, these studies reveal effects of OSM on endothelial cells and macrophages. Nonetheless additional studies are necessary to reveal its effect on the pathological mechanisms in MS and on the different immune cells involved.

1.3 Aims of the study

Despite significant progress in the development of therapies for MS, they only moderate the initial relapsing-remitting phase. For patients in the progressive phase of the disease there is an urgent need for therapies that not only modulate the immune response, but are also neuroprotective and promote CNS regeneration. LIF and OSM, two members of the gp130 cytokine family, have the potential to beneficially affect the immune response as well as CNS cells. These cytokines can exert similar effects as they both signal through the LIFR. OSM also signals through the specific OSMR and can thereby exert additional effects. LIF and OSM are produced in lesions of MS patients and are both shown to be neuroprotective. Moreover, LIF promotes oligodendrocyte survival and remyelination. Their effects on immune cell subtypes involved in the pathogenesis of MS remains unclear. Therefore, we determine in this study the effect of LIF and OSM on the autoimmune response. Moreover, we elucidate the role of OSMR signaling during de- and remyelination. This study provides novel insights into the role of LIF and OSM in CNS pathology, additionally to defining their therapeutic potential for MS and other neuroinflammatory diseases.

AIM 1: Determine the immunomodulatory role of LIF and OSM during neuro-inflammation

Over the last years, studies addressing the immunomodulatory properties of LIF and OSM have generated contradicting results. Therefore, the effect of LIF on the immune response is investigated in **chapter 2**, while the role of OSM is determined in **chapter 3**. We first define how these cytokines can modulate the autoimmune response by determining which immune cells express the receptors in MS patients and comparing this to healthy controls. Moreover, in chapter 2 we focus on the effect of LIF on the differentiation towards the different T helper

subtypes. Recent studies in mice suggest an inhibitory effect on Th17 differentiation, while supporting Treg generation. As it is unclear whether LIF has similar effects in humans, this is studied using circulating immune cells of healthy subjects as well as MS patients. Finally, the effects revealed *in vitro* are confirmed by therapeutic application using LV-mediated expression in EAE, a preclinical animal model of MS.

The effect of OSM on the autoimmune response is largely unknown. Besides defining the receptor expression on immune cells in chapter 3, we elucidate the effect of OSM on the polarization of human macrophages towards a M1 or M2 phenotype and on the cytotoxicity of $CD8^+$ T cells. Moreover, we determine in EAE whether therapeutic OSM treatment, when immune cells already infiltrated the CNS, can still affect disease.

AIM 2: Define the role of OSMR signaling in de- and remyelination

Multiple studies demonstrated protective effects of LIF on oligodendrocyte survival and demyelination [176, 179-182]. Moreover, recently it was reported that LIF enhances OPC proliferation and remyelination [187]. By activating the LIFR, OSM may exert similar beneficial effects. However, the role of OSMR signaling in these processes is unknown. In **chapter 4** we investigate the effect of OSMR signaling on demyelination and in **chapter 5** its role during remyelination is unraveled. The cuprizone mouse model is used, in which there is selective oligodendrocyte depletion and subsequent demyelination accompanied by OPC proliferation, microglial activation and astrogliosis. Remyelination is initiated by omitting cuprizone from the diet. We define whether the OSMR is upregulated during de- and remyelination, after which its impact on these processes is revealed using OSMR knock-out mice. Additionally, the therapeutic potential of OSM during de- and remyelination is demonstrated using LV-mediated overexpression in the CNS.

2

LIF tips the immune balance towards regulatory T cells in multiple sclerosis

Based on:

Leukemia inhibitory factor tips the immune balance towards regulatory T cells in multiple sclerosis

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Abstract

MS is an inflammatory demyelinating disease of the CNS, for which current treatments are unable to prevent disease progression. Based on its neuroprotective and neuroregenerating properties, LIF, a member of the IL-6 cytokine family, is proposed as a novel candidate for MS therapy. However, its effect on the autoimmune response remains unclear. In this study, we determined how LIF modulates T cell responses that play a crucial role in the pathogenesis of MS. We demonstrate that expression of the LIFR was strongly increased on immune cells of MS patients. LIF treatment potently boosted the number of Tregs in $CD4^+$ T cells isolated from healthy controls and MS patients with low serum levels of IL-6. Moreover, IL-6 signaling was reduced in the donors that responded to LIF treatment *in vitro.* Our data together with previous findings revealing that IL-6 inhibits Treg development, suggest an opposing function of LIF and IL-6. In a preclinical animal model of MS we shifted the LIF/IL-6 balance in favor of LIF by CNS-targeted overexpression. This increased the number of Tregs in the CNS during active autoimmune responses and reduced disease symptoms. In conclusion, our data show that LIF downregulates the autoimmune response by enhancing Treg numbers, providing further impetus for the use of LIF as a novel treatment for MS and other autoimmune diseases.

2.1 Introduction

MS is a chronic disabling disease of the CNS, characterized by multifocal inflammatory infiltrates, demyelination, axonal loss and neurodegeneration. Autoreactive Th1 and Th17 cells are thought to be the main effector cells in MS, whereas Tregs function to maintain self-tolerance and prevent autoimmunity [74]. Loss of suppressive function of circulating $CD4^+$ Tregs is demonstrated in MS patients [46-48]. As a result of the regulatory defects, Th1 and Th17 cells are hyperactive and cross the blood-brain barrier to initiate an inflammatory response leading to demyelinated lesions. Available therapies suppress the immune response, however they mainly moderate the initial relapsing-remitting phase and are unable stop disease progression [231]. Therefore, there is a high need for novel therapies that modulate the immune response and provide neuroprotection or even promote neural regeneration. LIF, a member of the IL-6 cytokine family, has been proposed as a promising candidate for MS therapy [194, 232, 233], as it promotes survival of neurons and oligodendrocytes, and stimulates neurite outgrowth [162, 170, 176]. In an animal model of MS, EAE, LIF treatment ameliorates clinical symptoms by preventing demyelination [179, 180] and by limiting axonal damage and loss [174]. Furthermore, in a toxin-induced demyelination model LIF stimulates proliferation of OPCs and enhances hippocampal remyelination [187].

Besides these promising neuroprotective and repair promoting effects in preclinical models of MS, LIF may also directly modulate the autoimmune response thereby providing dual disease ameliorating mechanisms. LIF induces an anti-inflammatory phenotype in macrophages [191, 192]. Moreover, in studies on graft rejection LIF was associated with transplantation tolerance, while its family member IL-6 was associated with allo-rejection [193]. In contrast to LIF, IL-6 is a potent proinflammatory cytokine driving Th17 differentiation [33, 34, 196].

The immunomodulatory properties of LIF remain largely unknown and are a hurdle for its application in the clinic. In this study, the therapeutic potential of LIF is further revealed by studying its effect on the T cell compartment. We demonstrate that in MS patients the LIFR was strongly increased on circulating T cells. Moreover, while LIF did not promote Th1, Th2 and Th17 differentiation *in vitro*, it enhanced the number of Tregs in donors with low serum levels of IL-6. Therapeutic application in EAE doubled the number of Tregs in the CNS and ameliorated clinical symptoms. Our combined human and *in vivo* mouse data reveal that LIF downregulates the autoimmune response and provide novel treatment strategies for MS patients.

2.2 Materials and methods

2.2.1 Study subjects

For measuring LIFR expression, blood samples were collected from 22 healthy controls, 41 untreated and 43 treated patients with clinically definite MS (Table 2.1). Patients received treatment with IFN-β (Avonex®, Rebif®, Betaferon®), glatiramer acetate (Copaxone®) or Natalizumab (Tysabri®). Healthy controls, untreated and treated MS patients were age and gender matched (Table 2.1). For additional *in vitro* assays, blood samples of 15 healthy controls and 12 untreated MS patients were used. Blood samples were collected in collaboration with the University Biobank Limburg (UBiLim). This study was approved by the Medical Ethical Committee of the University Hospital K.U.Leuven and informed consent was obtained from all study subjects. For immunohistochemistry, frozen brain tissue from 4 chronic active MS patients was obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands).

Table 2.1. Study subjects used for analysis of the LIFR on circulating immune cells

	Treated MS patients	**Untreated MS patients**	**Healthy controls**
Number	n = 43	n = 41	n = 22
Age (years)	45.09	46.63	39.59
Male/Female ratio	15/28 (0.54)	13/28 (0.46)	9/13 (0.69)
Disease duration (years)	12.00	12.37	NA
EDSS	3.35	3.87	NA
MS type			
- RR	31	23	NA
- CP	9	15	NA
Treatment			
- IFN-β	24	NA	NA
- Glatiramer acetate	8	NA	NA
- Natalizumab	11	NA	NA

MS, multiple sclerosis; EDSS, expanded disability status scale; RR, relapsing remitting; CP, chronic progressive; IFN-β; interferon beta; NA, not applicable.

2.2.2 EAE induction

Female 10 week old C57BL/6J mice (Harlan, Horst, the Netherlands) were immunized subcutaneously with myelin oligodendrocyte glycoprotein 35-55 peptide (MOG_{35-55}) emulsified in complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis according to manufacturer's guidelines (Hooke Laboratories, Lawrence, USA). Directly after immunization and 24h later, mice were intraperitoneally injected with pertussis toxin. Mice were daily weighted and evaluated for neurological signs of disease using a standard 5-point scale; 0: no symptoms; 1: limp tail; 2: hind limp weakness; 3: complete hind limp paralysis; 4: complete hind limp paralysis and partial front leg paralysis; 5: death. All animal procedures were in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by the Hasselt University ethics committee.

2.2.3 Lentiviral vector injection

LV encoding LIF (LV-LIF) and LV encoding enhanced green fluorescent protein (eGFP) were constructed as described in [180]. When mice showed EAE symptoms for 3 to 4 days they were stereotactically injected with LV-LIF or LV-eGFP in the right lateral ventricle. Mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg), after which they were placed in a stereotactic head frame (Stoelting, IL, USA), a midline incision of the skin was made and a small hole was drilled in the skull. At a rate of 0.25 µl/min 4 µl of concentrated vector ($4.8*10^7$ pg p24/ml) was injected using a 10 µl Hamilton syringe with a 30-gauge needle. Coordinates for injection into the lateral ventricle were anteroposterior 0.2 mm, lateral 1.0 mm and dorsoventral -1.8 mm using bregma as a reference.

2.2.4 Cell culture and T helper cell differentiation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation (Cedarlane lympholyte, Sheffield, UK).

PBMCs were cultured in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Hyclone Europe, Erembodegem, Belgium), 1% nonessential amino acids, 1% sodium pyruvate, 50 U/ml penicillin and 50 mg/ml streptomycin (all Life technologies, Merelbeke, Belgium). To measure LIFR expression after activation and IFN-β treatment, cells were stimulated with 2 μg/ml anti-CD3 (clone 2G3, BIOMED, Diepenbeek, Belgium), 2 μg/ml CpG2006 (ODN2006, InvivoGen, Toulouse, France) and 1000 U/ml recombinant human IFN-β (R&D systems, Abingdon, UK).

For differentiation toward Th1, Th2 and Th17 cells, $CD4^+$ T cells were isolated from PBMCs by immunomagnetic isolation (Easysep, Stemcell technologies, Grenoble, France). $CD4^+$ T cells were activated with anti-CD3/CD28 beads (Life technologies). Cytokines and neutralizing antibodies to induce differentiation were added: for Th1 differentiation 10 ng/ml IL-12 and 5 μg/ml anti-IL-4 antibody, for Th2 differentiation 200 ng/ml IL-4, 5 μg/ml anti-IFN-γ antibody and 5 μg/ml anti-IL-12 antibody (Affymetrix, Vienna, Austria) and for Th17 differentiation 25 ng/ml IL-23, 5 μg/ml anti-IL-4 and 5 μg/ml anti-IFN-γ antibody (all R&D systems). 25 ng/ml LIF (Millipore, Overijse, Belgium) was added alone or in combination with the differentiation-inducing cytokines and neutralizing antibodies.

To determine the effect of LIF on Tregs, memory $CD4^+$ T cells ($CD45RO^+$) were isolated from PBMCs using magnetic bead labeling (Miltenyi Biotec, Leiden, The Netherlands). Cells were treated with 25 or 250 ng/ml LIF (Millipore) for 3 days.

2.2.5 Flow cytometry

To determine LIFR expression, isolated PBMCs were incubated with anti-gp130-FITC (Abcam, Cambridge, UK) and anti-LIFRβ-PE antibodies (R&D systems), combined with PerCP labeled antibodies specific for the immune cell subsets, CD3, CD4, CD8, CD14 and CD19 (BD Biosciences, Erembodegem, Belgium). For FOXP3 staining memory $CD4^+$ T cells were surface stained with anti-CD25-FITC (BD

Biosciences), followed by fixation and permeabilization using human FOXP3 buffer set (BD Bioscience) and intracellular staining using anti-FOXP3-PE (BD Biosciences). Individuals were classified as responders when a minimal increase in the percentage of $FOXP3^{+}CD25^{high}$ Tregs of 1.2 was measured. Samples were run on FACSCalibur and analyzed using CellQuest Software (BD Biosciences). To determine the extent of phosphorylated STAT3 (pSTAT3) after IL-6 treatment, isolated memory $CD4^{+}$ T cells were allowed to rest for 2h, followed by incubation with 25 ng/ml IL-6 (BD Biosciences) for 15 min. Cells were fixated and permeabilized using Cytofix™ fixation buffer and Phosflow™ Perm Buffer III respectively, and intracellular staining was performed with anti-pSTAT3-Alexa Fluor 647 (BD Biosciences). Fluorescence intensity was measured using FACSAriaII and analyzed using FACSDiva software 6.1.3 (BD Biosciences).

Mononuclear cells were isolated from the spinal cord of EAE mice 7 days after stereotactic injection of LV-LIF or LV-eGFP. Briefly, animals were transcardially perfused with Ringer's solution. Spinal cords were dissected and incubated in RPMI containing 175 U/ml collagenase (Sigma-Aldrich, Bornem, Belgium) for 1h at 37°C, followed by 30% percoll density centrifugation (GE Healthcare). Single cell suspensions were obtained by passing the samples through a 70 µm filter. To determine the number of Tregs, cells were surface stained using anti-CD4-PerCP (BD biosciences) and anti-CD25-Alexa Fluor 488 antibodies (Affymetrix), followed by fixation and permeabilization using Mouse FOXP3 buffer set (BD Bioscience) and intracellular staining with anti-FOXP3-PE (BD Biosciences). To measure IFN-γ, IL-17 and IL-4 cells were stimulated for 4h with 25 ng/ml phorbol-12-myristate-13-acetate (PMA), 1 µg/ml calcium ionomycin (both Sigma-Aldrich) and Golgiplug (BD Biosciences). Surface staining was performed with anti-CD4-PerCP (BD Biosciences), followed by fixation and permeabilization using the Cytofix/Cytoperm™ Solution Kit (BD Biosciences) and intracellular staining with anti-IFN-γ-APC, IL-17-PE and IL-4-Alexa Fluor 488 (all Affymetrix). FACSAriaII was

used to measure fluorescence intensity and FACSDiva software 6.1.3 for analysis (BD Biosciences).

2.2.6 Immunohistochemistry

Ten micrometer cryosections of 4 MS patients were cut on the Leica CM3050S cryostat (Leica Microsystems, Wetzlar, Germany). For 3,3' diaminobenzidine (DAB) staining, cryosections were fixed in acetone, blocked with 10% goat serum and incubated with human LIFRβ antibody (Santa Cruz biotechnology, Dallas, Texas). Binding of the antibody was visualized using a goat anti-rabbit HRP-labeled antibody, followed by DAB substrate (both Dako, Heverlee, Belgium). For fluorescent staining sections were fixated, blocked and incubated with antibodies against human LIFRβ, IBA-1 (Wako, Neuss, Germany) and CD4 (AbD serotec, Düsseldorf, Germany). Binding of these primary antibodies was visualized with Alexa 488 or 555-conjugated secondary antibody (Life technologies) and nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI, Life technologies). Autofluorescence was blocked using 0,3% Sudan Black in 70% ethanol.

Mice were transcardially perfused with Ringer's solution 20 days after LV injection, spinal cords dissected and snap frozen in liquid nitrogen. Ten micrometer sections were made using the Leica CM3050S cryostat. Sections were fixed in acetone, blocked with Protein Block Dakocytomation (Dako) and incubated with anti-mouse CD3 and F4/80 antibodies (AbD serotec). Immunoreactivity was visualized using Alexa 555 secondary antibodies and nuclear staining was performed with DAPI (both Life technologies). Microscopic analysis was performed using an Eclipse 80i microscope (Nikon, Amstelveen, the Netherlands) and for image collection the Nis-Elements Basic Research version 2.3 microscopy software was used.

2.2.7 Quantitative PCR

RNA was prepared from the Th1, Th2 and Th17 cultures using the High Pure RNA Isolation Kit (Roche, Almere, The Netherlands) according to manufacturer's instructions. Conversion of RNA to cDNA was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Quantitative PCR was conducted on a StepOnePlus Real-Time PCR detection system (Applied Biosystems, Gaasbeek, Belgium) using universal cycling conditions (20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C). The PCR reaction consisted of fast SYBR green master mix (Applied Biosystems), 10µM of forward and reverse primers (Eurogentec, Seraing, Belgium), RNase free water and 12.5 ng template cDNA. Primers used are shown in Table 2.2. The expression was normalized using the two most stable reference genes and converted to fold change as compared to cells only incubated in culture medium using comparative Ct method.

To isolate RNA from the spinal cord of healthy and EAE mice the RNease Lipid Tissue Mini Kit (Qiagen, Venlo, The Netherlands) was used. Conversion of RNA to cDNA was performed using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, USA). Quantitative PCR was conducted as described above. Primers used to measure IL-6 were: Fw CAC-TTC-ACA-AGT-CGG-AGG-CT and Rv CTG-CAA-GTG-CAT-CAT-CGT-TGT.

Table 2.2. Primers for qPCR of human Th1, Th2 and Th17 cultures

Gene	Forward primer	Reverse primer
IFN-γ	GGG-GCC-AAC-TAG-GCA-GCC-AAC	AAG-CAC-TGG-CTC-AGA-TTG-CAG-GC
Tbet	GAG-GAC-TAC-GCG-CTA-CCC-GC	TGG-GAA-CAT-CCG-CCG-TCC-CT
IL-4	ACA-GCC-TCA-CAG-AGC-AGA-AGA-CTC	AAC-TGC-CGG-AGC-ACA-GTC-GC
GATA3	AGG-CCC-GGT-CCA-GCA-CAG-AA	TGG-CTG-CAG-ACA-GCC-TTC-GC
IL-17	ATG-GCC-CAG-CCA-TGG-TCA-AGT-A	GCA-CAG-GCG-GGC-AAC-TCT-CA
RORγt	CTG-GAC-CAC-CCC-CTG-CTG-AGA	GGA-AGA-AGC-CCT-TGC-ACC-CCT-CA

2.2.8 MS-qPCR for FOXP3i1

Genomic DNA was prepared from frozen pellets of $CD4^+$ memory T cells stimulated with 25 ng/ml LIF for 3 days using the PureLink Genomic DNA Mini Kit (Life Technologies) and treated with sodium bisulfite using the Epitect Bisulfite Conversion Kit (Qiagen). Real-time PCR amplification of methylated and demethylated FOXP3i1 sequence was performed on the bisulfite-converted DNA. Primers used were (sense/antisense/Taqman probe, all 5' to 3', with LNA-modified, bases underlined): CTCTTCTCTTCCTCCGTAATATCG/GTTATTGACG TTATGGCGGTC/AAACCCGACGATCCGAC for methylated FOXP3i1 sequences, and TCTACCCTCTTCTCTTCCTCCA/GATTTTTTTGTTATTGATGTTATGGT/AAACCCAACACAT CCAACCA for demethylated FOXP3i1 sequences. The proportion of cells with demethylated FOXP3i1 was calculated as follows: (number of demethylated FOXP3i1 sequences/(number of demethylated FOXPi1 sequences + number of methylated FOXP3i1 sequences)) x number of X chromosomes per cell.

2.2.9 ELISA

Concentration of IL-6 in the plasma of healthy controls and MS patients was measured using a commercially available quantitative human IL-6 ELISA (eBiosciences) according to manufacturer's instructions. Plasma was collected from the blood samples used to measure percentages of Tregs after LIF treatment.

2.2.10 Statistical analysis

Statistical analysis of LIFR expression of healthy controls, treated and untreated MS patients was performed using a non-parametric Mann-Whitney test. Repeated measures ANOVA was used to determine LIFR expression after different time points of activation. Dunn's multiple comparison test was used to define the effect of LIF treatment on the percentage of $FOXP3^+CD25^{high}$ T cells. Unpaired T-

test was used to reveal differences in LIFR expression, pSTAT3 and IL-6 serum concentration between responders and non-responder MS patients, and differences in the number of CD3, F4/80, FOXP3, IFN-γ, IL-17 and IL-4-positive cells between LV-eGFP and LV-LIF treated mice. Differences in EAE scores were analyzed using Wilcoxon matched pairs test. Analyses were performed using GraphPad Prism version 5.01.

2.3 Results

2.3.1 In MS patients LIFR expression is strongly upregulated on T cells, B cells and monocytes

To define whether LIF is able to modulate the ongoing autoimmune response in MS patients, we analyzed which circulating immune cell subsets express the LIFR and compared this to healthy controls. LIF binds to the LIFRβ subunit, which then recruits gp130 to form a heterodimer that induces signaling. Expression of LIFRβ and gp130 was measured by flow cytometry on immune cells isolated from the blood. In MS patients, higher percentages of $CD3^+$ T cells (19.92% vs 1.91%), $CD4^+$ T helper cells (19.06% vs 2.29%), $CD8^+$ cytotoxic T cells (16.51% vs 1.86%), $CD19^+$ B cells (11.18% vs 5.02%) and $CD14^+$ monocytes (62.82% vs 37.30%) express LIFRβ-gp130 as compared to healthy controls (Fig. 2.1A-E). When analyzing both subunits separately, gp130 is consistently expressed on all immune cells in healthy controls, while the LIFRβ subunit is only expressed by low percentages of immune cells (Fig. 2.1F-J). In MS patients however, the LIFRβ subunit is strongly upregulated on the different immune cells subsets (Fig. 2.1F-J). These data show that gp130 is constitutively expressed and that in MS patients expression of the specific LIFRβ subunit is augmented on circulating immune cells.

To determine whether activation of immune cells is responsible for the upregulated LIFR expression, we stimulated PBMCs with anti-CD3 antibody to activate T cells or with CpG to activate B cells. Activation of T or B cells induced a clear increase in LIFRβ-gp130 expression (Fig. 2.1K-M). Next, we defined whether current MS therapies, such as IFN-β, glatiramer acetate and natalizumab, aimed to suppress aberrant immune responses also downregulate LIFR expression. All immunosuppressive treatments strongly reduced the percentage of circulating immune cells expressing LIFRβ-gp130 as compared to untreated MS patients: $CD3^+$ T cells (1.59% vs 19.92%), $CD4^+$ T helper cells (1.29% vs 19.06%), $CD8^+$

cytotoxic T cells (1.93% vs 16.51%), $CD19^+$ B cells (3.63% vs 11.18%) and $CD14^+$ monocytes (27.95% vs 62.82%) (Fig. 2.2A-E). The reduction in receptor expression was detected for both subunits, LIFRβ and gp130 (Fig. 2.2F-J) and no difference in receptor expression was found between the different treatments (Fig. 2.2K-O). Taken together, these data suggest that activation of the immune system in MS patients enhances LIFR expression, while immunosuppressive treatment lowers receptor expression. To confirm this, T cells were ex vivo activated in absence or presence of the immunosuppressive agent IFN-β. Indeed, activation of T cells induced significant upregulation of LIFRβ-gp130, while activation of IFN-β-treated T cells did not lead to a significant increase (Fig. 2.1N).

To determine whether augmented LIFR expression is also evident on infiltrated autoreactive immune cells in MS lesions, we analyzed LIFRβ expression in post-mortem MS lesion material using immunohistochemistry. In the lesion, and especially in the active rim of the lesion, strong expression of LIFRβ was found (Fig. 2.3A). Macrophages and microglia (Fig. 2.3B) were positive for the receptor; as confirmed by a double staining with IBA-1 (Fig. 2.3C). In addition, we found a small subset of perivascular $CD4^+$ T helper cells expressing LIFRβ (Fig. 2.3D). Thus, during autoimmune disease circulating and CNS infiltrating immune subsets strongly upregulate their LIFR making these cells susceptible to LIF signaling.

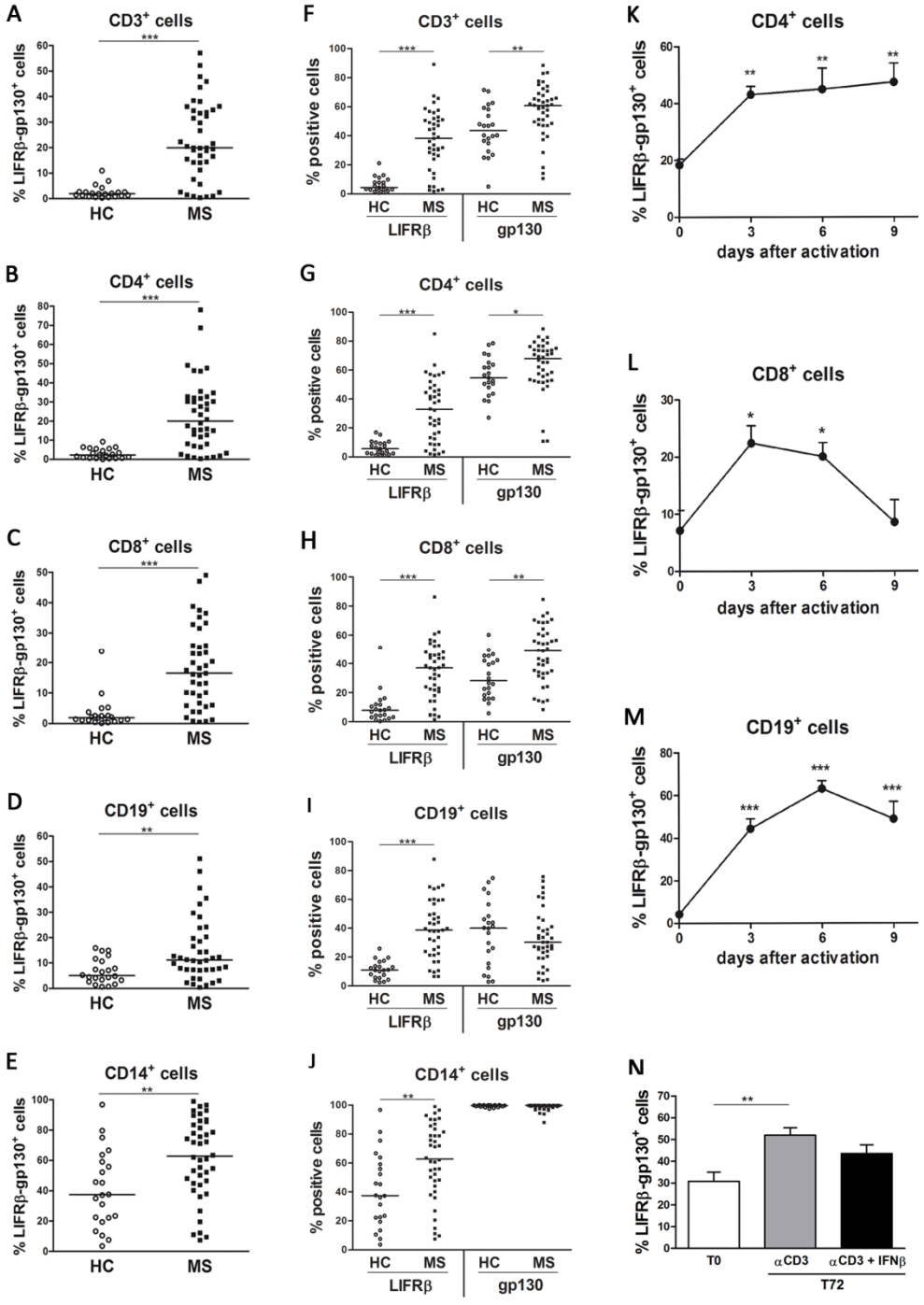
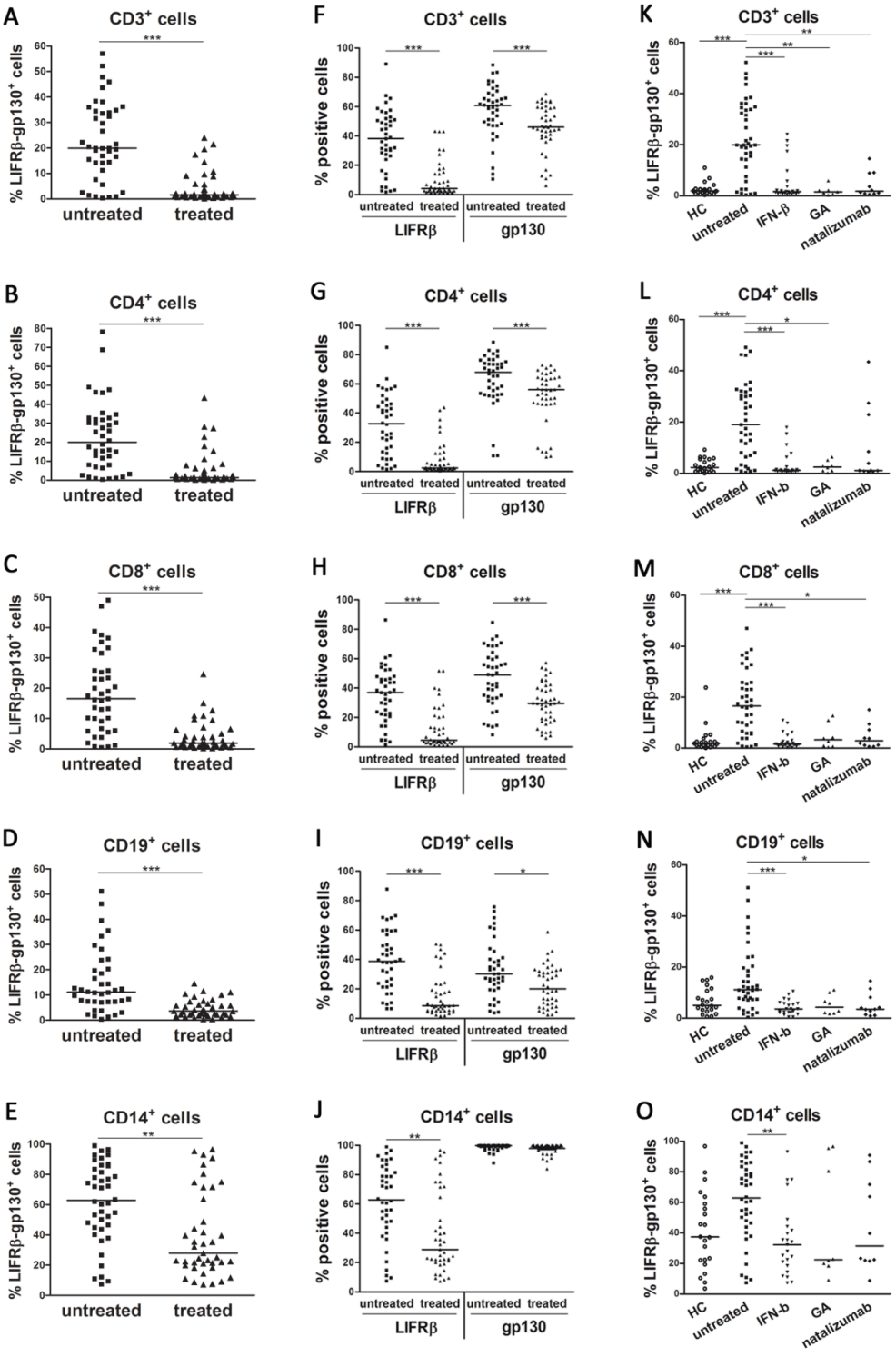


Fig. 2.1. Expression of the LIFR is highly increased on immune subsets of MS patients. (A-E) Percentage of $CD3^+$ T cells (A), $CD4^+$ T helper cells (B), $CD8^+$ cytotoxic T cells (C), $CD19^+$ B

cells (D) and $CD14^+$ monocytes (E) co-expressing LIFRβ and gp130 were analyzed in healthy controls (n=22) and untreated MS patients (n=41) using flow cytometry. **(F-J)** Expression of the subunits LIFRβ and gp130 separately on $CD3^+$ (F), $CD4^+$ (G), $CD8^+$ (H), $CD19^+$ (I) and $CD14^+$ cells (J) in healthy controls and untreated MS patients. Dots represent the percentage of positive cells in each donor and bars represent median per group. **(K-M)** LIFRβ-gp130 expression was measured on $CD4^+$ T helper cells (K), $CD8^+$ cytotoxic T cells (L) and $CD19^+$ B cells (M) of healthy subjects (n=4) after activation of PBMCs with anti-CD3 antibody (2 μg/ml) (K-L) or CpG (2 μg/ml) (M). **(N)** Percentage $CD4^+$ T cells expressing LIFRβ-gp130 following activation of PBMCs of healthy donors for 3 days (T72) with anti-CD3 antibody in absence or presence of IFN-β (1000 U/ml) (n=8). Percentages are compared to expression levels at day 0 (T0). Data are depicted as mean ±SEM. *p<0.05, **p<0.01, ***p<0.001. α-CD3, anti-CD3 antibody.


A
CD3+ cells
% LIFRβ-gp130+ cells
untreated
treated
F
CD3+ cells
% positive cells
LIFRβ
gp130
K
CD3+ cells
HC
IFN-β
GA
natalizumab
B
CD4+ cells
G
CD4+ cells
L
CD4+ cells
IFN-b
C
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N
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E
CD14+ cells
J
CD14+ cells
O
CD14+ cells

Fig. 2.2. Immunosupressive MS treatments strongly reduce LIFR expression on circulating immune subsets. (A-E) Percentage of $CD3^+$ T cells (A), $CD4^+$ T helper cells (B), $CD8^+$ cytotoxic T cells (C), $CD19^+$ B cells (D) and $CD14^+$ monocytes (E) expressing LIFRβ-gp130 in treated (n=43) and untreated (n=41) MS patients measured using flow cytometry. **(F-J)** Expression of the subunits, LIFRβ and gp130, on $CD3^+$ (F), $CD4^+$ (G), $CD8^+$ (H), $CD19^+$ (I) and $CD14^+$ cells (J) in untreated and treated MS patients. **(K-O)** Percentage of $CD3^+$ (K), $CD4^+$ (L), $CD8^+$ (M), $CD19^+$ (N) and $CD14^+$ cells (O) expressing LIFR-gp130 were analyzed in healthy controls (n=22), untreated (n=41), IFN-β (n=24), glatiramer acetate (n=8) and natalizumab (n=11) treated MS patients using flow cytometry. Dots represent the percentage of positive cells in each donor and bars represent median per group. *p<0.05, **p<0.01, ***p<0.001. GA, glatiramer acetate.

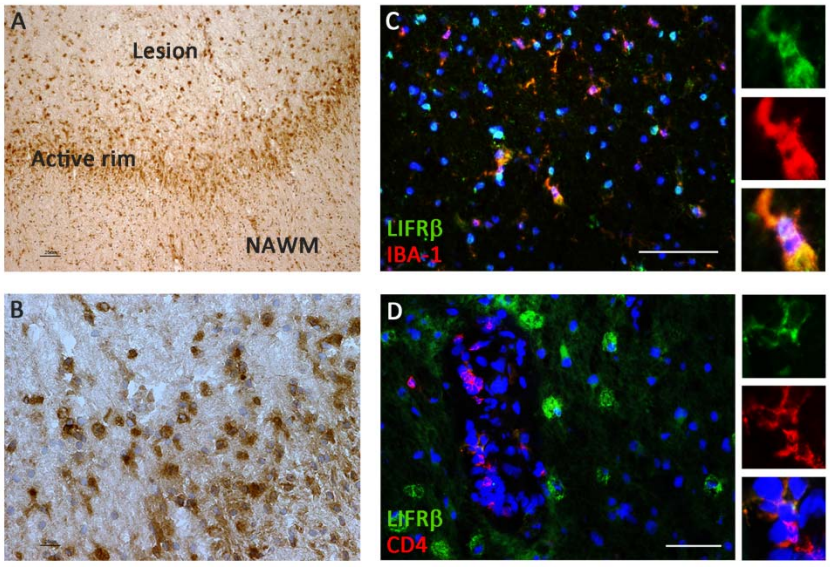


Fig. 2.3. In MS lesions, LIFRβ is expressed by macrophages/microglia and perivascular T cells. Immunohistochemical analysis for the LIFRβ was performed on brain sections of chronic active MS patients (n=4). **(A,** magnification **B)** Strong LIFRβ expression was found in the lesion and especially in the active rim. **(C-D)** Fluorescent double staining for LIFRβ (green) and IBA-1 (C) or CD4 (D) (red) was also performed on these brain sections. Magnifications of double positive cells are depicted on the right. Scale bars represent 25µm (A-B) and 50 µm (C-D) NAWM, normal appearing white matter.

2.3.2 LIF enhances Treg numbers in human memory CD4$^+$ T cells depending on the LIF/IL-6 balance

As CD4$^+$ T cells are considered to play an essential role in the pathogenesis of MS, we unraveled the effect of LIF on the differentiation of the different T helper subtypes. CD4$^+$ T cells were isolated from the blood of healthy subjects and differentiated toward Th1, Th2 and Th17 cells. Co-incubation with LIF did not affect differentiation toward Th1, Th2 and Th17 cells, neither did LIF treatment itself induce Th1, Th2 or Th17 cells (Fig. 2.4A-F). In contrast, treatment of memory CD4$^+$ T cells with LIF for 3 days increased the number of FOXP3$^+$CD25^{high} Tregs in a subpopulation of healthy controls and untreated MS patients (Fig. 2.5A-B). Similar responses were detected in healthy donors and MS patients, 40% of the healthy controls responded to LIF (4/10) and 33% of the MS patients (4/12). As FOXP3 upregulation can also be observed upon activation of T cells [234], we measured demethylation of CpG nucleotides located in the first intron of FOXP3 (FOXP3i1), which is a highly specific marker for human Tregs [235]. A positive correlation between the percentage of FOXP3$^+$CD25^{high} Tregs and the percentage of cells with demethylated FOXP3i1 was observed in LIF-treated CD4$^+$ T cells (Fig. 2.5C).

Next, we aimed to reveal why some individuals responded to LIF and others did not by determining differences in LIFR expression and signaling in responders versus non-responders. Responders displayed a trend towards higher percentages of CD4$^+$ T helper cells expressing LIFRβ-gp130 as compared to non-responders (p-value=0.0505, Fig. 2.5D). In addition, significantly more responder-derived CD4$^+$CD25^{high}CD127^{low} Tregs expressed LIFRβ-gp130 (Fig. 2.5D). Previous studies demonstrated that its family member IL-6 inhibits Treg development *in vitro* as well as *in vivo* [33, 195, 197, 236]. Moreover, it is suggested that LIF and IL-6 oppose each other in inducing Th17 differentiation [194]. To reveal whether there is an influence of IL-6 on LIF responsiveness, we assessed IL-6 signaling in the responders and non-responders via the Janus kinases/signal transducer and

activator of transcription (JAK/STAT) pathway by measuring pSTAT3. IL-6 treatment of memory CD4$^+$ T cells of the LIF responders resulted in a reduced percentages of cells positive for pSTAT3 compared to the non-responders (Fig. 2.5E-F). Additionally, in the non-responders higher serum levels of IL-6 were detected, while in the responders low IL-6 levels were measured (Fig. 2.5G). Taken together, while *in vitro* LIF treatment does not affect Th1, Th2 or Th17 differentiation, it enhances Treg numbers in subjects with low serum levels of IL-6.

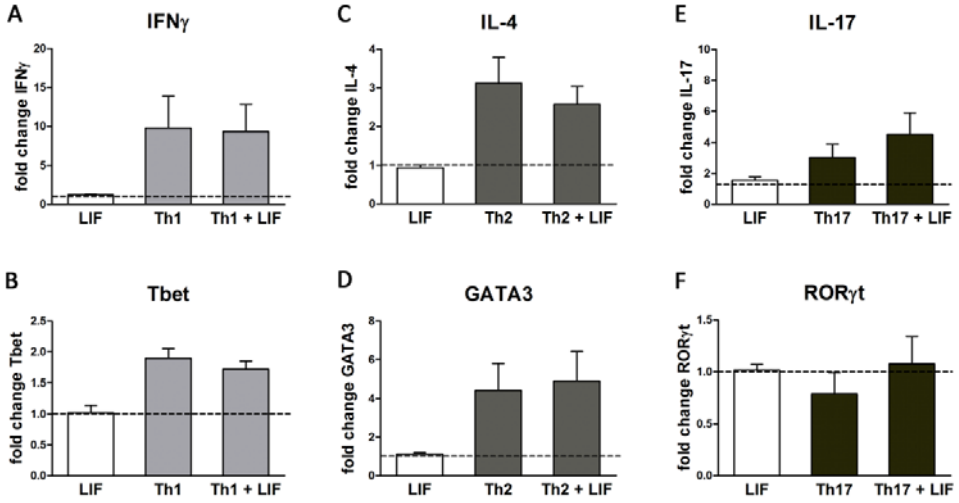


Fig. 2.4. LIF does not affect differentiation of human CD4$^+$ T cells toward Th1, Th2 and Th17 cells. (A-F) CD4$^+$ T cells were isolated from the blood of healthy subjects (n=5), after which they were incubated with IL-12 (10 ng/ml) and anti-IL-4 antibody (5 µg/ml) to induce Th1 differentiation (A-B), IL-4 (200 ng/ml), anti-IFNγ (5 µg/ml) and anti-IL-12 antibody (5 µg/ml) for Th2 differentiation (C-D), and IL-23 (25 ng/ml), anti-IL-4 (5 µg/ml) and anti-IFNγ antibody (5 µg/ml) for Th17 differentiation (E-F). LIF (25 ng/ml) was added to these cultures with and without the differentiation-inducing cytokines and neutralizing antibodies. mRNA was isolated from these cells to measure Th1 differentiation factors, IFN-γ (A) and Tbet (B); Th2 factors, IL-4 (C) and GATA3 (D), and Th17 factors, IL-17 (E) and RORγt (F) using QPCR. The expression was normalized using the two most stable reference genes and converted to fold change as compared to cells only incubated in culture medium using the $2^{-\Delta\Delta CT}$ method.

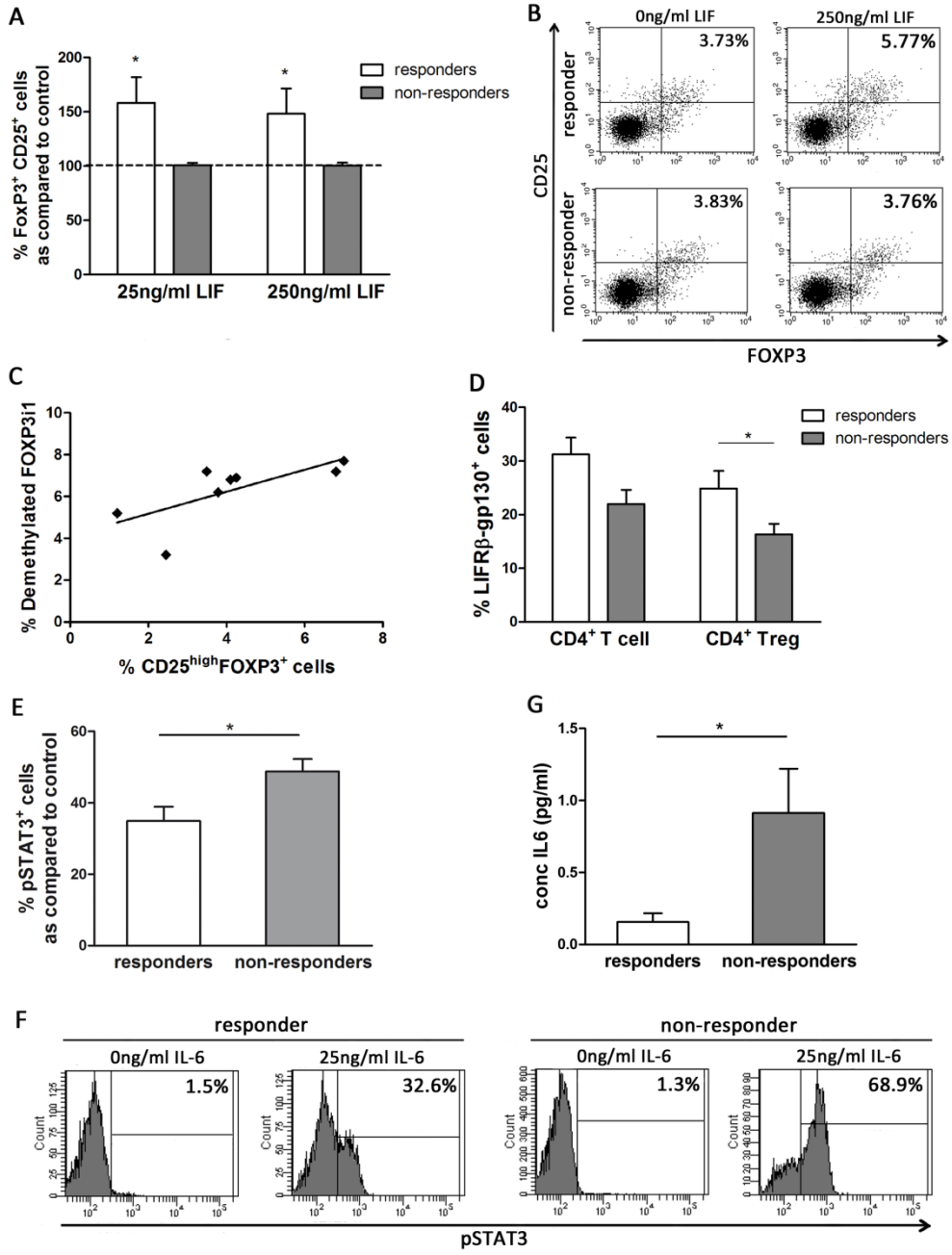


Fig. 2.5. LIF increases the number of Tregs in memory CD4⁺ T cells of healthy controls and untreated MS patients with low IL-6 serum levels. (A-B) Memory CD4⁺ T cells were isolated from the blood of healthy subjects (n=10) and untreated MS patients (n=12) and were incubated with 25 or 250 ng/ml LIF for 3 days. The number of FOXP3⁺CD25^{high} cells

was measured using flow cytometry. Subjects were classified as responders when a minimal increase in the number of $FOXP3^{+}CD25^{high}$ cells of 1.2 was detected. Of the 10 healthy controls, 4 were classified as responders and 6 as non-responders. In the untreated MS patients, 4 were responders and 8 non-responders. Data are expressed as mean per group as compared to control (0 ng/ml LIF). **(C)** FOXP3i1 demethylation was measured in $CD4^{+}$ memory T cells treated with 25 ng/ml LIF for 3 days and correlated to the percentages of $FOXP3^{+}CD25^{high}$ Tregs (p-value=0.0279, r^2=0.5031) (n=8). **(D)** LIFRβ-$gp130^{+}$ cells were measured in $CD4^{+}$ T cells and $CD4^{+}CD25^{high}CD127^{low}$ Tregs of the responding and non-responding donors by flow cytometry. **(E-F)** Memory $CD4^{+}$ T cells of responders and non-responders were treated with 25 ng/ml IL-6 for 15 min and the percentage of cells positive for pSTAT3 was measured using flow cytometry. **(G)** The concentration of IL-6 was measured in plasma of responders and non-responders by ELISA. Data are expressed as mean ±SEM, *p<0.05.

2.3.3 Therapeutic LIF administration boosts the number of CNS infiltrated Tregs and ameliorates EAE symptoms

Our human data suggest that shifting the LIF/IL-6 balance in favor of LIF increases the number of Tregs and could thus restore immune tolerance and consequently limit the inflammatory damage. We tested this hypothesis in EAE, a preclinical mouse model of MS, by therapeutic overexpression of LIF using LVs. LVs were intrathecally injected thereby transducing especially the ependymal cells lining the cerebrospinal fluid-filled spaces and choroid plexus cells [180], resulting in a continuous secretion of LIF in the cerebrospinal fluid and spread throughout the CNS. EAE was induced in C57BL/6J mice by immunization with MOG_{35-55}. Three days after clinical onset, when IL-6 levels are highly upregulated (Fig. 2.6A), mice were stereotactically injected with LV-LIF or LV-eGFP. CNS-targeted LIF expression after disease onset significantly reduced EAE symptoms as compared to LV-eGFP injected mice (Fig. 2.6B). In the control group disease symptoms increased, while in the LIF-treated mice there was no increase and the mice recovered more

rapidly. No difference in the number of $CD3^+$ infiltrating T cells or $F4/80^+$ macrophages/microglia was found in the spinal cord after the second relapse (Fig. 2.6C-F). Interestingly, CNS-targeted LIF treatment doubled the percentage of $FOXP3^+CD25^+$ Tregs (Fig. 2.6G,K) and reduced the percentage of IFN-γ producing Th1 cells present in the spinal cord 7 days after LV injection (Fig. 2.6H,L). No effect of LIF treatment was detected on $IL4^+$ Th2 and $IL17^+$ Th17 cells (Fig. 2.6I,J,L). These findings demonstrate that favoring the balance to LIF during autoimmune disease increases the number of Tregs in the CNS and reduces disease burden.

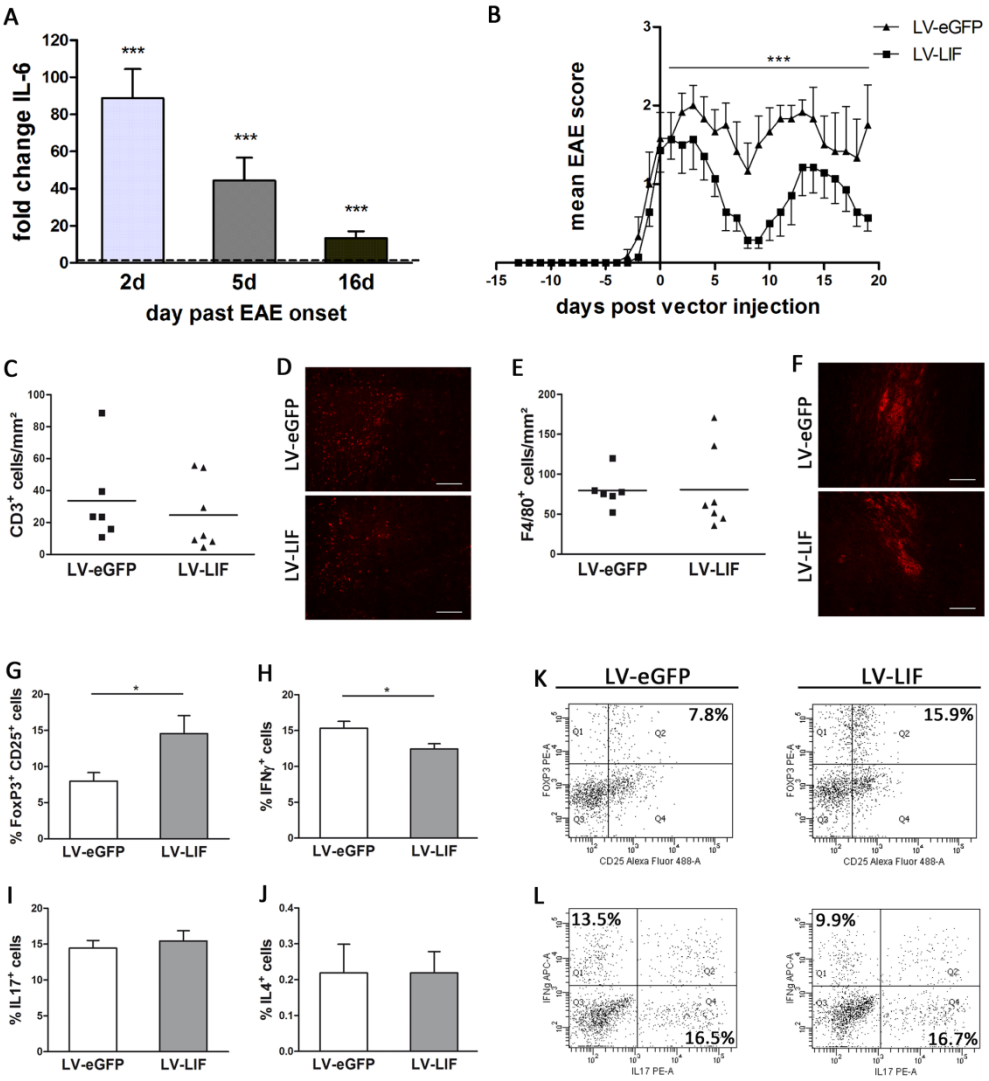


Fig. 2.6. CNS-targeted LIF expression after disease onset induces a regulatory phenotype in infiltrating T cells and ameliorates EAE symptoms. C57BL/6J mice were immunized with MOG_{35-55} in CFA. **(A)** At onset (day 2), at peak of disease symptoms (day 5) and in the chronic phase (day 16), spinal cords were isolated (n=5 per group) and mRNA expression of IL-6 was measured by QPCR. **(B)** When mice displayed EAE symptoms for 3 to 4 days LV-LIF or LV-eGFP was stereotactically injected into the right lateral ventricle. Clinical scores in the LV-LIF (n=7) and LV-eGFP-injected mice (n=6), representative of 3 independent experiments. **(C-F)** Quantification and representative pictures of the number of infiltrating $CD3^+$ T cells (C-D) and $F4/80^+$ macrophages/microglia (E-F) measured using

immunohistochemistry in the spinal cord at day 20 after vector injection in LIF (n=7) and eGFP mice (n=6). **(G-L)** Quantification and representative dot plots of the number of Tregs ($CD4^+CD25^+FOXP3^+$; G,K), Th1 cells ($CD4^+IFN\text{-}\gamma^+$; H,L), Th17 cells ($CD4^+IL\text{-}17^+$; I,L) and Th2 cells ($CD4^+IL\text{-}4^+$; J) in the spinal cord at day 7 after injection with LV-LIF (n=8) or LV-eGFP (n=8) using flow cytometry. The percentage of cells was measured as percentage of positive cells within the $CD4^+$ gate. Data are expressed as mean ±SEM, *p<0.05 and ***p<0.001. Scale bars represent 200 µm (D) and 100 µm (F).

2.4 Discussion

LIF treatment is a promising therapeutic strategy to protect neurons and oligodendrocytes from inflammatory damage in MS lesions and to promote remyelination, a strategy that would enable treatment of progressive MS patients. Here, we show that this cytokine can also limit autoimmune-mediated damage by enhancing Treg numbers.

First, we demonstrated a highly upregulated LIFR expression on circulating T cells, B cells and monocytes of untreated MS patients. The gp130 subunit was ubiquitously expressed on all immune cell subsets, while the specific LIFRβ subunit was expressed on low percentages of immune cells in healthy controls but strongly enhanced in MS patients. Moreover, we revealed that activation of immune cells upregulates the LIFR, while current immunosuppressive treatments reduce receptor expression. In active lesions of MS patients, LIFRβ was expressed on macrophages/microglia and perivascular T cells. Elevated levels of LIFRβ are also reported on neurons in the motor cortex of MS patients [163]. Moreover, LIF itself is increased in the serum, cerebrospinal fluid and lesions of MS patients [161, 162]. We believe that the upregulation of the LIFR and its ligand in MS patients is an endogenous response to heal inflammatory CNS lesions based on our findings that LIF downregulates aberrant immune responses by increasing Tregs numbers, in addition to its established neuroprotective properties.

We demonstrated that LIF enhanced the number of Tregs in healthy controls and MS patients who had reduced serum levels of IL-6. Moreover, in the LIF responders reduced IL-6 induced pSTAT3 signaling was found. Studies on graft rejection in mice already demonstrated that LIF is associated with transplantation tolerance and supports the expression of FOXP3 [193, 200]. IL-6, in contrast, limits the generation of Tregs *in vitro* and *in vivo* in mice [33, 197]. Both family members signal through gp130, IL-6 binds the IL-6Rα subunit which recruits a homodimer of gp130, whereas the LIFR is composed of a LIFRβ and gp130 subunit [144]. IL-6 was

previously shown to strongly inhibit transcription of the LIFRβ subunit [193], which further supports their opposing function and may explain the reduced LIFR expression in the $CD4^+$ T cell compartment of the non-responders.

We further reveal that CNS-directed overexpression of LIF *in vivo* doubled Treg numbers and reduced clinical symptoms of EAE. In parallel, the number of Th1 cells was reduced, whereas no effect on Th17 cell number was detected. Whether the LIF induced expansion of Tregs directly suppressed local Th1 cells is difficult to proof in our setup. If indeed Tregs would directly suppress pathogenic Th cells in the spinal cord, a similar reduction of Th17 cells would be expected, which was not the case. Alternatively, infiltrating Th1 cells may be re-educated locally and adapt to a regulatory phenotype under influence of locally provided cues, including LIF. Both the cytokine signature encountered by infiltrating T cells and the route of entry to the CNS may orchestrate CNS immunity. Indeed, Schwartz and colleagues recently showed that complex interactions of CNS-specific T effector and regulatory cells with the choroid plexus are able to instruct resolution of neuroinflammation [237]. Together, our data suggest that LIF can restore immune tolerance leading to reduced inflammatory damage and allowing CNS repair.

This study revealed that LIF is a promising therapeutic candidate as besides the previously reported neuroprotective and neuroregenerating properties it can also suppress the autoimmune response. Some concerns regarding the application of LIF in the clinic still have to be resolved. Phase I studies demonstrated the short half-life of LIF in serum indicating that repetitive injections would be needed [238, 239]. In addition, the limited potential to cross the blood-brain barrier means that high doses need to be administered to reach the target organ, risking systemic side effects. Delivery approaches such as LIF-loaded nanoparticles directed against $CD4^+$ T cells can increase efficiency of LIF treatment as shown in a non-human primate model [200]. Alternatively, CNS-targeted LIF expression by means of

lentiviral vectors would maximize neuroprotection, in addition to modulating the infiltrating immune cells. Based on our findings, the potency of LIF as a therapy may be further augmented by combining it with compounds that block IL-6 signaling.

In conclusion, we are the first to show that LIF enhances the number of Tregs *in vitro* in humans. Moreover, LIF treatment in a preclinical model of MS augments Treg numbers during active autoimmune responses and ameliorated clinical symptoms. The beneficial effects of LIF on the immune system demonstrated in this study in combination with the neuroprotective and neuroregenerating properties reported earlier provide great opportunities for the treatment of MS patients in the early as well as the progressive stage of the disease.

3

The immunomodulatory properties of OSM

Abstract

OSM, a member of the IL-6 cytokine family, is produced in lesions of MS patients. OSM can signal through the LIFR as well as its specific OSMR. LIFR signaling promotes the survival of glial cells and neurons, and induces an anti-inflammatory phenotype in macrophages. OSMR signaling is also neuroprotective, however the effects on the immune cells remain unclear. In this chapter, we investigate the effect of OSM on immune cells that are involved in the pathogenesis of MS. We reveal that in MS patients the OSMR was strongly upregulated on circulating T cells and B cells. Moreover, activation of immune cells enhanced OSMR expression, while immunosuppressive treatments lower the receptor expression. No effect of OSM was found on the polarization of human macrophages towards a M1/M2 phenotype or on the cytotoxicity of human $CD8^+$ T cells *in vitro*. Furthermore, OSM treatment after EAE onset, when immune cells have infiltrated the CNS and initiated damage, did not affect disease symptoms. This study reveals that during autoimmune disease the receptor for OSM is upregulated on immune cells, however the exact role remains elusive.

3.1 Introduction

In lesions of MS patients, OSM is produced by activated glia and infiltrating immune cells [215]. OSM is a member of the IL-6 cytokine family, which shares a receptor with its family member LIF. LIFR signaling promotes neuronal and oligodendrocyte survival, and stimulates axonal regeneration and remyelination [170, 174, 176, 180, 187, 232]. Moreover, LIF induces an anti-inflammatory phenotype in macrophages [191, 192]. By activating the LIFR, OSM can exert identical beneficial effects.

In addition, OSM also signals through its specific receptor, OSMR. In contrast to the extensively studied effects of LIFR signaling, the role of OSMR signaling in pathology remains largely unknown. A neuroprotective role of OSMR signaling is revealed, as OSM limits neuronal death caused by excitotoxicity [217, 218]. Moreover, OSM treatment reduces lesion size, promotes neurite outgrowth and functional recovery after spinal cord injury [216]. In EAE, we recently showed that CNS-targeted OSM expression strongly suppresses disease development, much more potent than LIF (Slaets, in preparation). This was the result of an anti-inflammatory and repair-promoting M2 phenotype induced by OSM, which prevented autoreactive T cells responses. This M2 polarizing effect of OSM is also demonstrated in OSMRβ knock-out mice, in which adipose tissue macrophages were polarized to a M1 phenotype and adipose tissue inflammation was augmented [229]. OSM treatment, in contrast, polarized these macrophages to a M2 phenotype and increased insulin sensitivity [229]. Thus, beneficial effects of OSM on macrophages are described in mice models, however studies on human cells or other immune cells which are involved in the pathogenesis of MS are lacking.

In this chapter, we demonstrate that the OSMR was strongly upregulated on circulating immune cells of MS patients. Activation of immune cells enhanced OSMR expression, while immunosuppressive treatment reduced receptor

expression. In contrast to data shown in mice, we did not detect an effect of OSM on the polarization of human macrophages to a M1 or M2 phenotype. In addition, OSM did not affect cytotoxicity and cytokine production of human $CD8^+$ T cells. Finally, therapeutic CNS-targeted OSM treatment had no effect on EAE symptoms. This study provides new insights into the regulation of the OSMR during autoimmune disease and the effects of OSM on the immune cells.

3.2 Materials and Methods

3.2.1 Study subjects

For characterization of OSMR expression on immune cells, blood samples were collected form 22 healthy controls, 41 untreated and 37 treated MS patients. Patients received treatment with IFN-β (Avonex®, Rebif®, Betaferon®), glatiramer acetate (Copaxone®) or Natalizumab (Tysabri®). Treated, untreated and healthy subjects were age and gender matched (Table 3.1). For *in vitro* assays, blood samples of 21 healthy donors were used. Blood samples were collected in collaboration with the University Biobank Limburg (UBiLim) and this study was approved by the Medical Ethical Committee of the University Hospital K.U.Leuven.

Table 3.1. Study subjects used for analysis of the OSMR on circulating immune cells

	Treated MS patients	**Untreated MS patients**	**Healthy controls**
Number	n = 37	n = 41	n = 22
Age (years)	43.68	46.63	39.59
Male/Female ratio	12/25 (0.48)	13/28 (0.46)	9/13 (0.69)
Disease duration (years)	11.29	12.37	NA
EDSS	3.31	3.87	NA
MS type			
- RR	28	23	NA
- CP	8	15	NA
Treatments			
- IFN-β	19	NA	NA
- Glatiramer acetate	7	NA	NA
- Natalizumab	11	NA	NA

MS, multiple sclerosis; HC, healthy control; EDSS, expanded disability status scale; RR, relapsing remitting; CP, chronic progressive; IFN-β; interferon beta; NA, not applicable.

3.2.2 Cell culture

PBMCs were isolated from whole blood using density gradient centrifugation (Cedarlane lympholyte). PBMCs were cultured in RPMI-1640 medium (Lonza) supplemented with 10% FCS (Hyclone Europe), 1% nonessential amino acids, 1% sodium pyruvate, 50 U/ml penicillin and 50 mg/ml streptomycin (all Life technologies). To measure OSMR expression after activation cells were stimulated with 2 µg/ml anti-CD3 antibody (clone 2G3, BIOMED) or 2 µg/ml CpG2006 (ODN2006, InvivoGen). To measure the cytokine production of $CD8^+$ T cells, these cell were isolated from PBMCs using immunomagnetic isolation (Easysep, Stemcell technologies) and treated with 25 ng/ml OSM (R&D systems) for 24h. Cytokine mRNA levels were measured with qPCR.

For M1/M2 macrophage differentiation, $CD14^+$ monocytes were isolated from PBMCs by immunomagnetic isolation (Easysep, Stemcell technologies). $CD14^+$ cells were cultured for 6 days in RPMI-1640 medium (Lonza) supplemented 10% autologous serum, 50 U/ml penicillin and 50 mg/ml streptomycin (Life technologies) to induce maturation towards macrophages. After 6 days cytokines were added to induce differentiation: for M1 differentiation 20 ng/ml IFN-γ (eBiosciences) and 10 ng/ml LPS (Sigma-Aldrich) for 24h and for M2 differentiation 100 ng/ml IL-4 (eBiosciences) for 48h. OSM (25 ng/ml, R&D systems) was added alone or in combination with the differentiation-inducing cytokines. mRNA levels of M1 and M2 markers were measured by qPCR.

3.2.3 Flow cytometry

To determine OSMR expression, isolated PBMCs were incubated with anti-gp130-FITC (Abcam) and anti-OSMRβ-PE antibodies (eBiosciences) combined with PerCP labeled antibodies specific for the immune cells subsets, CD3, CD4, CD8, CD14 and CD19 (all BD Biosciences).

To measure the effect of OSM on antigen presentation of monocytes, PBMCs isolated from the blood of 3 healthy subjects were pre-treated with 0, 10 or 100 ng/ml OSM (R&D systems) for 24h, followed by activation with 100 ng/ml LPS (Sigma-Aldrich) for 18h. Before (T0) and after activation (T18) cells were harvested and incubated with anti-CD14-PerCP antibody combined with anti-CD80-FITC and CD86-PE or CD40-FITC and HLA-DR-PE antibodies (all BD Biosciences).

To measure degranulation of $CD8^+$ T cells, PBMCs of 6 healthy subjects were pre-treated with 100 ng/ml OSM (R&D systems) for 20h, followed by activation by anti-CD3 antibody (clone 2G3, BIOMED) for 4h and incubation with anti-CD107a-FITC antibody and GolgiStop (both BD biosciences). As control, culture medium without any stimulus was used. After 4h cells were harvested and incubated with anti-CD8-PerCP (BD Biosciences), OSMRβ-PE (eBiosciences), LIFRβ-PE antibodies (R&D systems). Samples were measured using FACSCalibur and analyzed using CellQuest Software (BD Biosciences).

3.2.4 Quantitative PCR

RNA was prepared from the M1 and M2 cultures and from the $CD8^+$ T cell cultures using the High Pure RNA Isolation Kit (Roche) according to manufacturer's instructions. Conversion of RNA to cDNA was performed using the Reverse Transcription System (Promega). qPCR was conducted on a StepOnePlus Real-Time PCR detection system (Applied Biosystems) using universal cycling conditions: 20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C. The PCR reaction consisted of fast SYBR green master mix (Applied Biosystems), 10 µM of forward and reverse primers (Eurogentec), RNase free water and 12.5ng template cDNA. Primers used for qPCR are shown in Table 3.2. Gene expression was normalized using the two most stable reference genes and converted to fold change as compared to cells only incubated in culture medium using comparative Ct method.

Table 3.2. Primers for qPCR of M1/M2 cultures and $CD8^+$ T cell cultures

Gene	Forward primer	Reverse primer
CD40	TCAAGCAGATTGCTACAGGGG	ACAGCTTGTCCAAGGGTGAC
CCR7	ACCTGGGGAAACCAATGAA	TGTGGTGTTGTCTCCGATGT
IL-1β	GATGAAGTGCTCCTTCCAGG	GCATCTTCCTCAGCTTGTCC
TNF-α	AGCCCATGTTGTAGCAAACC	TGAGGTACAGGCCCTCTGAT
CD206	AAATGTTGAAGGGACGTGGC	CTCCAAAACCCAGAAGACGC
CCL22	TTACGTCCGTTACCGTCTGCμ	GAAGGTTAGCAACACCACGC
CCL18	GCCAGGTGTCATCCTCCTAA	CAGGCATTCAGCTTCAGGTC
IL-10	GCTGTCATCGATTTCTTCCC	ATAGAGTCGCCACCCTGATG
IFN-γ	GGGGCCAACTAGGCAGCCAAC	AAGCACTGGCTCAGATTGCAGGC
IL-17	ATGGCCCAGCCATGGTCAAGTA	GCACAGGCGGGCAACTCTCA
IL-4	ACAGCCTCACAGAGCAGAAGACTC	AACTGCCGGAGCACAGTCGC
TGF-β	GTGGAAACCCACAACGAAAT	CACGTGCTGCTCCACTTTTA

3.2.5 EAE induction

Female 10 week old C57BL/6J mice (Harlan) were immunized subcutaneously with MOG_{35-55} emulsified in CFA containing Mycobacterium tuberculosis according to manufacturer's guidelines (Hooke Laboraties). Directly after immunization and 24h later, mice were intraperitoneally injected with pertussis toxin. Mice were daily weighted and evaluated for neurological signs of disease using a standard 5-point scale; 0: no symptoms; 1: limp tail; 2: hind limp weakness; 3: complete hind limp paralysis; 4: complete hind limp paralysis and partial front leg paralysis; 5: death. All experiments were approved by the Hasselt University ethics committee according to European guidelines.

3.2.6 Lentiviral vector construction and administration

Murine OSM gene transcript (NM_001013365) was isolated from RAW 264.7 cells (ATCC, Molsheim, France) after stimulation with 100 ng/ml LPS (Sigma-Aldrich) for 24h. Complementary DNA was cloned into a lentiviral transfer plasmid containing

a central polypurine tract sequence, the SIN-18 deletion and the woodchuck hepatitis posttranscriptional regulatory element [240]. HIV-1 derived vector particles were produced by a triple transient transfection of 293T cells as described previously [180]. Briefly, cells were transfected with a second-generation packaging plasmid, a plasmid encoding the glycoprotein G of vesicular stomatitis virus and a transfer plasmid encoding the OSM gene under control of a cytomegalovirus promoter. This mixture combined, with a 1.42 µM polyethyleneimine solution in 150 mM NaCl, was added to 293T cells in OPTI-MEM (Life technologies) free of serum. Vector particles in the supernatant were collected and filtered 48h and 72h post transfection and concentrated using Vivaspin 15 columns (Vivascience, Hannover, Germany). p24 antigen content was measured using HIV-1 p24 Core Profile ELISA (DuPont, Dreieich, Germany).

When mice showed EAE symptoms for 3 to 4 days they were stereotactically injected with LV-OSM, LV-eGFP or saline in the right lateral ventricle. Mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg), after which they were placed in a stereotactic head frame (Stoelting, IL, USA). A midline incision of the skin was made and a small hole was drilled into the skull. Coordinates for injection into the lateral ventricle were anteroposterior 0.2 mm, lateral 1.0 mm and dorsoventral -1.8 mm using bregma as a reference. At a rate of 0.25 µl/min, 4 µl of concentrated vector ($3{,}43*10^7$ pg p24/ml) was injected using a 10 µl Hamilton syringe with a 30-gauge needle.

3.2.7 Statistical analysis

Statistical analysis of OSMR expression on immune cells of healthy controls, treated and untreated MS patients was performed using a non-parametric Mann-Whitney test. Repeated measures ANOVA was used to examine OSMR expression at different time points after activation. Analyses were performed using GraphPad Prism version 5.01.

3.3 Results

3.3.1 In MS patients, the OSMR is highly upregulated on circulating immune cells

To determine which immune cells can respond to OSM, we measured its receptor expression on T cells, B cells and monocytes isolated from the blood of healthy donors using flow cytometry. Human OSM can signal through the specific OSMR, composed of the OSMRβ and gp130 subunit, as well as the LIFR, composed of LIFRβ and gp130. In the previous chapter we already demonstrate expression of the LIFR on immune cells of healthy subjects and MS patients, therefore we focus on the expression of the OSMR. In healthy controls, approximately half of the $CD14^+$ monocytes expressed both OSMRβ and gp130, while lower percentages of T cells and B cells expressed the receptor complex (8.0% of the $CD3^+$ T cells, 12.8% $CD4^+$ T helper cells, 6.7% $CD8^+$ cytotoxic T cells and 11.3% $CD19^+$ B cells) (Table 3.3). In all cell populations most cells expressed gp130, whereas OSMRβ was present on a low percentage of cells, indicating that especially expression of the specific receptor subunit determines responsiveness to OSM.

Table 3.3. Percentages of circulating immune cell subsets expressing the OSMR

	OSMRβ	gp130	OSMRβ-gp130
$CD3^+$ T cells	15.2 ±4.7	44.0 ±3.5	8.0 ±3.0
$CD4^+$ T helper cells	20.9 ±5.7	53.1 ±3.6	12.8 ±4.6
$CD8^+$ cytotoxic T cells	20.0 ±6.1	30.7 ±3.1	6.7 ±2.7
$CD19^+$ B cells	24.0 ±5.2	36.5 ±4.8	11.3 ±2.9
$CD14^+$ monocytes	52.0 ±8.7	98.9 ±0.4	51.8 ±8.7

Mean percentage of $CD3^+$, $CD4^+$, $CD8^+$ or $CD19^+$ or $CD14^+$ cells expressing OSMRβ, gp130 or both measured in healthy controls (n=22) ±SEM.

To reveal whether the receptor expression is altered in MS patients, their expression levels were compared to those of healthy controls. In MS patients, higher percentages of $CD3^+$ T cells (28.40% vs 0.26%), $CD4^+$ T helper cells (25.40% vs 1.83%), $CD8^+$ cytotoxic T cells (20.93% vs 0.71%) and $CD19^+$ B cells (18.16% vs 4.27%) expressed OSMRβ-gp130 as compared to healthy subjects (Fig. 3.1A-D). In $CD14^+$ monocytes no significant difference in receptor expression was detected (79.24% vs 37.59%) (Fig. 3.1E). When both subunits were analyzed separately, especially the specific OSMRβ subunit was found to be strongly upregulated on the immune cell subsets studied (Fig. 3.1F-J). To reveal whether the aberrant activation of immune cells in MS patients augments the receptor expression, PBMCs isolated from the blood of healthy subjects were activated with anti-CD3 antibody or CpG to activate T cells or B cells respectively. Activation strongly enhanced OSMRβ-gp130 expression on $CD4^+$ and $CD8^+$ T cells and on $CD19^+$ B cells (Fig. 3.1K-M). These data reveal that during autoimmune disease the OSMR is strongly upregulated on circulating immune cells, which is probably due to activation by the ongoing immune response.

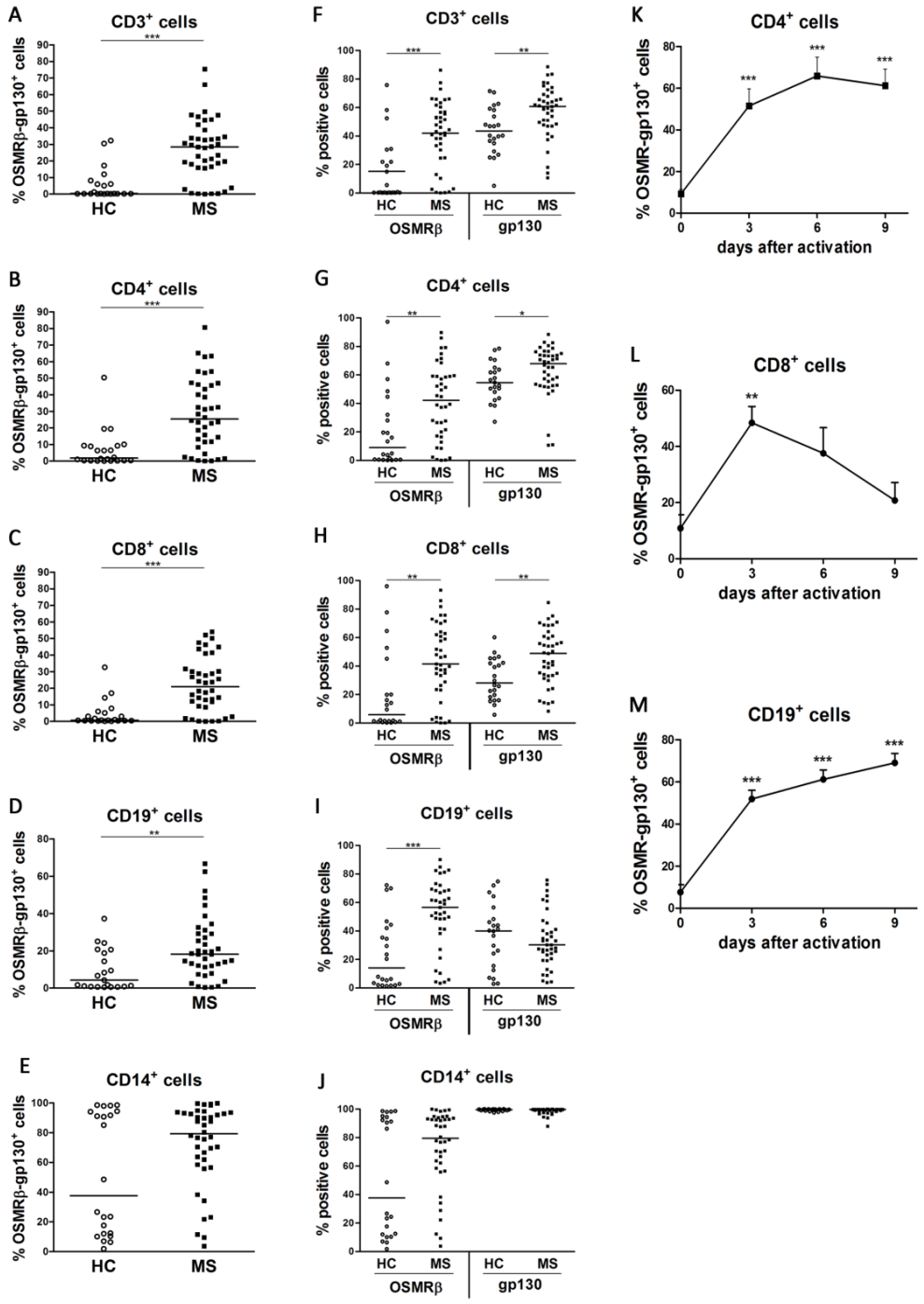
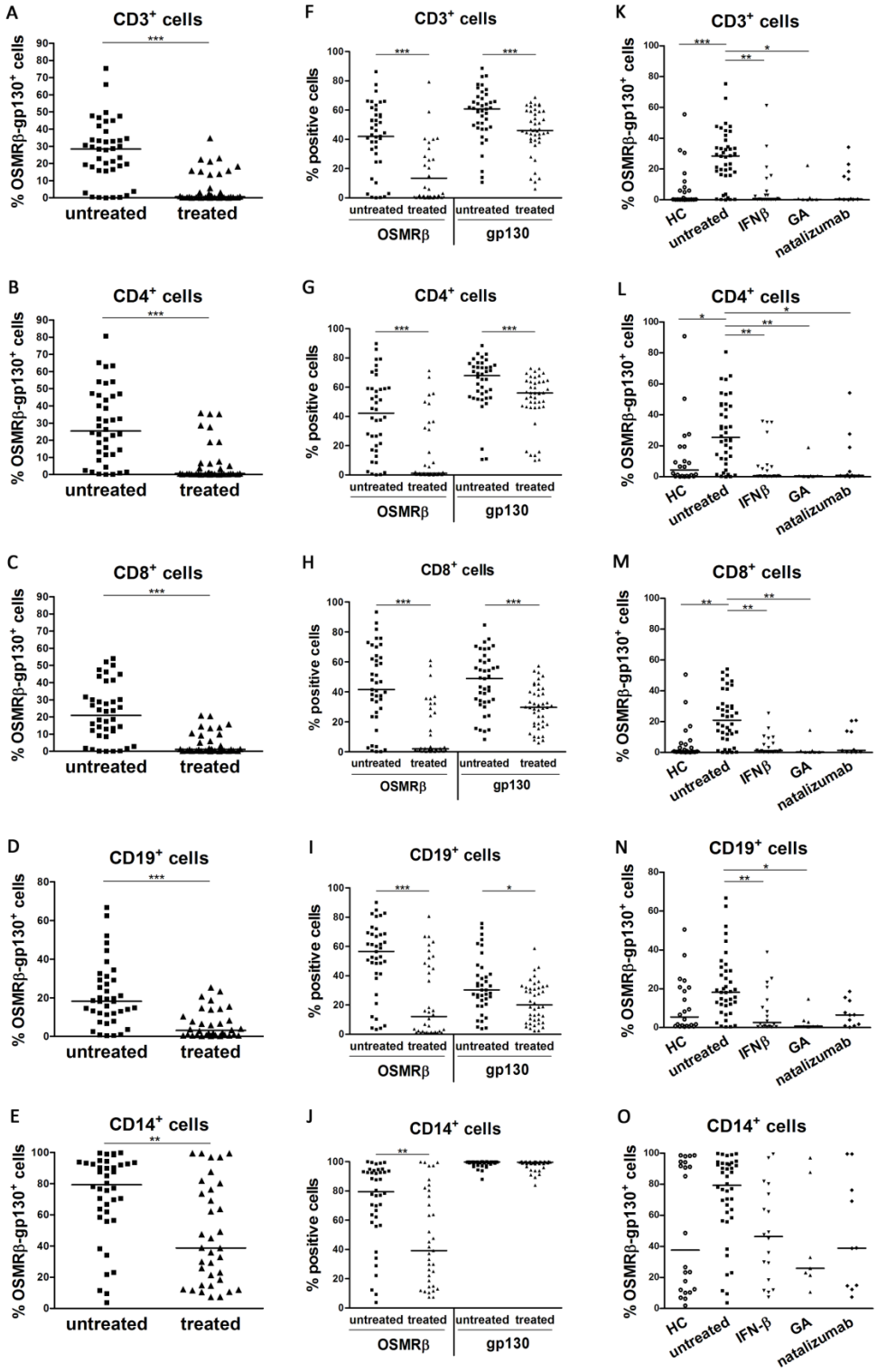


Fig. 3.1. In MS patients, expression of the OSMR is increased on circulating T cells and B cells. **(A-E)** Percentage of $CD3^+$ T cells (A), $CD4^+$ T helper cells (B), $CD8^+$ cytotoxic T cells (C)

$CD19^+$ B cells (D) and $CD14^+$ monocytes (E) co-expressing OSMRβ and gp130 were measured in PBMCs of healthy controls (n=22) and untreated MS patients (n=41) using flow cytometry. **(F-J)** Expression of the subunits OSMRβ and gp130 separately on $CD3^+$ (F), $CD4^+$ (G), $CD8^+$ (H), $CD19^+$ (I) and $CD14^+$ cells (J) of healthy controls and untreated MS patients. Dots represent the percentage of positive cells in each donor and bars represent median per group. **(K-M)** Expression of OSMRβ-gp130 after activation of PBMCs of healthy controls with anti-CD3 antibody (2 µg/ml) (K-L) or CpG (2 µg/ml) (M) on $CD4^+$ T helper cells (K), $CD8^+$ cytotoxic T cells (L) and $CD19^+$ B cells (M) (n=4). Data are depicted as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. α-CD3, anti-CD3 antibody.

3.3.2 Immunosuppressive treatments reduce OSMR expression on circulating immune cells

To determine the influence of immunosuppressive MS therapies on OSMR expression, the receptor was measured on circulating immune cells of MS patients receiving treatment with IFN-β, glatiramer acetate and natalizumab. Treatment strongly reduced the percentage of $CD3^+$ T cells (0.53% vs 28.40%), $CD4^+$ T helper cells (0.53% vs 25.40%), $CD8^+$ cytotoxic T cells (1.04% vs 20.93%), $CD19^+$ B cells (3.09% vs 18.16%) and $CD14^+$ monocytes (38.72% vs 79.24%) expressing OSMRβ-gp130 as compared to untreated MS patients (Fig. 3.2A-E). Expression of both subunits, OSMRβ and gp130, was reduced (Fig. 3.2F-J). No difference in receptor expression was detected between the different treatments (Fig. 3.2K-O). Together these data indicate that during autoimmune disease OSMR expression is strongly upregulated on circulating immune cells, whereas immunosuppressive therapies lower the receptor expression.


A
CD3+ cells
% OSMRβ-gp130+ cells
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J
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O
CD14+ cells
IFN-β

Fig. 3.2. Immunosuppressive MS treatments reduce OSMR expression on circulating T cells, B cells and monocytes. (A-E) Flow cytometric analysis of the percentage of $CD3^+$ T cells (A), $CD4^+$ T helper cells (B), $CD8^+$ cytotoxic T cells (C), $CD19^+$ B cells (D) and $CD14^+$ monocytes (E) expressing OSMRβ-gp130 in untreated (n=41) and treated (n=37) MS patients. **(F-J)** Expression of the subunits, OSMRβ and gp130, on $CD3^+$ (F), $CD4^+$ (G), $CD8^+$ (H), $CD19^+$ (I) and $CD14^+$ cells (J) in untreated and treated MS patients. **(K-O)** Percentage of $CD3^+$ (K), $CD4^+$ (L), $CD8^+$ (M), $CD19^+$ (N) and $CD14^+$ cells (O) expressing OSMRβ-gp130 analyzed in healthy controls (n=22), untreated (n=41), IFN-β (n=19), glatiramer acetate (n=7) and natalizumab (n=11) treated MS patients using flow cytometry. Dots represent the percentage of positive cells in each donor and bars represent median per group. *p<0.05, **p<0.01, ***p<0.001. GA, glatiramer acetate.

3.3.3 OSM has no effect on M1/M2 polarization or antigen presentation of human macrophages

As macrophages are abundantly present in MS lesions and our data indicate that high percentages of these cells express the OSMR, we determined the effect of OSM on the polarization of human macrophages to a M1 and M2 phenotype and on antigen presentation. M1 polarization was induced by IFN-γ and LPS, while M2 polarization was induced by IL-4. Co-incubation with OSM did not affect M1 differentiation, as measured by CD40, CCR7, IL-1β, TNF-α mRNA levels, or M2 differentiation, as measured by CD206, CCL22, CCL18, IL-10 (Fig. 3.3A-H). OSM treatment alone did also not promote M1 or M2 polarization (Fig. 3.3A-H). The effect of OSM on antigen presentation by circulating monocytes was determined by measuring surface expression of CD86, CD80, CD40, HLA-DR using flow cytometry. OSM treatment did not affect the expression these antigen presenting molecules (Fig. 3.3I-L). These data indicate that OSM does not affect the phenotype or the expression of antigen presenting molecules by human macrophages/monocytes.

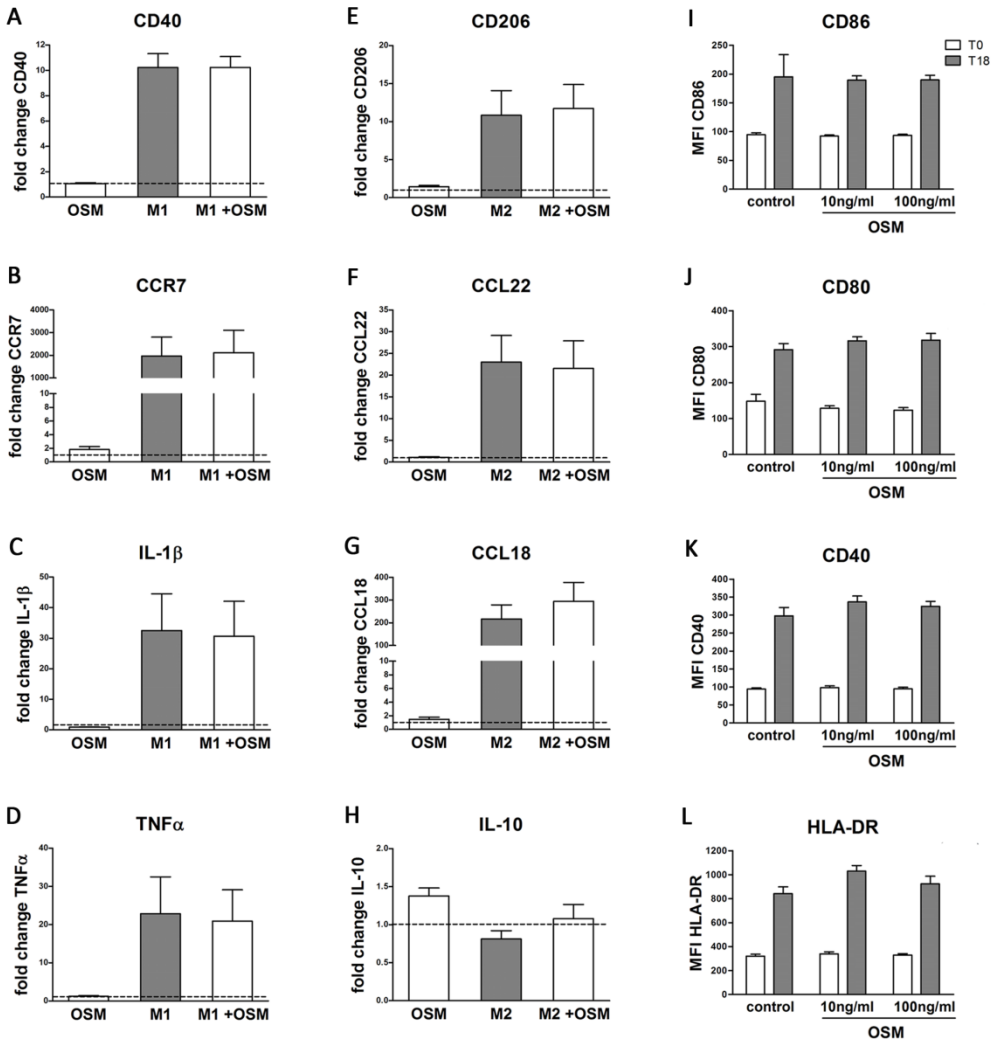

Fig. 3.3. OSM does not affect M1/M2 differentiation or expression of antigen presenting molecules on human macrophages. **(A-H)** $CD14^+$ monocytes isolated from the blood of healthy subjects (n=7) were cultured for 6 days to induce maturation towards macrophages. M1 differentiation was induced by 20 ng/ml IFN-γ and 10 ng/ml LPS for 24h and M2 differentiation by 100 ng/ml IL-4 for 48h. OSM (25 ng/ml) was added alone or in combination with the differentiation cytokines. mRNA levels of M1 markers CD40 (A), CCR7 (B), IL-1β (C), TNF-α (D), and M2 markers CD206 (E), CCL22 (F), CCL18 (G), IL-10 (H) were measured by qPCR. Expression was converted to fold change as compared to cells only incubated in culture medium. **(I-L)** PBMCs isolated from blood of healthy subjects

(n=3) were pre-treated with 0, 10 or 100 ng/ml OSM for 24h, followed by activation with 100 ng/ml LPS for 18h. Expression of CD86 (I), CD80 (J), CD40 (K) and HLA-DR (L) was measured on $CD14^+$ monocytes using flow cytometry before (T0) and 18h after activation (T18). Data are depicted as mean ± SEM.

3.3.4 OSM does not affect degranulation and cytokine production of $CD8^+$ T cells

As $CD8^+$ cytotoxic T cells play an important role in MS pathogenesis and the receptor for OSM is upregulated on these cells during disease, we determined the effect of OSM on their degranulation and cytokine production. Degranulation of $CD8^+$ T cells was determined by measuring CD107a expression. This protein is present on the inside of cytotoxic granules and after degranulation it is expressed on the cell surface. PBMCs isolated from the blood of healthy subjects were pre-treated with OSM, after which they were activated with anti-CD3 antibody and expression of CD107a was measured using flow cytometry. OSM treatment did not affect the percentage of degranulating $CD107a^+CD8^+$ T cells (Fig. 3.4A). Moreover, in cells positive for OSMRβ or LIFRβ no difference was found in the percentage of $CD8^+$ degranulating cells after OSM treatment (Fig. 3.4B-C). To measure the effect of OSM on cytokine production by $CD8^+$ T cells, these cells were isolated from the blood of healthy subjects and treated with OSM for 24h. OSM had no effect on IFN-γ, TNF-α, IL-17, IL-4, TGF-β or IL-10 mRNA levels (data not shown). These data indicate that OSM does not affect the cytotoxicity or cytokine production of human $CD8^+$ T cells.

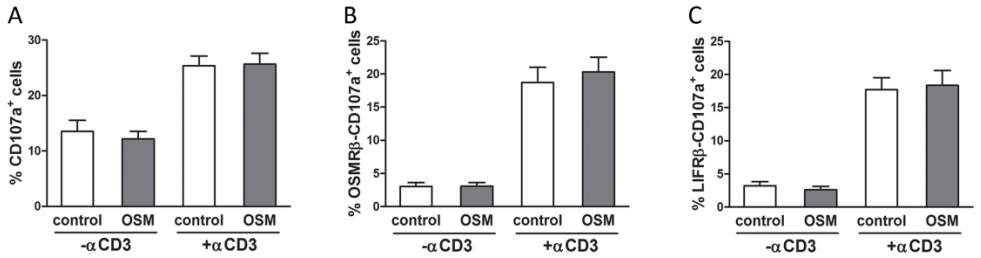

Fig. 3.4. OSM has no effect on the cytotoxicity of human CD8$^+$ T cells. (A-C) PBMCs of healthy subjects (n=6) were pre-treated with 100 ng/ml OSM for 20h, activated by anti-CD3 antibody (+αCD3) or not activated (-αCD3) as control, and 4h later CD107a expression was measured by flow cytometry. Percentage of CD8$^+$ cells (A), CD8$^+$ OSMRβ$^+$ cells (B) and CD8$^+$LIFR$^+$ cells (C) expressing CD107a. Data are depicted as mean ±SEM. αCD3, anti-CD3 antibody.

3.3.5 Therapeutic OSM treatment does not affect EAE symptoms

A previous study in our group demonstrated that prophylactic OSM treatment strongly suppresses EAE development (Slaets, in preparation). In the study, OSM was expressed locally in the CNS using LVs. To reveal whether OSM treatment has an effect when auto-reactive immune cells already infiltrated the CNS and initiated damage, we stereotactically injected LV-OSM three days after EAE onset. LVs were used as OSM, similar to LIF, has a limited potential to cross the blood-brain barrier and a short half-life in serum. LVs were intrathecally injected to induce a continuous production of OSM into to CSF. Therapeutic OSM treatment did not affect EAE symptoms as compared to the control groups that received intrathecal LV-eGFP or saline injections (Fig. 3.5A). In addition, no effect of OSM on weight loss was detected (Fig. 3.5B). These data demonstrate that CNS-targeted OSM treatment after disease onset has no effect on EAE symptoms.

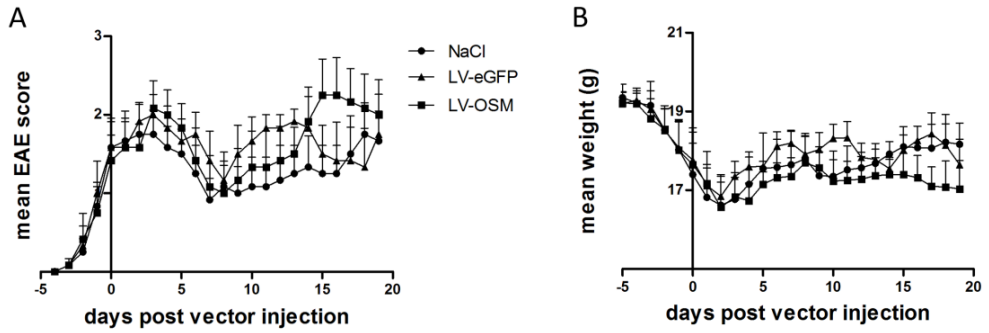

Fig. 3.5. CNS-targeted OSM treatment after disease onset has no effect on EAE symptoms. (A-B) EAE was induced in C57BL/6J mice with MOG_{35-55} in CFA. LV-OSM, LV-eGFP or saline was stereotactically injected in the lateral ventricle of C57BL/6 mice 3 or 4 days after disease onset. Clinical scores (A) and weights (B) were daily measured in the different groups. Data are depicted as mean values ±SEM.

3.4 Discussion

OSM is produced in lesions of MS patients [215], however its role in autoimmune disease is still unclear. In this study we reveal that in MS patients the OSMR was strongly enhanced on circulating immune cells. No functional effects of OSM were found on human macrophages and cytotoxic T cells *in vitro*. Moreover, therapeutic OSM treatment did not affect EAE symptoms.

We demonstrate that in healthy subjects the OSMR was present on high percentages of circulating macrophages, while lower percentages of T cells and B cells expressed the receptor. Gp130 was ubiquitously expressed on all immune cell subsets, while the specific OSMRβ subunit was present on low percentages of cells. Interestingly, in untreated MS patients OSMRβ-gp130 was strongly upregulated on circulating T cells and B cells. This strong increase was especially detected for the OSMRβ subunit. Moreover, we show that activation of circulating immune cells enhanced OSMRβ-gp130 expression. Immunosuppressive treatments, in contrast, potently lower the receptor. Expression of OSMRβ is already demonstrated on neurons, pointing towards its neuroprotective role [241, 242]. Our data indicate that during autoimmune disease circulating immune cells strongly enhance their OSMR expression. In the previous chapter we revealed similar results for LIFR expression on immune cells. This could indicate that immune cells of untreated MS patients are more sensitive for signaling by OSM through both the OSMR and LIFR.

Furthermore, we show that OSM did not affect the cytotoxicity and cytokine production of human $CD8^+$ T cells. Moreover, OSM had no effect on M1 or M2 differentiation, or antigen presentation of human macrophages. This is in contrast to a recent study in our group in EAE and a report on mouse adipose tissue macrophages, that showed that OSM induces M2 polarization (Slaets, in preparation) [229]. This discrepancy could be due to the difference in signaling, in mice OSM only signals through the OSMR, while in humans OSM signals through

both the OSMR and LIFR. Activation of the LIFR or OSMR could inhibit signaling through the other receptor by for example activation of negative regulators such as SOCS1 and SOCS3. Additionally, OSM could affect other functions of these immune cells, including the phagocytosis of macrophages or the direct cytotoxicity of $CD8^+$ T cells. Moreover, it would be interesting to perform these assays on immune cells isolated from untreated MS patients, as we revealed that their immune cells display enhanced OSMR expression.

Further research should also define the effects of OSM on other immune cells that play a role in the MS pathogenesis. For example, its effect on antibody and cytokine production by B cells or the development of plasma cells should be investigated. In addition, it should be determined whether OSM affects maturation of DCs or their antigen presentation and cytokine production. In the previous chapter we demonstrated that LIFR signaling boosts the number of Tregs, while it did not affect Th1, Th2 and Th17 development. OSM could exert identical effects in humans by activating the LIFR, but could also exert additional synergistic or opposing effects by OSMR activation. Thus additional research is required to address whether OSMR signaling affects generation of the different Th subsets.

Finally, we showed that therapeutic CNS-targeted expression of OSM does not affect EAE symptoms. A previous study in our group demonstrated that prophylactic CNS-targeted OSM expression strongly inhibits disease development (Slaets, in preparation). However, in that study LVs were injected in the striatum, while in the present study injections were made in the lateral ventricle. Injection in the striatum induces local tissue remodeling and alters BBB permeability accompanied with strong infiltration of lymphocytes and macrophages. These macrophages/microglia were found to have a M2 phenotype, thereby limiting the autoreactive T cell response and preventing EAE. Prophylactic injection of LV-OSM in the lateral ventricle results in delayed disease onset, however, 7 days after EAE

onset no difference in clinical symptoms is detected compared to the control group (unpublished data). This is in agreement with the present study, in which OSM injection in the lateral ventricle after disease onset does not affect EAE symptoms. Thus, it seems that injection in the striatum induces a potent anti-inflammatory response in the CNS which is less pronounced with injection in the lateral ventricle. Whether therapeutic injection of LV-OSM in the striatum can affect disease remains to be determined. Taken together, this study reveals how the OSMR is regulated during MS. Although we failed to detect effects of OSM on human $CD8^+$ T cells and macrophages, further insights into the immunomodulatory properties of OSM will enhance our understanding of its role in the pathogenesis of MS and other neuroinflammatory diseases.

4

OSM protects against demyelination by inducing a protective microglial phenotype

Based on:

Oncostatin M protects against demyelination by inducing a protective microglial phenotype

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Submitted

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Abstract

MS is a chronic disabling disease of the CNS, in which destruction of myelin sheaths leads to disturbed axonal conduction. Available MS therapies modulate the immune response, but are unable to prevent neurological decline. Therefore, great efforts are made to develop therapies that limit demyelination and axonal degeneration. OSM, a member of the IL-6 cytokine family, is produced in demyelinating lesions of MS patients and stimulates neuronal survival. In this study, we reveal that the OSMR was robustly upregulated on microglia/macrophages and astrocytes in the cuprizone-induced demyelination model. While OSMR deficiency led to aggravated demyelination, CNS-targeted OSM treatment largely prevented demyelination. OSM treatment increased IL-4 expression and induced polarization of myeloid cells towards an anti-inflammatory M2 phenotype *in vivo*. This study reveals a previously uncharacterized and protective role for OSM during demyelination, and indicates that OSM is a promising therapeutic candidate to limit CNS damage in demyelinating diseases including MS.

4.1 Introduction

MS is a chronic demyelinating disease of the CNS, characterized by focal areas of inflammation, gliosis and axonal degeneration [4, 74]. The nerve insulating myelin sheath is destructed, resulting in disturbed neuronal signaling. This causes a variety of symptoms including muscle weakness, spasticity, visual and sensory disturbances [4]. Microglia activation and astrocyte reactivity are prominent features of demyelinated lesions. Both cell types contribute to demyelination by producing proinflammatory cytokines, chemokines, reactive oxygen species and glutamate [81, 243]. However, by secreting trophic factors and anti-inflammatory cytokines they may also limit the development of demyelinated lesions [81, 244]. Current MS therapies suppress the aberrant immune response, but cannot prevent consequent neurological disability. Therefore, there is a high need for novel therapies that limit demyelination and axonal degeneration to complement the currently available therapies.

OSM, a member of the IL-6 cytokine family, is produced in demyelinating lesions of MS patients by activated glia and infiltrating leukocytes [215]. OSM attenuates excitotoxicity induced neuronal death *in vivo* and *in vitro* [217, 218]. Moreover, OSM prevents death of primary cortical neurons after supplement withdrawal [216]. Following spinal cord injury OSM treatment limits the lesion size and promotes neurite outgrowth and functional recovery [216]. While the neuroprotective properties of OSM are well established, its role in demyelination is unknown.

In this study, we reveal that the OSMR is strongly upregulated during cuprizone-induced demyelination. In OSMR deficient mice demyelination is aggravated, whereas CNS-targeted OSM treatment strongly limits oligodendrocyte depletion and demyelination. Local overexpression of OSM increases IL-4 expression and induces polarization of microglia/macrophages to a M2 phenotype. These data reveal that OSM protects against demyelination and suggest that it is a promising

therapeutic candidate to prevent CNS damage in MS and other demyelinating disorders.

4.2 Materials and methods

4.2.1 Animals and cuprizone treatment

OSMR knock-out mice were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan and were generated as previously described [245, 246]. For experiments with CNS-targeted lentiviral overexpression, 5 week old male C57BL/6J mice were purchased from Harlan (Horst). Animals were housed in an accredited animal facility under a 12h light/dark cycle and had free access to food and water. Eight week old mice were fed a diet containing 0.2% cuprizone (Sigma-Aldrich), which was homogenously mixed into powdered rodent chow and renewed three times a week. All animal procedures were in accordance with the EU directive 2010/63/EU and were approved by the Hasselt University's Ethics Committee for Animal Experiments.

4.2.2 Lentiviral vector construction and administration

Murine OSM gene transcript (NM_001013365) was isolated from RAW 264.7 cells (ATCC) after stimulation with 100 ng/ml LPS (Sigma-Aldrich) for 24h. Complementary DNA was cloned into a lentiviral transfer plasmid containing a central polypurine tract sequence, the SIN-18 deletion and the woodchuck hepatitis posttranscriptional regulatory element [240]. HIV-1 derived vector particles were produced by a triple transient transfection of 293T cells as described previously [180]. Briefly, cells were transfected with a second-generation packaging plasmid, a plasmid encoding the glycoprotein G of vesicular stomatitis virus and a transfer plasmid encoding the OSM gene under control of a cytomegalovirus promoter. This mixture combined, with a 1.42 µM polyethyleneimine solution in 150 mM NaCl, was added to 293T cells in OPTI-MEM (Life technologies) free of serum. Vector particles in the supernatant were collected and filtered 48h and 72h post transfection and concentrated using

Vivaspin 15 columns (Vivascience). p24 antigen content was measured using HIV-1 p24 Core Profile ELISA (DuPont).

Six week old C57BL/6J mice were stereotactically injected with LV-OSM, LV-eGFP or saline in the striatum two weeks before starting cuprizone treatment. Mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg) and placed in a stereotactic head frame (Stoelting). A midline incision of the skin was made and a small hole was drilled into the skull. At a rate of 0.25 µl/min 4 µl of concentrated vector ($3.43*10^7$ pg p24/ml) was injected using a 10 µl Hamilton syringe with a 30-gauge needle. Coordinates for injection were anteroposterior 0.5 mm, lateral 2.2 mm and dorsoventral -2.0 mm using bregma as reference.

4.2.3 Histochemistry

Mice were transcardially perfused with Ringer's solution, their brains dissected and snap frozen in liquid nitrogen. Ten micrometer cryosections were cut using the Leica CM3050S cryostat (Leica Microsystems). For Luxol Fast Blue (LFB) staining, sections were fixed in acetone, followed by incubation in LFB solution at 56°C for 16h. Sections were then differentiated in 0.5% lithium carbonate solution and counterstained with cresyl violet (Sigma-Aldrich). Microscopic analysis was performed using an Eclipse 80i microscope (Nikon) and for image collection the Nis-Elements Basic Research version 2.3 microscopy software was used. LFB staining was used to quantify the myelinated area in the corpus callosum of 4 to 5 animals per group (4 to 5 sections per animal) using Nis-Elements software. Immunohistochemistry was performed by fixation of the cryosections in acetone, subsequent blocking with 10% goat serum and incubation with rat anti-OSMRβ (1:50, R&D systems), rabbit anti-IBA1 (1:350, WAKO), mouse anti-NeuN (1:100, Millipore), rat anti-F4/80 (1:100, AbD serotec), mouse anti-glial fibrillary protein (GFAP; 1:500, Sigma-Aldrich) and anti-CD206 antibodies (1:150, AbD serotec). Immunoreactivity was visualized using Alexa 488 or 555-conjugated secondary

antibodies (Life technologies) and nuclear staining was performed with DAPI (Life technologies). The GFAP$^+$ area and F4/80$^+$ area were measured based on intensity and the number of CD206$^+$ cells was counted in 4 to 5 animals per group (6 photos per animal) using the Nis-Elements software. For CC-1 immunostaining (APC, mouse, 1:200, Millipore) the Vector® mouse on mouse (M.O.M.™) Immunodetection Kit (Vector Laboratories, Peterborough, UK) was used according to the manufacturer's instructions. The number of CC1$^+$ cells in the corpus callosum was counted in 4 to 5 animals per group (9 photos per animal) based on the presence of nuclei using the Nis-Elements software.

4.2.4 Quantitative PCR

Brains of healthy and cuprizone-treated mice (n=5 per group) were isolated after transcardial perfusion with Ringer's solution. RNA was isolated from the brains using the RNease Lipid Tissue Mini Kit (Qiagen) according to manufacturer's instructions. Conversion of RNA to cDNA was performed utilizing qScript™ cDNA SuperMix (Quanta Biosciences). Quantitative PCR was performed using a StepOnePlus Real-Time PCR detection system (Applied Biosystems) and universal cycling conditions (20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C). The PCR reaction mix contained fast SYBR green master mix (Applied Biosystems), 10 µM forward and reverse primer (Eurogentec), RNase free water and 12.5 ng template cDNA. Primers (5'->3') used for qPCR were OSMRβ, Fw TCACAACTCCAGATGCACGC, Rv ACTTCTCCTTCACCCACTGAC; MBP, Fw GGCTGTGCCACATGTACAAGGACT, Rv TGGGATGGAGGTGGTGTTCGAGG. To measure IL-4 expression commercially available primers were used (Taqman Gene Expression Assays, Applied Biosystems). Expression was normalized using the two most stable reference genes and converted to fold change as compared to healthy mice using comparative Ct method.

4.2.5 Viability assay

The oligodendrocyte cell line, OliNeu, was plated at a density of 25.000 cells/well. Cells were pre-treated with 0, 5, 50, 200 ng/ml OSM (R&D systems) for 4h. Next, cell death was induced by adding 50 ng/ml TNF-α and IFN-γ (both Peprotech, London, UK) and after 72h cell viability was assessed using 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. MTT (Sigma-Aldrich) was added to the cells at a final concentration of 0.5 mg/ml. After 4h, the supernatant was removed and dimethyl sulfoxide and 0.1 M glycine (both Sigma-Aldrich) were added. The absorbance was recorded at 550 nm in a microplate reader.

4.2.6 Statistical analysis

All data are expressed as mean ±SEM, *=p<0.05, **=p<0.01 and ***p=<0.001. Statistical differences between groups were determined using an unpaired two-sample t test or one-way ANOVA with Tukey's multiple comparison test. Analyses were performed using GraphPad Prism 5.0 software (GraphPad software Inc.).

4.3 Results

4.3.1 OSMR expression is robustly upregulated during demyelination

We first measured the expression level of the OSMR in the brain at different time points of cuprizone-induced demyelination. mRNA expression of the OSMR was upregulated after 3 days of cuprizone diet (3.6-fold compared to healthy mice) and persisted during the entire demyelination period of 5 weeks (Fig. 4.1A). A peak in receptor expression was detected 7 days after starting the cuprizone diet (7.5-fold) (Fig. 4.1A). In healthy mice, high numbers of neurons expressed the OSMR as revealed by NeuN immunoreactivity (Fig. 4.1B). The OSMR was also detected on low numbers of $CC1^+$ oligodendrocytes, $GFAP^+$ astrocytes and $IBA1^+$ microglia (Fig. 4.1C-E). While cuprizone-induced demyelination did not affect OSMR expression on neurons or oligodendrocytes (data not shown), it was strongly upregulated on both astrocytes and microglia/macrophages (Fig. 4.1F-G). These data reveal that the OSMR is robustly increased upon demyelination, mainly on the astrocytes and microglia.

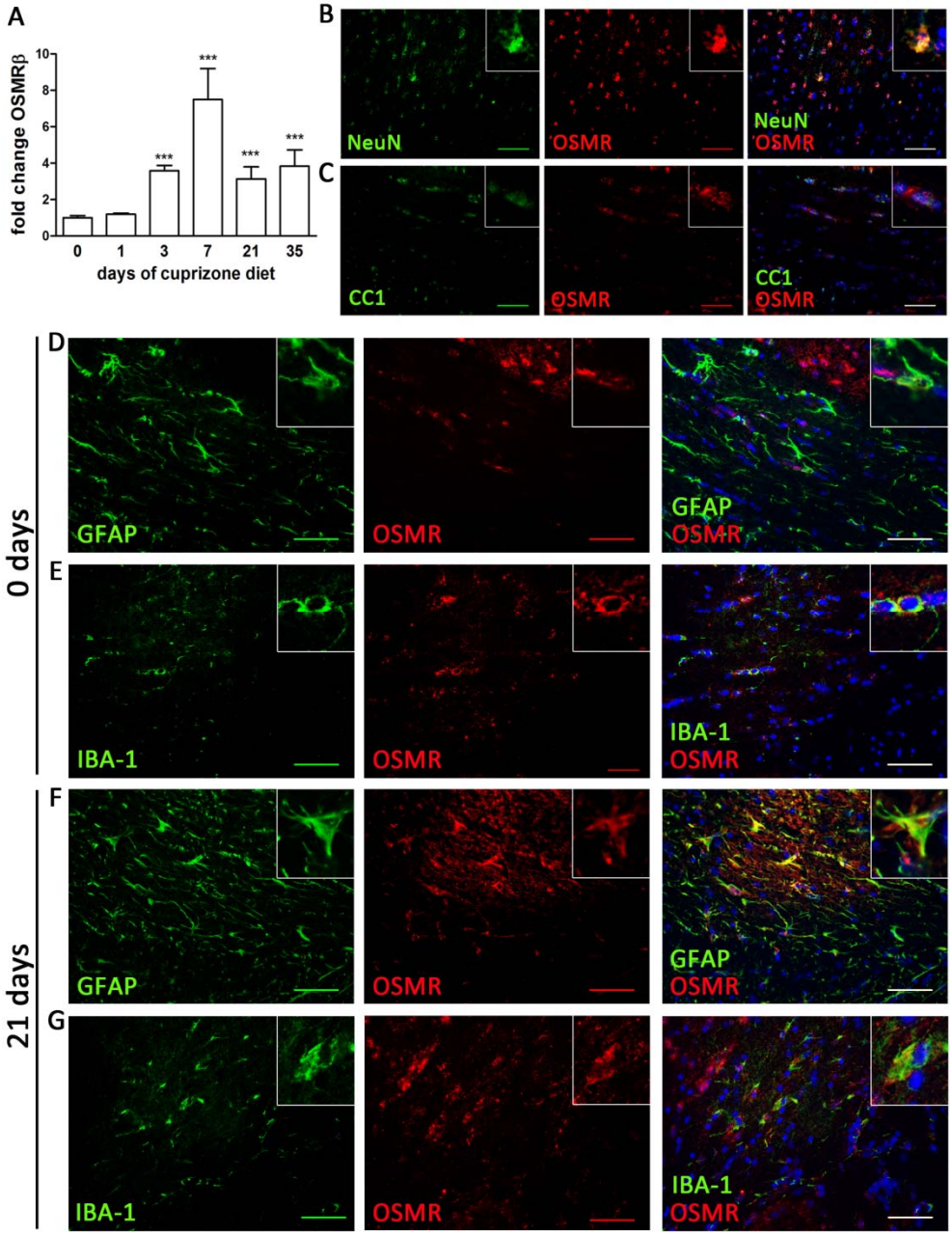

Fig. 4.1. Demyelination enhances OSMR expression on astrocytes and microglia. (A) OSMR mRNA levels were measured in the brain of mice receiving cuprizone diet for 0, 1, 3, 7, 21 and 35 days using QPCR (n=5 per time point). Expression levels were converted to fold change as compared OSMR expression in brains of healthy mice (0 days of cuprizone

diet). Data are depicted as mean ±SEM, ***p<0.001. **(B-C)** OSMR expression on $NeuN^+$ neurons (B) and $CC1^+$ oligodendrocytes (C) in healthy mice detected using immunohistochemistry. **(D-G)** OSMR expression on $GFAP^+$ astrocytes (D,F) and IBA^+ microglia/macrophages (E,G) in healthy mice (0 days; D-E) or 21 days (F-G) after start of the cuprizone diet. Magnifications of double positive cells are shown in upper right corner. Scale bars represent 50µm.

4.3.2 OSMR deficiency aggravates demyelination

To determine the involvement of OSMR signaling in demyelination, the cuprizone model was applied in OSMR knock-out mice. OSMR deficient mice displayed a stronger reduction of MBP mRNA expression after 7 and 21 days of cuprizone diet as compared to wild type mice, indicating that demyelination is exacerbated when animals lack the OSMR (Fig. 4.2A). Indeed, the myelinated area in the corpus callosum was more robustly reduced in OSMR knock-out mice compared to WT mice after 21 days of cuprizone diet (0.218 ±0.013 mm^2 vs 0.328 ±0.007 mm^2) (Fig. 4.2B-C). These data provide evidence that demyelination aggravates in absence of OSMR signaling.

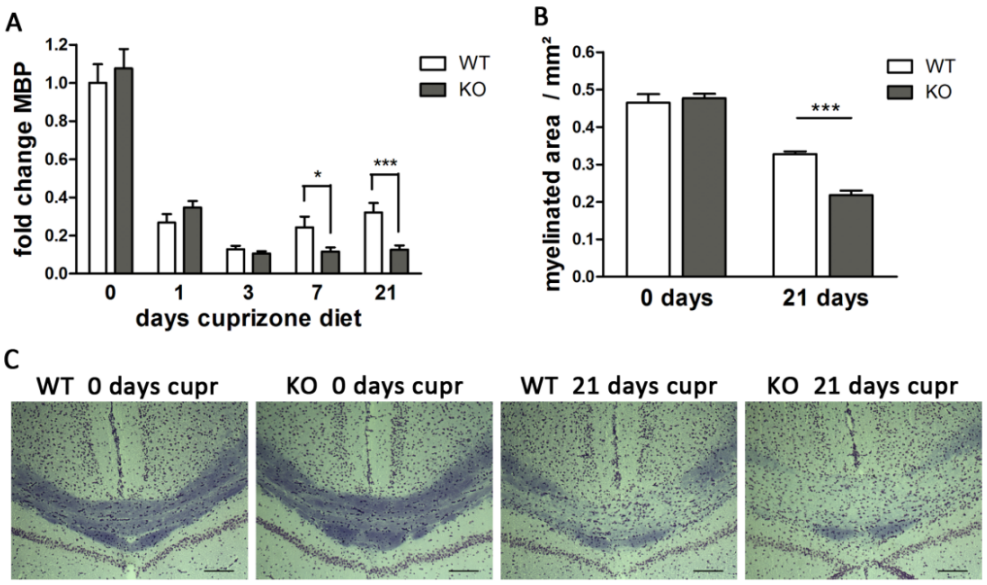

Fig. 4.2. OSMR deficient mice show enhanced demyelination following cuprizone challenge. (A) MBP mRNA expression in the brain of wild type and OSMR knock-out mice at day 0, 1, 3, 7 and 21 after cuprizone feeding (n=5 per group). Expression was converted to fold change as compared to MBP expression in brains of healthy wild type mice. **(B-C)** Quantification and representative pictures of the myelinated area measured in the corpus callosum using LFB staining in wild type and OSMR knock-out mice receiving standard diet (n=4 per group) or cuprizone diet for 21 days (n=5 per group). Data are depicted as mean ±SEM, *p<0.05, ***p<0.001. Scale bars represent 200 µm. WT, wild type; KO, knock-out; cupr, cuprizone.

4.3.3 CNS-targeted OSM treatment strongly limits demyelination

Since lack of OSMR signaling results in more pronounced demyelination, we next determined whether OSM treatment by means of LV-mediated delivery to the CNS can prevent cuprizone-induced demyelination. Two weeks before starting the cuprizone diet, LV-OSM was injected in the right striatum resulting in stable OSM expression in the striatum and the overlying corpus callosum. CNS-targeted OSM treatment strongly limited myelin loss in the corpus callosum (myelinated area;

0.521 ± 0.021 mm²) in comparison to the NaCl (0.273 ±0.031 mm²) and LV-eGFP (0.297 ±0.033 mm²) treated group (Fig. 4.3A-B). Moreover, OSM treatment significantly reduced mature oligodendrocyte death ($CC1^+$ cells; 362.4 ±37.4/mm²) compared to the control groups (NaCl: 129.8 ±15.8/mm², LV-eGFP: 127.9 ±19.5 mm²) (Fig. 4.3C-D). To reveal whether this was a direct protective effect of OSM on the oligodendrocytes, we induced TNF-α and IFN-γ mediated cell death in the oligodendrocyte cell line, OliNeu [247]. While cell viability decreased 72h after addition of TNF-α and IFN-γ, pretreatment with different concentrations of OSM was unable to prevent this (Fig. 4.3E). These data suggest that OSM treatment indirectly limits the depletion of mature oligodendrocytes and subsequent demyelination.

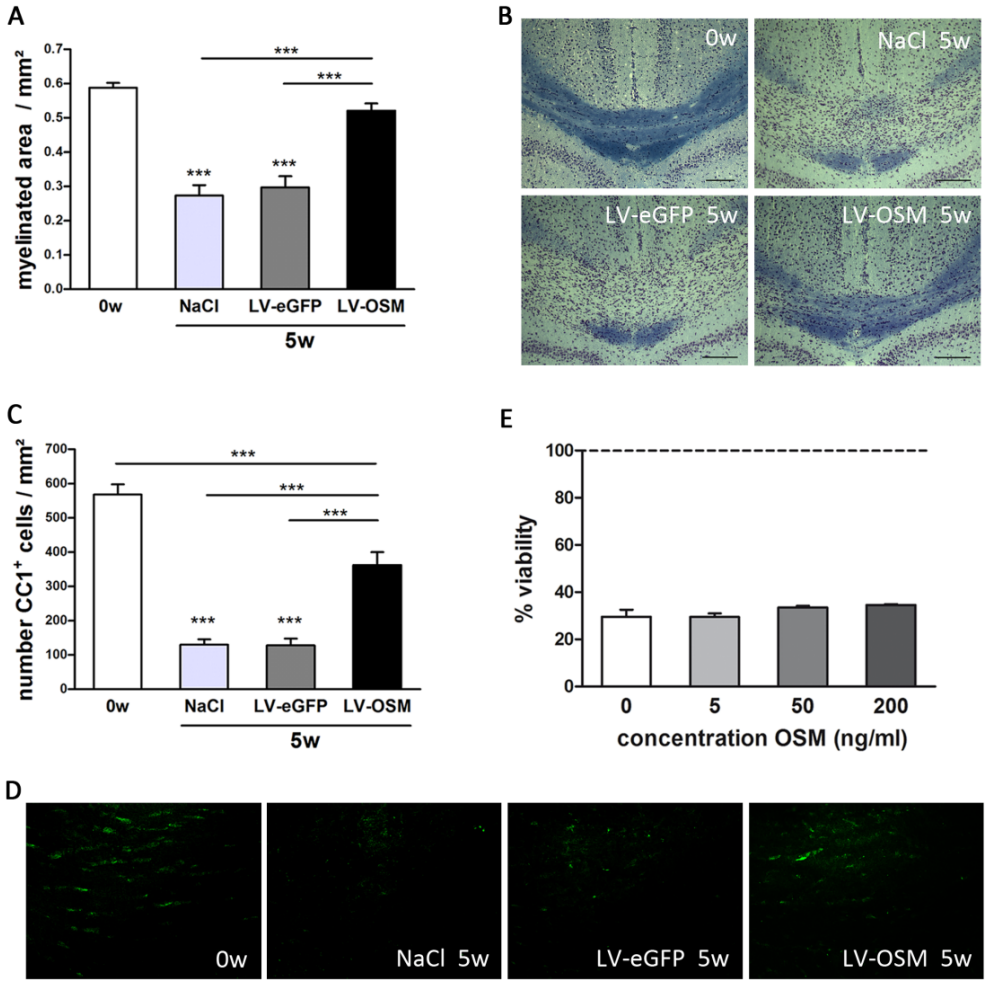

Fig. 4.3. CNS-targeted OSM treatment prevents cuprizone-induced demyelination. Two weeks after injection of NaCl, LV-eGFP or LV-OSM in the right striatum, mice received cuprizone diet for 5 weeks. **(A-B)** Quantification and representative pictures of the myelinated area in the corpus callosum measured using LFB staining (n=4 or 5 per group). **(C-D)** Quantification and representative pictures of the number of $CC1^+$ cells in the corpus callosum as analyzed by immunohistochemistry. **(E)** OliNeu cells were pretreated for 4h with 0, 5, 50, 200 ng/ml OSM, after which they were incubated with 50 ng/ml TNF-α and IFN-γ for 72h to induce cell death. Viability was measured using the MTT assay and is expressed as percentage relative to viability of cells in culture medium. Data are depicted as mean ±SEM, ***p<0.001. Scale bars represent 200 µm.

4.3.4 OSM treatment regulates the glial response following demyelination

Demyelination is accompanied by an extensive astroglial and microglial response that greatly influences the extent of demyelination [81, 243]. Indeed, strong astrogliosis was seen in the corpus callosum of the NaCl and LV-eGFP group ($GFAP^+$ area; 0.0129 ±0.0013 mm² and 0.0117 ±0.0013 mm² respectively) (Fig. 4.4A-B). LV-OSM treated mice showed a trend towards reduced astrogliosis (0.0077 ±0.0015 mm²) (Fig. 4.4A-B). Five weeks of cuprizone diet also induced a robust microglial reaction in the corpus callosum ($F4/80^+$ area; NaCl: 0.0503 ±0.0078 mm², LV-eGFP0.0510 ±0.0064 mm²), whereas this was strongly limited by CNS-targeted OSM treatment (0.0129 ±0.0051 mm²) (Fig. 4.4C-D).

Interestingly, locally at the site of injection OSM enhanced GFAP immunoreactivity (0.0427 ±0.0020 mm²) as compared to the contralateral site (0.0176 ±0.0012 mm²) and the injection site in the NaCl (0.0173 ±0.0010 mm²) and LV-eGFP group (0.0182 ±0.0010 mm²) (Fig. 4.4E-F). Moreover, microglia/macrophage accumulation was also increased locally at the site of OSM overexpression (0.0160 ±0.0031 mm² compared to 0.0001 ±0.00002 mm² contralateral, 0.0004 ±0.0002 mm² in the NaCl group and 0.0013 ±0.0005 mm² in the LV-eGFP group) (Fig. 4.4G-H). These data show that CNS-targeted OSM treatment induces an astroglial and microglial response at site of injection, while it prevents microglia/macrophage accumulation in the demyelinated corpus callosum.

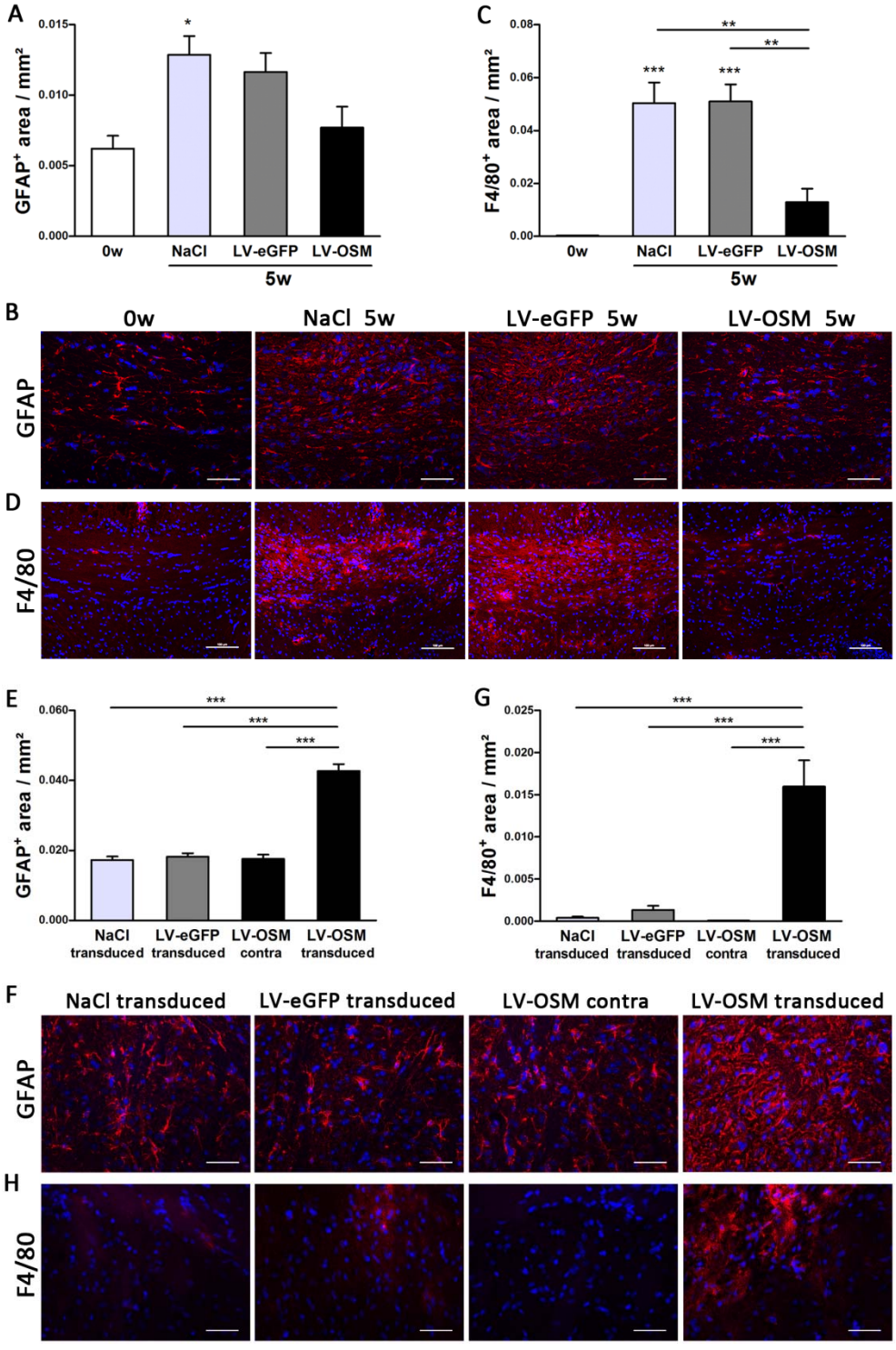
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Fig. 4.4. CNS-targeted OSM treatment regulates the glial response. (A-D) Quantification and representative pictures of GFAP (A-B) and F4/80 (C-D) immunoreactivity in the corpus callosum of healthy mice and NaCl, LV-eGFP and LV-OSM injected mice receiving cuprizone diet for 5 weeks. **(E-H)** Quantification and representative pictures of the $GFAP^+$ (E-F) and $F4/80^+$ (G-H) area at the site of injection in the NaCl, LV-eGFP and LV-OSM mice (transduced) and at the contralateral site in the LV-OSM mice (contra). Data are depicted as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001. Scale bars represent 50 μm (B, F, H) or 100 μm (D).

4.3.5 OSM treatment induces an anti-inflammatory M2 phenotype

Next, we aimed to define whether the OSM-induced glial response affected demyelination. Whereas, astrocytes and microglia can exacerbate demyelination by the production of proinflammatory cytokines, chemokines and nitric oxide, they have also been reported to protect oligodendrocytes against the demyelinating insult by producing trophic factors and anti-inflammatory cytokines [81, 244]. We measured mRNA expression of TNF-α, IL-6, IFN-γ, IL-1β, IL-4, TGF-β, IGF-1, LIF, CNTF, PDGF and NT-3 in the brain after 5 weeks of demyelination. No difference in expression of proinflammatory cytokines and trophic factors was found between the LV-OSM and LV-eGFP treated mice (data not shown). Interestingly, IL-4 mRNA levels were 3.5 times higher in the region of the corpus callosum of the OSM-treated mice compared to the LV-eGFP group (Fig. 4.5A). Moreover, increased immunoreactivity of the M2 macrophage marker CD206 was detected in the corpus callosum and at the injection site of OSM-treated mice compared to the control treatment groups (Fig. 4.5B-D). These data reveal that CNS-targeted OSM treatment stimulates IL-4 expression and M2 polarization during demyelination.

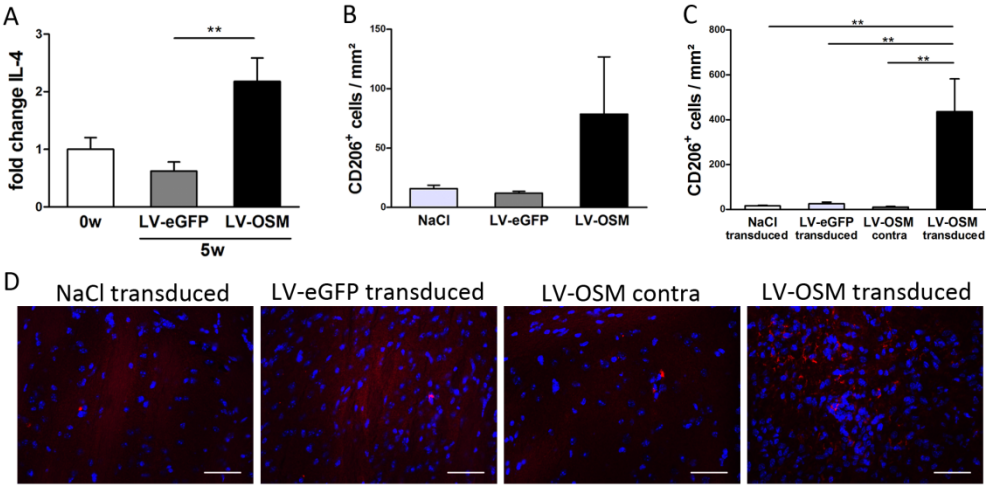

Fig. 4.5. CNS-targeted OSM treatment promotes IL-4 expression and M2 polarization. (A) IL-4 mRNA expression in the region of the corpus callosum in healthy mice and LV-eGFP and LV-OSM injected mice receiving cuprizone diet for 5 weeks (n=5 per group). **(B)** Quantification of $CD206^+$ cells in the corpus callosum of NaCl, LV-eGFP and LV-OSM mice after 5 weeks of cuprizone diet measured using immunohistochemistry. **(C-D)** Quantification and representative pictures of $CD206^+$ cells at the site of injection in NaCl, LV-eGFP and LV-OSM mice (transduced) and at the contralateral site in the LV-OSM mice (contra). Data are depicted as mean ±SEM, **p<0.01. Scale bars represent 50 µm.

4.4 Discussion

While the available MS therapies modulate the immune response, none have shown to directly prevent or limit demyelination and are thus unable to stop the neurological decline. In this study, we demonstrate that in absence of the OSMR, which was highly upregulated during demyelination, this process was aggravated. In line with this finding, CNS-targeted OSM treatment strongly limited oligodendrocyte death and demyelination. OSM was not directly oligoprotective, but upregulated IL-4 and induced accumulation of microglia/macrophages with an anti-inflammatory M2 phenotype.

We first show that the OSMR was robustly upregulated during cuprizone-induced demyelination. This is in agreement with previous reports that demonstrated OSMR upregulation after spinal cord injury [216] and striatial injection of LPS [220]. Next, we evaluated which cells are susceptible to OSMR triggering during demyelination. Whereas the OSMR was mainly expressed on neurons in healthy mice, demyelination robustly upregulated receptor expression on astrocytes and microglia/macrophages. *In vitro*, the OSMR is detected on primary human and murine astrocytes [248, 249], whereas it is undetectable in murine microglia [248]. In contrast, others supports the presence of the OSMR on microglia, as OSM induces activation of the NFκB pathway in primary microglial cultures [250]. Our findings indicate that in pathological conditions the OSMR is highly expressed on microglia and astrocytes.

We conclude this receptor upregulation is part of an endogenous protective response, since OSMR deficiency led to enhanced demyelination. The protective property of OSMR signaling was confirmed by CNS-targeted OSM treatment, which strongly limited mature oligodendrocyte death and subsequent demyelination. The microglial/macrophage response that accompanies demyelination [251, 252] was reduced by CNS-targeted OSM treatment. Interestingly, locally at the injection site OSM induced microglia/macrophage

accumulation. The microglia/macrophages present at the site of OSM overexpression and the corpus callosum were found to have an anti-inflammatory M2 phenotype. Additionally, we detected an upregulation of IL-4 expression in the OSM-treated mice. IL-4 is an anti-inflammatory cytokine and a potent inducer of M2 polarization [72]. In demyelinated MS lesions, IL-4 is produced by reactive astrocytes [253], whereas in the animal model of MS, EAE, microglia were found to express high levels of IL-4 mRNA [254]. IL-4 production in the CNS is reported to be essential for the induction and maintenance of M2 microglia/macrophages, and for controlling clinical symptoms in EAE [254]. While this is the first study demonstrating that OSM treatment can induce M2 polarization in the CNS, others showed that OSM treatment polarizes adipose tissue macrophages to a M2 phenotype [229]. Furthermore, hypoxic breast cancer cells release OSM, thereby attracting macrophages and promoting their M2 polarization [230]. In contrast to other members of the IL-6 cytokine family such as LIF, IL-11 and CNTF [176, 255, 256], OSM did not directly promote the survival of oligodendrocytes. Thus members of this cytokine family have overlapping as well as unique effects in the CNS.

In conclusion, this study identifies a novel and crucial role of OSM in the demyelination process. The beneficial effect on demyelination together with it neuroprotective properties reported earlier indicate that OSM is a promising therapeutic candidate to limit CNS damage in MS and other demyelinating disorders.

5

OSM receptor signaling is essential for remyelination

Based on:

Oncostatin M receptor signaling is essential for remyelination

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Submitted

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Abstract

OPCs migrate to demyelinated lesions in the CNS, differentiate into mature oligodendrocytes and restore myelin sheaths. This remyelination process is often insufficient in MS patients, resulting in disturbed neuronal signaling and leaving denuded axons vulnerable for degeneration. Successful remyelination is highly context-dependent as environmental cues dictate OPC differentiation and myelination. The molecular frame-work that regulates these processes is poorly understood. In this study, we identify OSM, a member of the IL-6 cytokine family, as a crucial contributor that potentiates remyelination. Expression of the OSMR was strongly upregulated during remyelination. While, in OSMR knock-out mice OPC proliferation was reduced and remyelination was completely abrogated, CNS-targeted OSM treatment increased the number of newly formed oligodendrocytes and induced remyelination. OSMR triggering stimulated a beneficial astroglial response and tissue inhibitor of metalloproteinase-1 (TIMP-1) produced by astrocytes was shown to be one of the determining factors involved in OSM-mediated remyelination. This study reveals a crucial role for astrocytic OSMR signaling during remyelination and suggests that OSM is a promising therapeutic candidate for MS and other demyelinating diseases.

5.1 Introduction

MS is a chronic disabling disease of the CNS, which leads to demyelinated lesions throughout the white and grey matter [4]. In response to environmental cues, OPCs proliferate and migrate to the lesion site, where they differentiate into oligodendrocytes and enwrap naked axons with new myelin sheaths [99]. This remyelination process restores saltatory conduction, protects axons from degeneration, and resolves functional deficits [94, 257, 258]. Despite the presence of OPCs in demyelinated lesions of MS patients, remyelination is variable and insufficient [259-261]. Astrocytes and microglia are crucially involved in the formation of oligodendrocytes and myelination of axons by producing neurotrophic factors, inhibitory molecules and inflammatory cytokines [78, 244]. While it is clear that a permissive environment is essential to allow robust remyelination, the molecular signals that regulate this are far from understood.

A possible important factor is OSM that is produced by microglia, hypertrophic astrocytes and infiltrated immune cells in demyelinated lesions [215]. OSM belongs to the gp130 cytokine family, which activates signaling pathways important for remyelination [187, 262]. OSM limits neuronal cell death caused by excitotoxicity *in vitro* and *in vivo* [217, 218]. Moreover, OSM promotes neurite outgrowth of primary cortical neurons [216]. Following spinal cord injury, OSM treatment reduces lesion size, promotes sprouting of nerve fibers and induces functional recovery [216]. While these reports established the neuroprotective properties of OSM, its role in remyelination remains elusive.

In this study, the cuprizone model is used as a reproducible and well-characterized model of demyelination in which mature oligodendrocytes are almost completely depleted. This is followed by remyelination when omitting cuprizone from the diet [115]. We demonstrate that the OSMR is highly upregulated during remyelination. In OSMR deficient mice remyelination is completely absent, whereas CNS-targeted OSM treatment induces remyelination.

We show that tissue inhibitor of metalloproteinase-1 (TIMP-1), which is produced by astrocytes, is one of the determining factors involved in OSM-mediated remyelination. In conclusion, this study reveals a previously uncharacterized and crucial role for OSMR signaling in remyelination and suggests that OSM is a promising potential therapy for MS and other demyelinating diseases

5.2 Materials and methods

5.2.1 Animals

OSMR knock-out mice were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan and were generated as previously described [245, 246]. For experiments with CNS-targeted lentiviral overexpression, 7 week old male C57BL/6J mice were purchased from Harlan (Horst). Animals were housed in an accredited animal facility under a 12h light/dark cycle and had free access to food and water. All animal procedures were in accordance with the EU directive 2010/63/EU and were approved by the Hasselt University ethics committee for animal experiments.

5.2.2 Cuprizone treatment

Eight week old mice were fed a diet containing 0.2% cuprizone (Sigma-Aldrich), which was homogenously mixed into powdered rodent chow and renewed three times a week. Two experimental set-ups were applied. First, OSMR knock-out and wild type mice received a cuprizone diet for 5 weeks to induce acute demyelination, followed by 1 or 2 weeks of standard diet to allow spontaneous remyelination. Second, to reveal the effect of CNS-targeted OSM treatment on remyelination, mice were fed with a cuprizone diet for 12 weeks to induce chronic demyelination, followed by 1 or 2 weeks of standard diet to allow remyelination. Chronic demyelination reduces the rate of subsequent spontaneous remyelination, allowing more sensitive detection of beneficial effects on the remyelination process. After 11 weeks of cuprizone diet, mice were stereotactially injected with LV-OSM or LV-eGFP. Analysis of mRNA levels was performed after 1 week of remyelination using QPCR and protein levels after 2 weeks of remyelination utilizing immunohistochemical analysis.

5.2.3 Lentiviral vector construction and administration

Murine OSM gene transcript (NM_001013365) was isolated from RAW 264.7 cells (ATCC) after stimulation with 100 ng/ml LPS (Sigma-Aldrich) for 24h. Complementary DNA was cloned into a lentiviral transfer plasmid containing a central polypurine tract sequence, the SIN-18 deletion and the woodchuck hepatitis posttranscriptional regulatory element [240]. HIV-1 derived vector particles were produced by a triple transient transfection of 293T cells as previously described [180]. Briefly, cells were transfected with a second-generation packaging plasmid, a plasmid encoding the glycoprotein G of vesicular stomatitis virus and a transfer plasmid encoding the OSM gene under control of a cytomegalovirus promoter. This mixture combined, with a 1.42 µM polyethyleneimine solution in 150 mM NaCl, was added to 293T cells in OPTI-MEM (Life technologies) free of serum. Vector particles in the supernatant were collected and filtered 48h and 72h post transfection and concentrated using Vivaspin 15 columns (Vivascience). p24 antigen content was measured using HIV-1 p24 Core Profile ELISA (DuPont).

After 11 weeks of cuprizone diet mice were stereotactically injected with LV-OSM or LV-eGFP in the right striatum. Mice were anesthetized with ketamine (75 mg/kg) and medetomidine (1 mg/kg) and placed in a stereotactic head frame (Stoelting). Next, a midline incision of the skin was made and a small hole drilled into the skull. Coordinates for injection were anteroposterior 0.5 mm, lateral 2.2 mm and dorsoventral -2.0 mm using bregma as reference. At a rate of 0.25 µl/min, 4 µl of concentrated vector ($3.43*10^7$ pg p24/ml) was injected using a 10 µl Hamilton syringe with a 30-gauge needle.

5.2.4 Histochemistry

Mice were transcardially perfused with Ringer's solution, their brains dissected and snap frozen in liquid nitrogen. Ten micrometer cryosections were cut using

the Leica CM3050S cryostat (Leica Microsystems). LFB staining was performed by fixing the cryosections in acetone, followed by incubation in LFB solution at 56°C for 16h. Sections were then differentiated in 0.5% lithium carbonate solution and counterstained with cresyl violet (Sigma-Aldrich). Microscopic analysis was performed using an Eclipse 80i microscope (Nikon) and for image collection the Nis-Elements Basic Research version 2.3 microscopy software was used. LFB staining was used to quantify the myelinated area in the corpus callosum of 4 to 5 animals per group (4 to 5 sections per animal) using Nis-Elements software.

For immunohistochemistry, sections were fixed in acetone, blocked with 10% goat serum and incubated with rabbit anti-NG2 (1:200, Millipore), rat anti-F4/80 (1:100, AbD serotec), mouse anti-GFAP (1:500, Sigma-Aldrich), rat anti-TIMP-1 (1:50, Abcam) and goat anti-OSM antibodies (1:50, R&D systems). Binding of the primary antibodies was visualized with Alexa 488 or 555-conjugated secondary antibodies and nuclear staining was performed with DAPI (both Life technologies). The number of $NG2^+$ cells was counted and the $GFAP^+$ and $F4/80^+$ area were measured based on intensity in 4 to 5 animals per group (6 photos per animal) using the Nis-Elements software. For CC-1 immunostaining (APC, mouse, 1:200, Millipore) the Vector® Mouse On Mouse Immunodetection Kit (Vector Laboraties) was used according to the manufacturer's instructions. The number of $CC1^+$ cells in the corpus callosum was counted in 4 to 5 animals per group (6 photos per animal) based on the presence of nuclei using the Nis-Elements software.

5.2.5 Quantitative PCR

Brains of healthy and cuprizone-treated mice (n=5 per group) were isolated after transcardial perfusion with Ringer's solution. RNA was isolated from the brains using the RNease Lipid Tissue Mini Kit (Qiagen) according to manufacturer's instructions. Conversion of RNA to cDNA was performed using qScript™ cDNA SuperMix (Quanta Biosciences). Quantitative PCR was performed utilizing a

StepOnePlus Real-Time PCR detection system (Life technologies) and universal cycling conditions (20s at 95°C, 40 cycles of 3s at 95°C and 30s at 60°C). The PCR reaction consisted of fast SYBR green master mix (Life technologies), 10µM forward and reverse primer (Eurogentec), RNase free water and 12.5 ng template cDNA. Primers (5'->3') used for qPCR were OSMRβ, Fw TCACAACTCCAGATGCACGC, Rv ACTTCTCCTTCA CCCACTGAC; MBP, Fw GGCTGTGCCACATGTACAAGGACT, Rv TGGGATGGAGGTGGTGTTCGAGG; TIMP-1, Fw GGACCTGGTCATAAGGGCTA, Rv TACCGGATATCTGCGGCATT; CNTF, Fw AGAGAGTGCATTTCACCCCG, Rv TCTGTTCCAGAAGCGCCATT; LINGO-1, Fw ACCTTCGCCTTCATCTCCAA, and Rv CAGGCAGAATAGGACAACGC. Expression was normalized using the two most stable reference genes and converted to fold change as compared to healthy mice using the comparative Ct method.

5.2.6 Transmission electron microscopy

Sample preparation for transmission electron microscopy (TEM) was performed as previously described with minor modifications [263]. Mice were transcardially perfused with Ringer's solution, followed by 2% gluteraldehyde in 0.05 M cacodylate buffer (pH 7.3) at 4°C. A coronal block of the corpus callosum (1 mm thick) within the anterioposterior coordinates from -0.3 to -1.5 mm was cut in midsagital plane. Tissue blocks were post fixed in 2% osmium tetroxide (Aurion, Wageningen, the Netherlands), dehydrated through graded concentrations of acetone and embedded in araldite epoxy resin (Aurion). To delineate the region of interest, semithin sections of 0.5 µm were cut using a Leica EM UC6 microtome (Leica, Groot-Bijgaarden, Belgium) and stained with 0.1% thionin and methylene blue solution for light microscopic selection. Ultrathin sections of 0.06 µm were mounted on 0.7% formvar-coated 50-µm mesh copper grids (Aurion) and automatically contrasted using a Leica EM AC20 (Leica) with 0.5% uranyl-acetate followed by a stabilized solution of lead citrate (both from Laurylab, Saint Fons,

France). TEM analysis was performed with a Philips EM208 transmission electron microscope (Philips, Eindhoven, The Netherlands) equipped with a Morada Soft Imaging System camera (Olympus SIS, Münster, Germany). The G-ratio was calculated as axon diameter/fiber diameter in 5 representative pictures per animal in which 50 fibers per image were analyzed using Image J software.

5.2.7 Isolation primary astrocytes

Brains were dissected from P2 C57BL/6J pups to obtain primary astrocytes, cortices isolated and the meninges removed. The cortices were then homogenized using incubation in papain solution at 37°C. The cell suspension was washed and triturated using sterile glass Pasteur pipettes. The mixed glial culture was transferred to a poly-L-lysine (5µg/ml) coated flask containing DMEM (Life technologies) supplemented with 10% FCS (Hyclone Europe), 50 U/ml penicillin and 50 mg/ml streptomycin (Life technologies) and incubated at 37°C and 8.5% CO_2. After 14 and 21 days non-adherent cells (oligodendrocytes and microglia) were removed by shaking the flask at 220 rpm overnight.

5.2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad software Inc.). Differences between group means were determined using an unpaired two-sample t test or one-way ANOVA with Tukey's multiple comparison test and all data depicted as mean ±SEM, *=p<0.05, **=p<0.01 and ***p=<0.001.

5.3 Results

5.3.1 OSMR signaling is crucial for remyelination

To determine the involvement of OSMR signaling in remyelination, we first measured the expression level of the OSMR in the brain of cuprizone-challenged mice. After 5 weeks of cuprizone-induced demyelination, mRNA expression of the OSMR was strongly upregulated, increasing 5.3-fold compared to healthy mice (Fig. 5.1A). This enhanced OSMR expression persisted during remyelination (3.3-fold after 1 week of standard diet) (Fig. 5.1A). To define the functional significance of OSMR upregulation, OSMR knock-out mice were used. Five weeks of cuprizone diet induced demyelination to a similar extent in both wild type and OSMR knock-out mice, as evidenced by a reduction in MBP mRNA expression in the brain (Fig. 5.1B). Moreover, the myelinated area in the corpus callosum was reduced in both wild type and knock-out mice (0.193 ±0.026 mm^2 and 0.165 ±0.009 mm^2 respectively compared to 0.547 ±0.042 mm^2 and 0.521 ±0.019 mm^2 in healthy mice) (Fig. 5.1C-D). In wild type mice, MBP mRNA expression was strongly upregulated after 1 week of remyelination, whereas this upregulation was completely absent in OSMR knock-out mice (Fig. 5.1B). Moreover, no increase in myelinated area in the corpus callosum was found in OSMR knock-out mice (0.253 ±0.022 mm^2) in contrast to what was found in wild type mice (0.452 ±0.016 mm^2) (Fig. 5.1C-D). Taken together, in absence of OSMR signaling remyelination is completely abrogated.

Five weeks of cuprizone diet reduced the number of $CC1^+$ mature oligodendrocytes in the corpus callosum of both wild type and OSMR knock-out mice (Fig. 5.1E). This depletion of mature oligodendrocytes was accompanied by a strong increase in the number of $NG2^+$ OPCs in wild type mice, while in the OSMR knock-out mice this was less pronounced (820.3 ±88.6/mm^2 vs 585.1 ±65.8/mm^2) (Fig. 5.1F). A robust increase in $CC1^+$ oligodendrocytes was found in wild type

mice, but not in OSMR knock-out mice following the 2 weeks remyelination period (766.5 ±54.0/mm² vs 439.7 vs 21.9/mm²) (Fig. 5.1E). In conclusion, lack of OSMR signaling resulted in decreased OPC numbers following acute demyelination, thereby reducing the numbers of newly formed mature oligodendrocytes and ultimately leading to failure of remyelination.

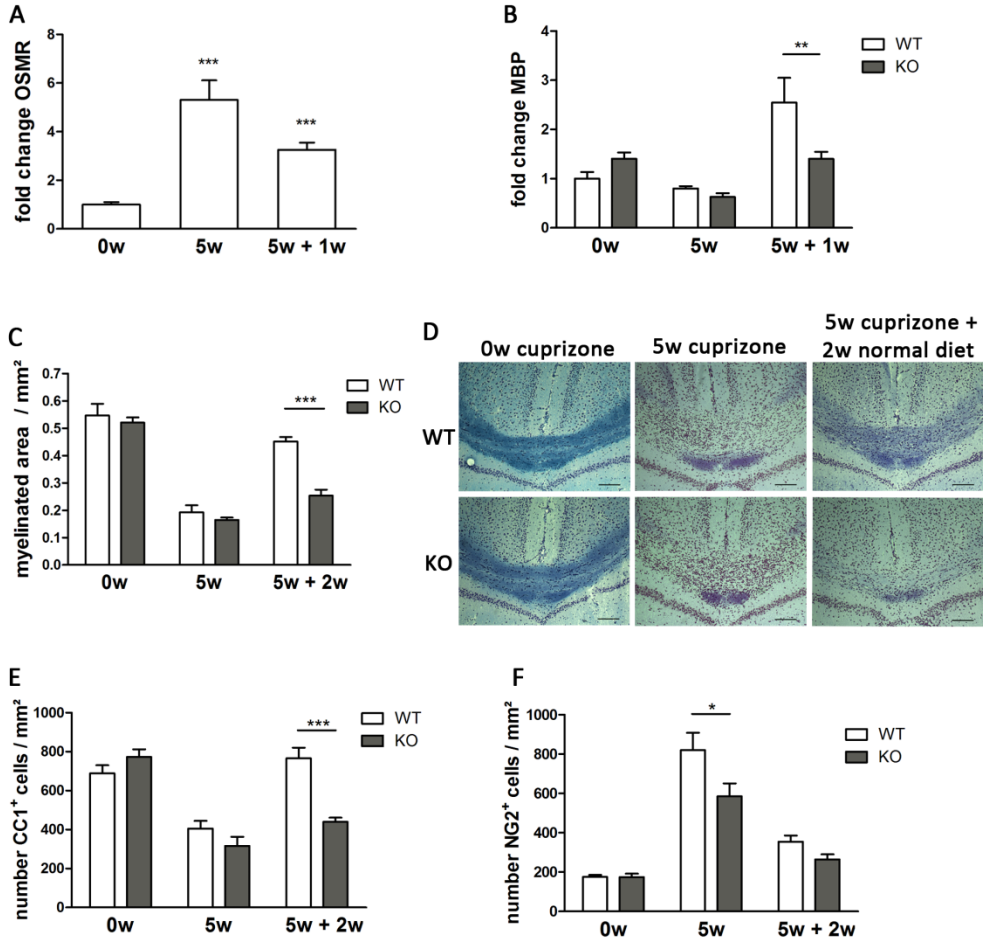

Fig. 5.1. OSMR signaling is essential for remyelination. (A) OSMR expression was measured in healthy mice (0 weeks cuprizone diet), after acute demyelination (5 weeks cuprizone diet) and during remyelination (5 weeks cuprizone diet followed by 1 week standard diet) using qPCR (n=5 per group). **(B)** MBP mRNA expression in the brain of wild type and OSMR knock-out mice receiving standard diet, after acute demyelination and

after remyelination measured using qPCR (n=5 per group). **(C-F)** Quantification and representative pictures of the LFB staining (C-D), $CC1^+$ cells (E) and $NG2^+$ cells (F) in the corpus callosum of wild type and OSMR knock-out mice receiving standard diet (n=4 per group), after acute demyelination (n=4 per group), and acute demyelination followed by remyelination (n=5 per group). Data are depicted as mean ±SEM, * $p<0.05$ and *** $p<0.001$. Scale bars represent 200 μm. WT, wild type; KO, knock-out.

5.3.2 CNS-targeted OSM treatment induces remyelination

Since OSMR deficiency abrogated remyelination, we next investigated whether OSMR triggering, through therapeutic application of a LV expressing OSM, is able to stimulate remyelination. Mice received a cuprizone diet for 12 weeks to induce chronic demyelination, resulting in pronounced depletion of oligodendrocytes and their progenitors, and thereby limiting the rate of spontaneous remyelination after cuprizone withdrawal [118]. LV-OSM, or control LVs (LV-eGFP), was injected in the right striatum after 11 weeks of cuprizone diet to ensure stable expression during the remyelination period (Fig. 5.2A). While administration of LV-eGFP did not induce remyelination in the corpus callosum (0.235 ±0.029 mm^2 vs 0.176 ±0.016 mm^2 after chronic demyelination), LV-OSM markedly enhanced the myelinated area (0.391 ±0.083 mm^2) (Fig. 5.2B-C). In line herewith, CNS-targeted OSM treatment significantly decreased the G-ratio (0.817 ±0.011 vs 0.884 ±0.004 after chronic demyelination) evidencing remyelination, while no reduction in G-ratio was detected in the control group (0.870 ±0.004) (Fig. 5.2D-E). In addition, the number of $CC1^+$ mature oligodendrocytes was enhanced, further reflecting the repair-inducing properties of OSM (404.7 ±22.9/mm^2 vs 314.4 ±23.9/mm^2 in control mice) (Fig. 5.2F). Collectively, these results show that CNS-targeted OSM treatment stimulates OPC differentiation and induces remyelination.

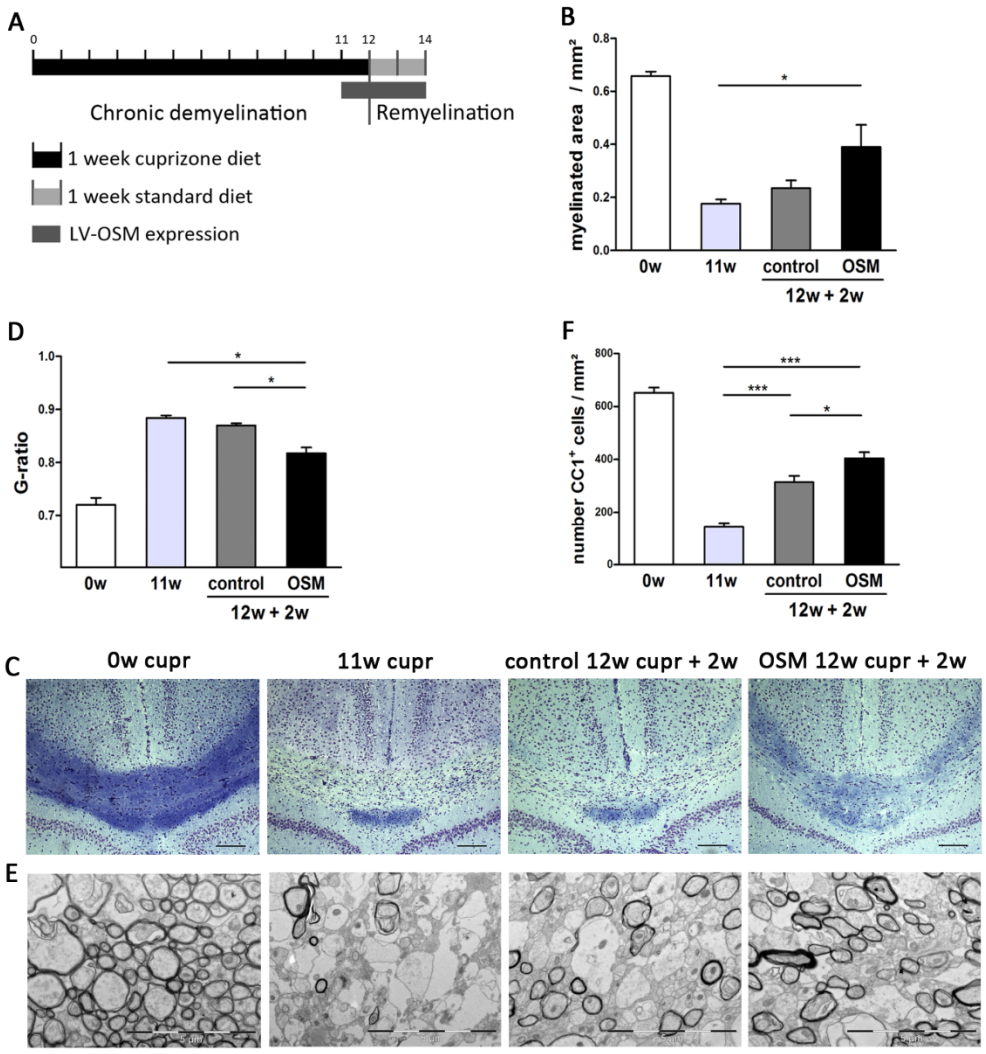

Fig. 5.2. CNS-targeted OSM treatment induces remyelination after chronic demyelination. (A) Schematic representation of cuprizone feeding; mice received cuprizone diet for 12 weeks to induce chronic demyelination, followed by 2 weeks of standard diet to allow remyelination. After 11 weeks, mice were injected with LV-OSM (OSM) or LV-eGFP (control) in the right striatum. **(B-C)** Quantification and representative images of LFB staining in the corpus callosum (n=4 or 5 per group). **(D-E)** Quantification of the G-ratio and representative TEM images of the corpus callosum (n=3 per group). **(F)** Quantification of $CC1^+$ cells measured using immunohistochemistry in the corpus callosum

(n=4 or 5 per group). Data are depicted as mean ±SEM, *p<0.05, **p<0.01 and ***p<0.001. Scale bars represent 200 µm (C) and 5 µm (E). Cupr, cuprizone.

5.3.3 OSMR signaling regulates the glial response during remyelination

Accumulating evidence indicates a crucial role for microgliosis and astrogliosis in both CNS damage and repair [78, 244]. Acute demyelination resulted in an increased F4/80 immunoreactivity, indicating microgliosis, in the corpus callosum of the OSMR knock-out mice compared to wild type mice (0.033 ±0.003 mm² and 0.022 ±0.004 mm² respectively) (Fig. 5.3A-B). This microgliosis was strongly reduced during remyelination in both the OSMR knock-out and wild type group (0.0012 ±0.0002 mm² and 0.0018 ±0.0003 mm²) (Fig. 5.3A-B). Astrogliosis, measured by GFAP immunoreactivity, was enhanced in the corpus callosum following both acute demyelination and remyelination with no difference between the OSMR knock-out and wild type mice (0.014 ±0.001 mm² vs 0.017 ±0.002 mm² respectively after 5 weeks cuprizone and 0.016 ±0.002 mm² vs 0.015 ±0.002 mm² after 2 weeks standard diet) (Fig. 5.3C-D). Interestingly, the astroglial response seen in the cortex of wild type mice after acute demyelination and remyelination (0.029 ±0.003 mm² and 0.032 ±0.002 mm² respectively) was completely absent in the OSMR knock-out mice (0.003 ±0.001 mm² and 0.003 ±0.001 mm²) (Fig. 5.3E-F). These results indicate that lack of OSMR signaling enhances microgliosis following acute demyelination and prevents the astroglial response in the adjacent grey matter.

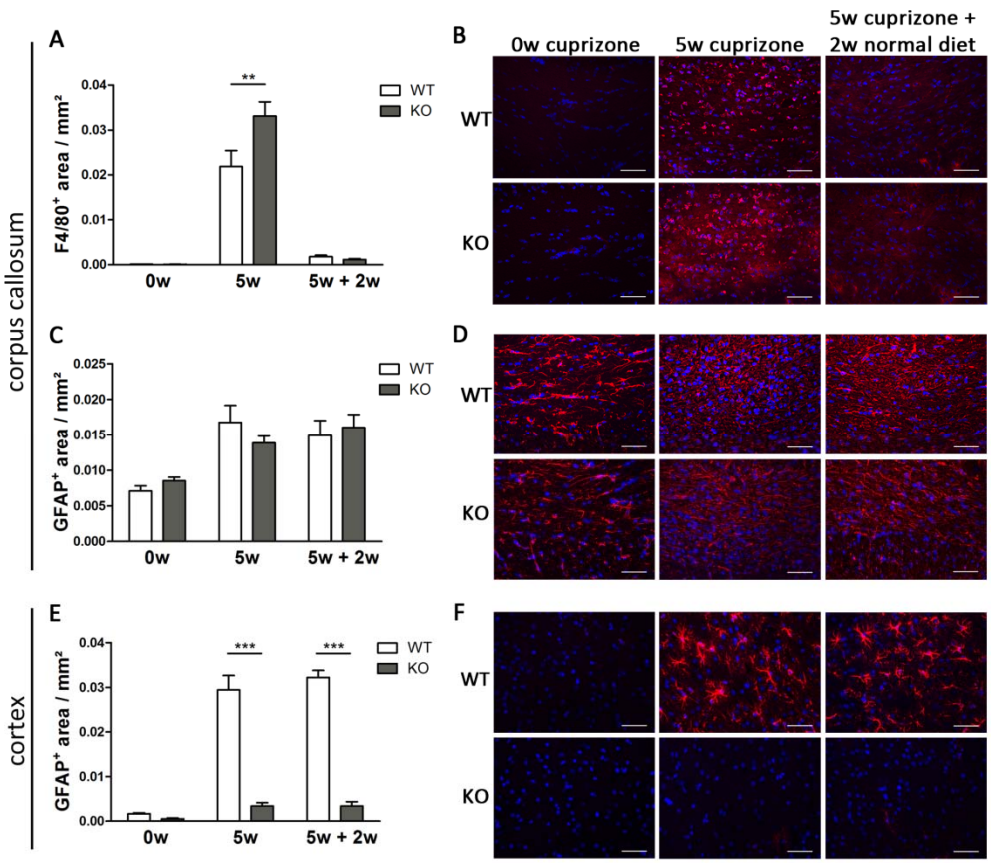

Fig. 5.3. OSMR signaling regulates the glial response following acute demyelination and remyelination. Wild type and OSMR knock-out mice received standard diet (n=4 per group), 5 weeks of cuprizone diet (n=4 per group) or 5 weeks cuprizone diet followed by 2 weeks of standard diet (n=5 per group). **(A-B)** Quantification and representative pictures of F4/80$^+$ area in the corpus callosum. **(C-F)** Quantification and representative pictures of the GFAP$^+$ area in the corpus callosum (C-D) and cortex (E-F) measured using immmunohistochemistry. Data are depicted as mean ±SEM, ** p<0.01 and *** p<0.001. Scale bar represents 50µm. WT, wild type; KO, knock-out.

Chronic demyelination induced microgliosis and astrogliosis in the corpus callosum and microgliosis diminished during the remyelination period, while astrogliosis persisted. CNS-targeted OSM treatment had no effect on the microglial or astroglial response in the corpus callosum as compared to the control mice (F4/80 immunoreactivity: 0.0032 ±0.0005 mm^2 vs 0.0041 ±0.0009 mm^2 and GFAP immunoreactity: 0.0464 ±0.0029 mm^2 vs 0.0464 ±0.0034 mm^2) (Fig. 5.4A-B). Interestingly, microgliosis was markedly increased locally at the site of OSM overexpression (0.0080 ±0.0030 mm^2), compared to the contralateral site (0.0007 ±0.0002 mm^2) and a trend was observed compared to the site of injection in the control mice (0.0020 ±0.0008 mm^2) (Fig. 5.4C-D). In addition, the astroglial response was enhanced at the site of OSM expression (0.050 ±0.009 mm^2) as compared to the contralateral site (0.028 ±0.001 mm^2) and the injection site in the control group (0.031 ±0.001 mm^2) (Fig. 5.4E-F). Thus, while CNS-targeted OSM treatment does not affect the microglial or astroglial response in the demyelinated corpus callosum, it induces a clear glial response at the site of overexpression.

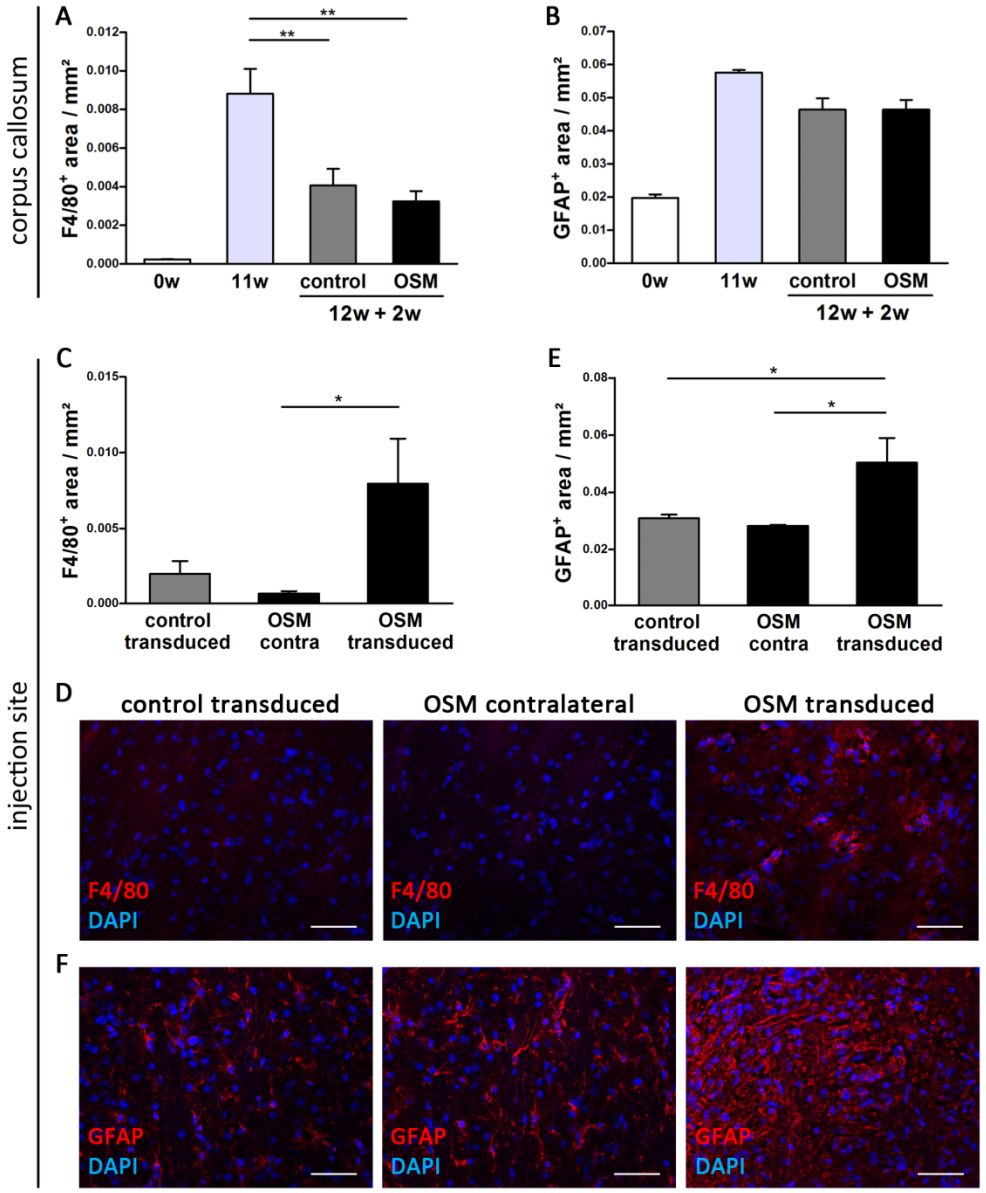

Fig. 5.4. CNS-targeted OSM treatment induces a local glial response. Mice were fed with standard diet, cuprizone diet for 11 weeks or cuprizone diet for 12 weeks followed by 2 weeks of standard diet and LV-eGFP (control) or LV-OSM (OSM) injection in week 11 (n=4 or 5 per group). **(A-B)** Quantification of the F4/80$^+$ area (A) and GFAP$^+$ area (B) measured using immunohistochemistry in the corpus callosum. **(C-F)** Quantification and representative pictures of the F4/80$^+$ area (C-D) and GFAP$^+$ area (E-F) measured at the

injection site of control mice (control transduced), contralateral site of OSM mice (OSM contra) and injection site of OSM-treated mice (OSM transduced). Data are depicted as mean ±SEM, *p<0.05, **p<0.01 and ***p<0.001. Scale bar represents 50µm.

5.3.4 OSMR signaling regulates astrocytic TIMP-1 production during remyelination

To elucidate how the OSM-induced glial response is involved in OSM-mediated remyelination, we measured mRNA expression of trophic factors (CNTF, LIF, IGF-1, TIMP-1), inhibitory molecules (Notch1, Jagged1, LINGO-1), pro- and anti-inflammatory cytokines (IL-1β, IL-6, TNFα, TGFβ), and M1/M2 markers (CD32, CD86, CD206) in the brain using qPCR. A strong increase in mRNA expression of TIMP-1 was observed in the brain of wild type mice after acute demyelination and remyelination, while in the OSMR knock-out mice this upregulation was completely absent (Fig. 5.5A). Moreover, CNS-targeted OSM treatment, following chronic demyelination, induced a three times higher TIMP-1 mRNA expression in the region of overexpression and the region of the corpus callosum compared to control mice (Fig. 5.5B). In the OSM-treated mice, an increased CNTF expression and a decreased LINGO-1 expression was also detected in the same areas (Fig. 5.5D,F), while no significant difference in both CNTF and LINGO-1 expression was found in the OSMR knock-out mice compared to wild type mice (Fig. 5.5C,E). These data suggest an important role for TIMP-1 as both endogenous OSMR signaling as well as OSM treatment strongly regulate its expression. TIMP-1 is an endogenous regulator of the proteolytic activity of MMPs and promotes oligodendrocyte differentiation and CNS myelination [264]. In the demyelinated corpus callosum of the OSM-treated mice, we detected astrocytes as producers of TIMP-1 during remyelination (Fig. 5.5G). Moreover, treatment of primary astrocytes with OSM strongly upregulated TIMP-1 mRNA (4.8-fold) and protein

expression (Fig. 5.5H-I). Taken together, OSMR signaling regulates astrocytic TIMP-1 production in demyelinated and remyelinating lesions.

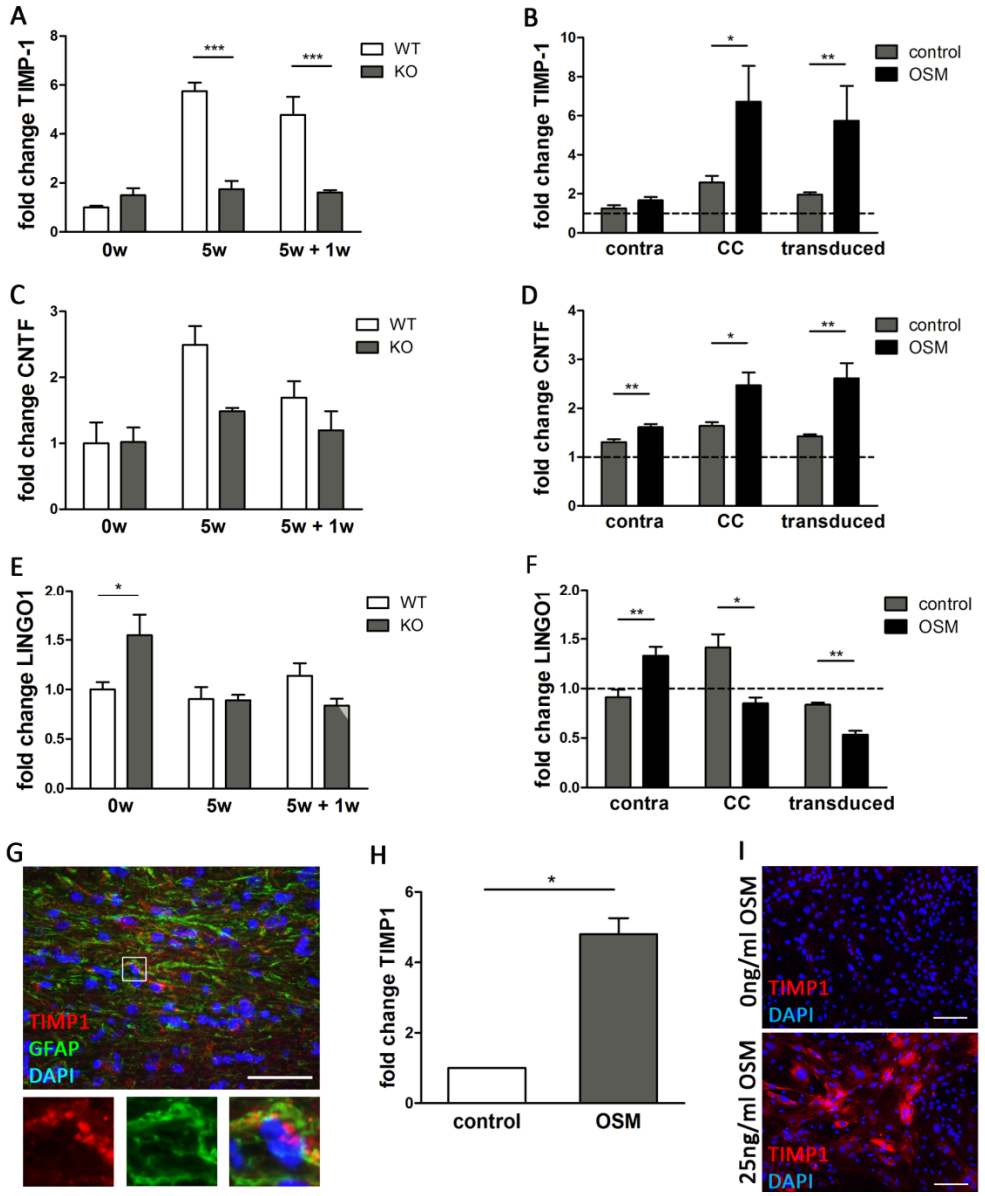

Fig. 5.5 OSMR signaling induces astrocytic TIMP-1 production. (A,C,E) mRNA expression of TIMP-1 (A), CNTF (C) and LINGO-1 (E) in the brain of wild type and OSMR knock-out mice receiving standard diet, after acute demyelination and following remyelination

measured using QPCR (n=5 per group). **(B,D,F)** mRNA expression of TIMP-1 (B), CNTF (D) and LINGO-1 (F) in the region of injection (transduced), contralateral region (contra) and region of the corpus callosum (CC) in LV-eGFP (control) and LV-OSM injected mice (OSM) after 12 weeks cuprizone followed by 1 week normal diet (n=5 per group). Expression is converted to fold change as compared to mice receiving normal diet (dotted line). **(G)** Double immunohistochemical staining for TIMP-1 (red) and GFAP (green) in the corpus callosum following chronic demyelination and remyelination. Magnifications of double positive cells are depicted below. **(H)** Primary astrocytes were treated with 0 ng/ml or 25 ng/ml OSM for 24h, after which TIMP-1 mRNA expression was measured by QPCR (n=3). **(I)** Representative picture of TIMP-1 protein expression in primary astrocytes treated for 72h with 0 ng/ml or 25 ng/ml OSM measured using immunohistochemistry. Data are depicted as mean ±SEM, *p<0.05, **p<0.01. Scale bar represents 50 μm (G) or 100 μm (I). WT, wild type; KO, knock-out.

5.4 Discussion

In this study, we defined that OSMR signaling is a crucial element in the remyelination process. We demonstrate that the OSMR was upregulated during remyelination. Expression of OSM and its receptor are increased in response to different types of CNS pathology, such as nerve injury, epileptic seizures, spinal cord injury and MS [213-216]. Whereas several of these studies already pointed to a direct neuroprotective effect of OSM, this is the first study showing that OSMR signaling is essential for remyelination. In OSMR knock-out mice, OPC numbers were reduced following acute demyelination, leading to a decrease in newly formed oligodendrocytes and subsequent failure of remyelination. In contrast, CNS-targeted OSM treatment following chronic demyelination enhanced the number of newly formed mature oligodendrocytes thereby inducing remyelination.

TIMP-1 was identified as one of the factors responsible for OSM-induced remyelination. In response to inflammatory or cuprizone-induced demyelination, TIMP-1 expression is robustly induced [265-268]. Here, we demonstrate that in absence of the OSMR upregulation of TIMP-1 was abrogated, indicating that endogenous OSMR signaling is crucial for inducing TIMP-1 in demyelinated and remyelinating lesions. Moreover, CNS-targeted OSM treatment strongly increased TIMP-1 expression. TIMP-1 is an inhibitor of MMPs, which are enzymes involved in extracellular matrix maintenance and remodeling [269]. TIMP-1 also fulfills MMP-independent functions in the CNS where it stimulates neuronal survival and synaptic plasticity [270, 271]. TIMP-1 treatment promotes differentiation of primary OPCs *in vitro*, and TIMP-1 deficiency delays myelin formation during development [264] and myelin recovery during EAE [266]. Therefore, we conclude that OSM is potentiating remyelination through upregulation of TIMP-1 that stimulates OPC differentiation and subsequent remyelination. As remyelination is a highly complex process, we do not exclude the involvement of other mediators

as exemplified by the observed upregulation of the trophic factor CNTF and reduction of the inhibitory molecule LINGO-1 following OSM overexpression [272, 273].

The main producers of TIMP-1 in our model are astrocytes, which have been reported to express a functional OSMR [274]. TIMP-1 is typically expressed by astrocytes that surround demyelinated lesions [265, 275]. Accumulating evidence indicates that astrocytes are key players in CNS repair [78, 83]. They are the most abundant cell type in the CNS and fulfill multiple functions such as maintaining metabolic homeostasis, regulation of synaptogenesis, formation of the blood-brain barrier, and providing trophic support. Following CNS damage, astrocytes produce factors that promote OPC proliferation, differentiation, and myelination [83, 184, 276]. In contrast to microgliosis, the astroglial response persists throughout both cuprizone-induced demyelination and remyelination [251, 277]. Interestingly, astrocyte activation was completely absent in the cortex of mice lacking the OSMR, indicating that the beneficial astroglial response occurring during demyelination is regulated by OSMR signaling. In addition, CNS-targeted OSM expression induced a local astroglial reaction at the site of overexpression. TIMP-1 expression was evident in the accumulating astrocytes and we confirmed that OSM induces TIMP-1 in primary astrocytes. Besides inducing remyelination, TIMP-1 has been reported to stimulate astrocyte proliferation and TIMP-1 deficiency leads to reduced astrocyte numbers during delayed myelination [264, 266]. These findings could explain the lack of astroglial response seen in the OSMR knock-out mice.

Taken together, our data support the notion that astrocytes play an essential role during remyelination and suggest that OSM-induced TIMP-1 production by these cells may be one of the major contributors in this process. The crucial role of OSMR signaling in potentiating remyelination, together with the well-established

neuroprotective properties reported, suggest that OSM is a promising therapeutic candidate for MS and other demyelinating CNS disorders.

6

SUMMARY AND GENERAL DISCUSSION

MS is a chronic disabling disease of the CNS, for which current therapies are able to delay disease progression but cannot prevent it [231]. Moreover, for patients in the progressive phase, available therapies largely fail to provide any benefit [278, 279]. Thus, there is an urgent need for novel therapies that can limit and resolve CNS degeneration besides modulating the immune response. Interesting candidates for dual mode therapy are two members of the gp130 cytokine family, namely LIF and OSM. These cytokines can exert similar effects as they both signaling through the LIFR, however human OSM can have additional effects by activating its specific OSMR [144]. In contrast, mouse OSM can only signal through the OSMR, making mice models an excellent tool to selectively study OSMR signaling [146]. In this thesis, the immunomodulatory properties of LIF and OSM were characterized in depth. In addition, the effect of OSM on de- and remyelination was investigated. In the following paragraphs, the main findings are summarized and discussed, and suggestions for future research are proposed.

PART 1: Immune modulation by LIF and OSM

How are the receptors for LIF and OSM regulated during autoimmunity?

Previous studies already demonstrated that LIF and OSM are enhanced in lesions of MS patients [162, 215]. To reveal how they regulate the autoimmune response, we defined in **chapter 2 and 3** on which immune cells the receptors are expressed during autoimmunity. In MS patients, LIFR and OSMR expression were strongly enhanced on circulating T cells, B cells and macrophages compared to healthy controls. Activation of immune cells upregulated LIFR and OSMR expression, while current immunosuppressive therapies potently reduced the receptors. In brain lesions of MS patients, high LIFR expression was detected in the rim of the lesion, especially on macrophages/microglia. The LIFR was also present on perivascular $CD4^+$ T helper cells. Thus, during autoimmunity immune cells upregulate the LIFR

and OSMR, making these cells susceptible for LIF and OSM signaling. The data discussed in the next paragraphs indicate that the upregulation of the receptors and their ligands during autoimmunity comprise an endogenous protective response to limit and resolve inflammation and CNS damage.

Do LIF and OSM affect the adaptive immune response?

T cells are considered as the main effector cells in the pathogenesis of MS. However, the role of LIF and OSM on the T cells responses are still unclear. In **chapter 2**, we did not detect an effect of LIF on the differentiation of human $CD4^+$ T cells to Th1, Th2 or Th17 cells *in vitro*. Therapeutic CNS-targeted LIF treatment in EAE did reduce pathogenic Th1 cell numbers, while not affecting the Th17 and Th2 cells. The reduction in Th1 cells may result from suppression by Tregs as their numbers were doubled. Still, a similar decrease in pathogenic Th17 cells would have been expected, which was not detected. Our findings are in contrast to the study by Zhang and colleagues, who reported that LIF inhibits Th17 differentiation in EAE and in human $CD4^+$ T cells [198]. Contrasting results of the EAE data could be explained by the difference in administration route for LIF, systemic versus CNS-targeted, or the time point of administration, prophylactic versus therapeutic. Other studies using the same preclinical model with systemic LIF treatment starting the day of EAE immunization, did also not detect an effect on Th17 cells [179].

In **chapter 2** we further demonstrated that LIF enhanced the number of Tregs in human $CD4^+$ T cells and in a preclinical model of MS. LIF augmented Treg numbers in $CD4^+$ T cells isolated from healthy controls and MS patients with low serum levels of IL-6. Moreover, in the donors that responded to LIF, IL-6 signaling was diminished. Therapeutic CNS-targeted LIF treatment in a preclinical animal model of MS doubled the number of Tregs in the CNS and ameliorated clinical symptoms. Our data are in line with a recent study on graft rejection, in which LIF

is associated with transplantation tolerance and supports expression of FOXP3 [193]. IL-6, in contrast, is associated with allo-rejection and represses FOXP3 expression [193]. Moreover, IL-6 is shown to prevent Treg differentiation *in vitro* and *in vivo* [33, 195, 197, 236]. Thus, LIF and IL-6 have an opposing function on Tregs. Taken together, our data indicate that LIF treatment of MS patients could modulate the autoimmune response by enhancing Treg numbers.

By signaling through the LIFR, OSM could exert similar beneficial effects as LIF on Tregs and Th1 cells. However, this needs to be confirmed in additional studies. By signaling through the OSMR, OSM could exert additional synergistic or opposing effects on the T cell response. In **chapter 3** we performed a similar set-up as for CNS-targeted LIF treatment in EAE. Therapeutic CNS-targeted OSM treatment had no effect on disease symptoms, suggesting that OSMR signaling probably does not affect Treg and Th1 cell numbers in the CNS.

In **chapter 3** we also demonstrated that OSM had no effect on the cytotoxicity and cytokine production of $CD8^+$ T cells, another T cell subset important in the pathogenesis of MS. The effect of LIF on $CD8^+$ T cells is still unclear. In conclusion, we showed that LIF can modulate the autoimmune response by augmenting Treg numbers, the effect of OSM on the T cell response requires further investigation.

Do LIF and OSM affect the innate immune response?

Macrophages and microglia are the most abundant immune cells in MS lesions [62]. In addition, we demonstrated that a high percentage of these cells expressed the LIFR and OSMR (**chapter 2 and 3**). Our group previously showed that LIF stimulates myelin uptake and reduces production of proinflammatory mediators, such as oxygen radicals and TNF-α, by macrophages *in vitro* [191]. Moreover, LIF promotes the generation of tumor-associated macrophages, which resemble M2 macrophages [192]. In **chapter 4,** we demonstrated that CNS-targeted OSM treatment limited cuprizone-induced demyelination by inducing M2 polarization.

In line with this, a recent study in our group revealed that CNS-targeted OSM treatment induces a M2 phenotype thereby redirecting infiltrating Th1/Th17 cells and preventing EAE development (Slaets, in preparation). Systemic OSM treatment polarizes adipose tissue macrophages to a M2 phenotype and increases insulin sensitivity, whereas in OSMR deficient mice these macrophages are polarized to a M1 phenotype and adipose tissue inflammation is augmented [229]. In contrast, we did not detect an effect of OSM on the differentiation of human macrophages to a M1 or M2 phenotype or on antigen presentation *in vitro* (**chapter 3**). It was proposed that this could be due to the fact that OSM also activates the LIFR in humans making it difficult to study selective OSMR signaling. Blocking LIFR signaling using antibodies directed against the receptor could address this. It is also possible that the effect on M2 polarization found in cuprizone-induced demyelination, in EAE and in the adipose tissue macrophages was the result of an indirect effect of OSM on the macrophages/microglia. Additional studies are required to confirm this. Taken together, these data indicate that both LIF and OSM induce an anti-inflammatory phenotype in macrophages/microglia.

Is the autoimmune response differently regulated by LIFR and OSMR signaling?

Although LIF and OSM closely resemble each other, the fact that they regulate the autoimmune response differently is clearly demonstrated in the multiple EAE studies conducted. The initial studies using prophylactic LIF treatment either systemic or CNS-targeted have shown that LIF protects oligodendrocytes thereby limiting demyelination, while not finding effects on the immune cells [179, 180]. Recently, Zhang and colleagues reported that systemic LIF treatment starting the day of immunization inhibits Th17 differentiation [198]. In all reports, LIF treatment attenuates clinical symptoms of EAE. In contrast, our group showed that prophylactic CNS-targeted OSM treatment strongly prevents disease

development. This was attributed to its effects on the endothelial cells and polarization of macrophages/microglia to a M2 phenotype (Slaets, in preparation). In **chapter 2**, we revealed that therapeutic CNS-targeted LIF treatment enhanced the number of Tregs in the CNS and reduced EAE symptoms. In **chapter 3,** we performed a similar set-up for CNS-targeted OSM treatment and no effect on disease symptoms was found suggesting that OSMR signaling does not affect Treg numbers in the CNS. In conclusion, these studies indicate that LIFR signaling exerts its effects on the autoimmune response by regulating Tregs and Th17 cells, while OSMR signaling modulates macrophages/microglia and endothelial cells. The diverse effects of LIFR and OSMR signaling could be explained by the presence of the functional receptors on different cell types or differences in expression levels of the receptors. In addition, they possibly activate distinct signaling pathways. Further research should address which signaling pathways are induced by LIFR and OSMR activation in the different cell types.

PART 2: Role of LIF and OSM in de- and remyelination

Does LIFR and OSMR signaling affect oligodendrocyte survival?

Damage to myelin sheaths and oligodendrocytes is the key pathological hallmark of MS. LIF limits oligodendrocyte apoptosis induced by IFN-γ and TNF-α *in vitro* [162, 176]. Moreover, in EAE and the cuprizone-induced demyelination model LIF treatment prevents oligodendrocyte death and demyelination [179, 181]. In **chapter 4**, we revealed a protective role of OSMR signaling in demyelination, since OSMR deficiency aggravated this process. In addition, CNS-targeted OSM treatment strongly limited oligodendrocyte death and demyelination. In contrast to LIF and other members of the gp130 cytokine family such as IL-11 and CNTF [176, 255, 256], OSMR signaling did not directly promote oligodendrocyte survival. In the OSM-treated mice, we demonstrated enhanced IL-4 expression

and polarization of microglia/macrophages to a M2 phenotype which could explain the reduced oligodendrocyte death and demyelination. Thus, both LIFR and OSMR signaling protect against oligodendrocyte death and demyelination, though by different mechanisms.

Does LIFR and OSMR signaling affect OPC proliferation, differentiation and myelination?

OPCs play a key role in remyelination, as in response to molecular signals they proliferate and migrate to demyelinated lesions, where they differentiate into mature oligodendrocytes and restore myelin sheaths [99]. Despite the presence of OPCs in demyelinated lesions of MS patients, remyelination is mostly insufficient [259-261]. As it is a highly complex process, the molecular framework that regulates OPC proliferation, differentiation and myelination is still incompletely understood. Recently it was demonstrated that CNS-targeted LIF administration directly stimulates proliferation of the OPC pool [187]. Following cuprizone-induced demyelination, LIF administration restores oligodendrocyte numbers and promotes remyelination [187], whereas LIF deficiency impairs remyelination [181]. In **chapter 5** we revealed an essential role for OSMR signaling in remyelination, since OSMR deficiency completely abrogated this process. Moreover, triggering the receptor by CNS-targeted OSM treatment enhanced oligodendrocyte differentiation and induced remyelination. The repair-promoting effect OSM was attributed to astrocytic TIMP-1 production. TIMP-1 regulates the proteolytic activity of MMPs and is shown the promote oligodendrocyte differentiation and CNS myelination [264, 266]. In response to inflammatory of cuprizone-induced demyelination, TIMP-1 is robustly expressed [265-268]. In OSMR deficient mice TIMP-1 upregulation was completely absent in response to de- and remyelination, indicating that OSMR signaling is essential for the enhanced TIMP-1 production in demyelinated and remyelinating lesions. CNS-

targeted OSM treatment augmented TIMP-1 production in the chronically demyelinated CNS. Moreover, it is likely that other factors are involved in boosting remyelination as exemplified by increased CNTF and decreased LINGO-1 expression in the OSM-treated mice. In conclusion, we showed that OSMR signaling is essential for remyelination, but in contrast to LIFR signaling it exerts its protective effect on the OPCs and remyelination in an indirect manner.

Does LIFR and OSMR signaling affect the glial response during CNS damage and repair?

Microglia and astrocytes are crucially involved in CNS damage and repair [78, 81, 243, 244]. LIF can affect CNS de- and regeneration by inducing an anti-inflammatory phenotype in macrophages/microglia [191, 192]. In **chapter 4**, we showed that during demyelination the OSMR was strongly upregulated on microglia/macrophages and astrocytes. Moreover, we demonstrated that OSM induced its beneficial effects on de- and remyelination by modulating microgliosis and astrogliosis, prominent processes during demyelination in the cuprizone model (**chapter 4 and 5**). CNS-targeted OSM treatment significantly reduced the microglial response during demyelination. Interestingly, locally at the site of OSM overexpression a microglial and astroglial reaction was induced. The accumulated microglia/macrophages were demonstrated to have a M2 phenotype. In addition, CNS-targeted OSM treatment enhanced IL-4 expression. IL-4 is an anti-inflammatory cytokine and a potent inducer of the M2 phenotype [72]. A previous study in our group also revealed that CNS-targeted OSM treatment induces M2 polarization thereby preventing EAE development (Slaets, in preparation). Thus, OSM limits demyelination by enhancing IL-4 expression and inducing M2 polarization.

During remyelination the astroglial response persisted, while microgliosis was strongly reduced. CNS-targeted OSM treatment had no effect on the astroglial

and microglial reaction in the corpus callosum, while at the site of OSM overexpression an astroglial and microglial response was induced. No enhanced IL-4 production or M2 polarization was detected, which is probably due to the difference in time point of OSM overexpression. To study the effect of OSM on demyelination, LVs were injected before starting the cuprizone diet, whereas the effect on remyelination was investigated by injecting LVs after chronic demyelination. Instead, TIMP-1 was identified as an important factor responsible for OSM-induced remyelination. We showed that astrocytes produced TIMP-1 in response to de- and remyelination, and confirmed that OSM induced TIMP-1 in primary astrocytes. Interestingly, the astroglial reaction seen in the cortex following de- and remyelination was completely absent in OSMR deficient mice, indicating that OSMR signaling is essential for modulating the astroglial reaction during these processes. TIMP-1 is also reported to stimulate astrocyte proliferation [264, 266], which could explain the lack of astrogliosis in the cortex of the OSMR deficient mice. Taken together, our studies confirm that astrocytes and microglia play an essential role in de- and remyelination, and indicate that OSMR signaling is crucially involved in regulating their beneficial effects.

Part 3: Therapeutic avenues for LIF and OSM: where are we?

Are LIF and OSM promising therapeutic candidates?

MS is a complex disease in which various immune cells and CNS cells are involved. Current MS therapies only suppress the immune system. LIF modulates the autoimmune response by boosting the number of Tregs (**chapter 2**) and by promoting an anti-inflammatory phenotype in macrophages (Fig. 6.1) [191, 192]. In addition, LIF stimulates survival of neurons and oligodendrocytes, and promotes axonal regeneration and remyelination (Fig. 6.1) [170, 174, 176, 180, 187]. Thus, its beneficial effects on both the immune system and CNS cells make

LIF a promising therapeutic candidate for patients in both the early and the progressive phase of the disease.

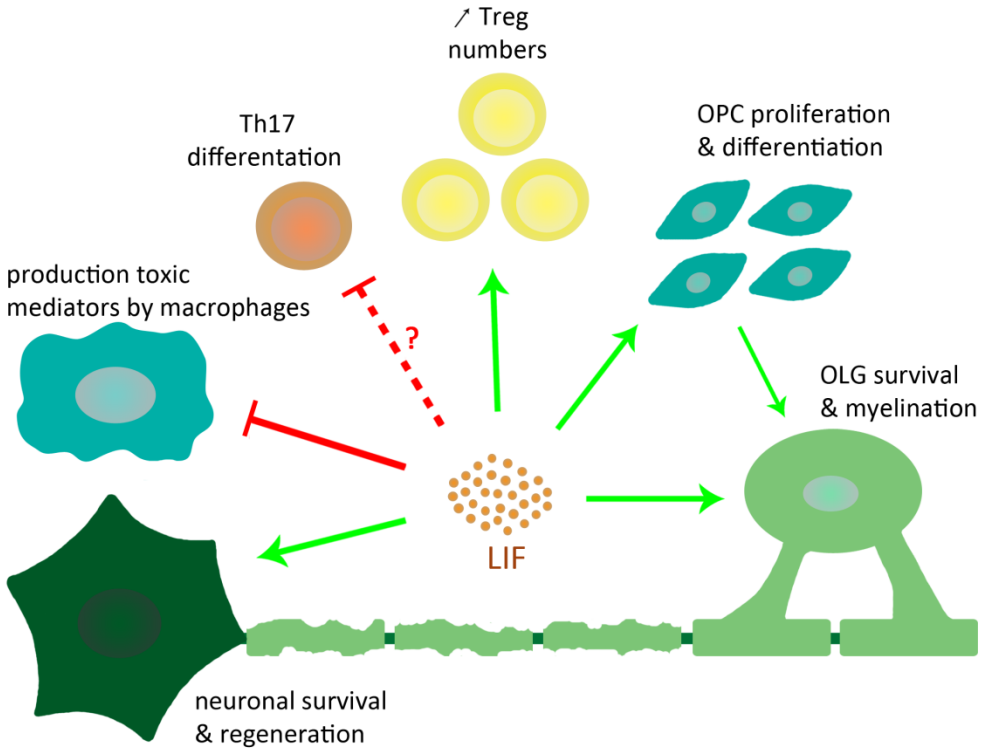

Fig. 6.1. Schematic representation of the effects of LIF on the pathogenesis of MS. In demyelinated MS lesions, LIF is produced. LIF stimulates neuronal survival and regeneration, OPC proliferation and differentiation, and oligodendrocyte survival and myelination. Additionally, LIF limits the production of toxic mediators by macrophages and inhibits Th17 differentiation. Finally, LIF boosts the number of Tregs. OLG, oligodendrocytes.

OSM could have a superior therapeutic potential compared to LIF by signaling through both the LIFR and OSMR. Whereas, the role of LIFR signaling in the pathogenesis of MS is extensively studied, far less studied are devoted to the effects of OSMR signaling. Neuroprotective and neuroregenerating properties of OSMR signaling are demonstrated (Fig. 6.2) [216-218]. In **chapter 4**, we show that OSMR signaling limits demyelination by enhancing IL-4 expression and inducing M2 polarization. Moreover, OSMR signaling is crucial for remyelination (**chapter 5**) (Fig. 6.2). Although, these promising data on both the immune cells and CNS cells support the therapeutic potential of OSM, additional research is required. In this thesis, LVs were used to express OSM in the CNS. Injection of LV-OSM in the striatum induced an astroglial reaction, accumulation of microglia/macrophages and tissue remodeling locally at the site of overexpression (**chapter 4 and 5**). Although, we demonstrated that this was a protective and anti-inflammatory response, additional studies need to define whether lower doses of OSM induce similar beneficial effects without the profound tissue remodeling. Moreover, studies on cultures of human cells are essential to determine the effect of the combined LIFR and OSMR signaling. Thus, whereas further research is required, we demonstrated protective effects of LIF and OSM on the immune response and de- and remyelination, thereby providing further impetus to explore the therapeutic potential of LIF and OSM for MS patients.

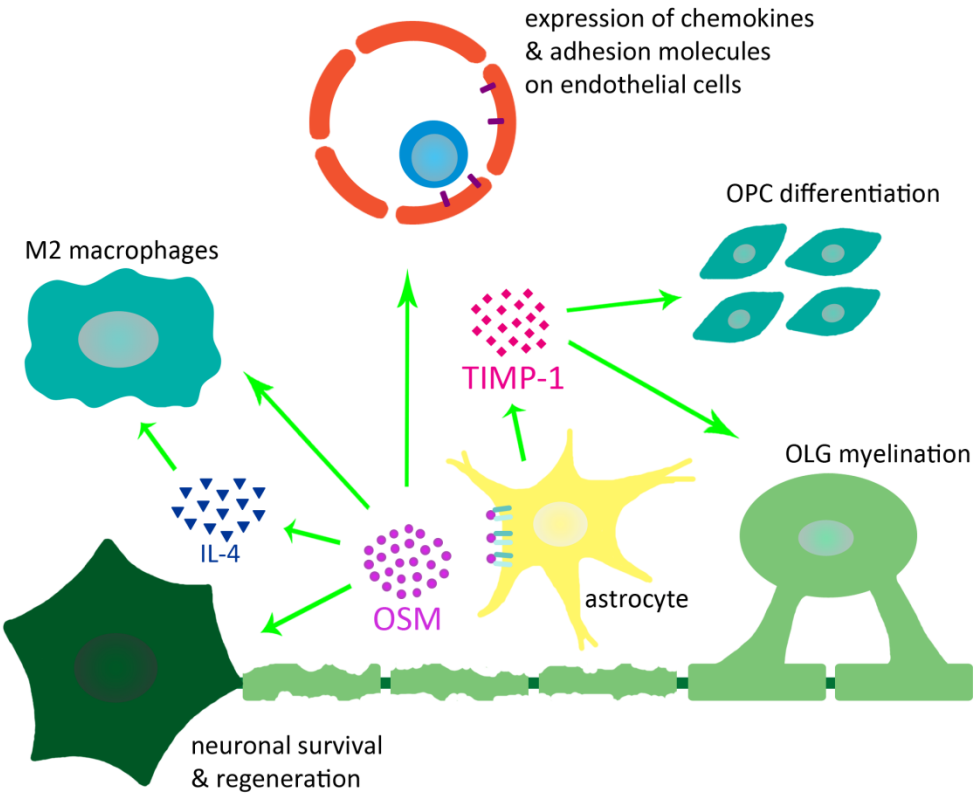

Fig. 6.2. Schematic representation of the effects of OSM on the pathogenesis of MS. In demyelinated MS lesions, OSM is produced. OSM augments the expression of adhesion molecules and chemokines such as ICAM-1, E-selectin, CXCL1, CXCL5, CCL21 on endothelial cells. Moreover, OSM promotes neuronal survival and regeneration. OSM induces IL-4 production and stimulates M2 polarization. Finally, OSM induces TIMP-1 production by astrocytes, thereby promoting OPC differentiation and myelination. OLG, oligodendrocytes.

Translation of LIF and OSM therapy to the clinic

Our studies and others indicate that LIF and OSM are promising therapeutic candidates for MS therapy, however several concerns regarding their clinical application need to be resolved. Systemic treatment with recombinant LIF, Emfilermin, is already tested in phase I and II studies in cancer patients, where it is demonstrated to be well tolerated and safe (2 to 16 μg/kg) [239, 280]. Some patients display side effects such as impotence, fever, dizziness, hypotension and rigors. The phase I study revealed a short half-life (1-5 hours) in serum which is independent of the dose [239]. This short half-life is also shown in a pharmacokinetics study in women undergoing *in vitro* fertilization and embryo transfer [238]. As a result of the short half-life, frequent injections would be needed. Additionally, LIF and OSM have a limited potential to cross BBB, indicating that high doses are needed to induce effects in the CNS, thereby risking systemic side effects. Other delivery approaches, such as viral vectors, liposomes and nanoparticles could circumvent these problems. Viral vectors are highly efficient gene delivery systems. Approximately 70% of gene therapy clinical trials performed so far uses modified viruses such as adenoviruses, adeno-associated viruses (AAVs), retroviruses and lentiviruses to deliver genes [281]. Adenoviruses carry double-stranded DNA, which is not incorporated in the genome of host cells upon transfection. Therefore the genes are not replicated when a cell divides and readministration is essential for long-term treatment [282]. AAVs are non-pathogenic DNA viruses that infect both dividing and non-dividing host cells [283]. Their low immunogenicity and low cytotoxicity make them attractable for gene therapy [283]. Retroviruses and their subclass lentiviruses integrate into the host cell genome and induce stable and long-term expression. While retroviruses require cell division for transfection, lentiviruses can also integrate into the genome of non-dividing cells such as neurons [284, 285]. A widely used LV, which is applied in this thesis, is derived from the human-immunodeficiency virus type 1

(HIV-1) [240]. CNS-targeted expression of LIF or OSM by means of viral vectors would enable to maximize neuroprotection and neuroregeneration, additionally to modulating the infiltrating immune cells. However, viral vectors have several constrains such as immunogenicity, carcinogenesis, difficulty of vector production and limited DNA packaging capacity [281].

Non-viral delivery systems have the potential to address some of these limitations. However, the major challenge is the transport of large, fragile molecules to the target cells or into the nucleus of the cells without degradation. Non-viral delivery systems including liposomes, polymers and nanoparticles can deliver genes as well as proteins. Recently, nanoparticles loaded with recombinant LIF and coated with anti-CD4 antibodies were used in mice receiving allograft transplantation. The LIF-loaded CD4-targeted nanoparticles support survival of vascularized heart grafts [200]. Moreover, in a non-human primate model *in vitro* LIF-loaded CD4-targeted nanoparticles induce expansion of $FOXP3^+$ Tregs [200]. Nanoparticles protect from rapid degradation, enable prolonged delivery through sustained release and allow to target specific cell types [200].

Last decade great advances in viral and non-viral delivery systems have been made. Multiple phase I and II clinical trials using these systems have been conducted. Moreover, in 2012 the European commission approved the first AAV-based gene therapy, Glybera®, to treat familial lipoprotein lipase deficiency [286, 287]. This approval was a major milestone for gene therapy and raises hope that progress made in the next years provides further impetus to explore CNS-targeted delivery of LIF and OSM for MS patients.

Final conclusion

The results obtained in this thesis provide previously uncharacterized and essential insights into the role of LIF and OSM in the pathogenesis of MS and further support their therapeutic potential. We reveal that the receptor for both cytokines is highly upregulated on immune cells of MS patients. LIF boosts the number of Tregs in human $CD4^+$ T cells and in a pre-clinical animal model of MS. OSMR signaling strongly limits demyelination by inducing IL-4 expression and M2 polarization. Moreover, OSMR signaling is crucial for the induction OPC differentiation and remyelination. As LIF and OSM are produced in lesions of MS patients, this suggests that they attempt to limit the autoimmune response and CNS damage, as well as stimulating regeneration. However, the produced levels can probably not counter they aberrant inflammatory reaction and degeneration prevails. Boosting the levels of LIF and OSM could thus be of high therapeutic value for MS patients. To further elucidate their therapeutic potential additional research is needed to define the effect of human OSM since it signals through both the LIFR and OSMR, and could thus exert additional beneficial effects compared to LIF. Moreover, the ideal delivery system for these cytokines, as well as the dosing and timing has to be determined in preclinical models. Thus, although significant challenges remain, this thesis reveals novel beneficial effects of both LIF and OSM in the pathogenesis of MS.

7

NEDERLANDSE SAMENVATTING

Multiple sclerose (MS) is een chronische aandoening van het centraal zenuwstelsel (CZS). De beschermende myelinelaag rond de zenuwvezels wordt aangetast en dit resulteert in een verstoorde impulsgeleiding waardoor symptomen zoals krachtverlies, gevoelsstoornissen, coördinatiemoeilijkheden, blaasproblemen en vermoeidheid kunnen optreden [4]. MS beïnvloedt de levensverwachting nauwelijks, maar heeft wel een immense impact op de levenskwaliteit. De ziekte start meestal tussen de 20 en 40 jaar en is de meest voorkomende oorzaak van niet-traumatische neurologisch invaliditeit bij jongvolwassenen [6]. De geschatte prevalentie in Europa is 83 per 100 000 inwoners [5]. De exacte oorzaak van de ziekte is nog niet gekend, maar studies tonen aan dat zowel omgevingsfactoren als genetische aanleg een bijdrage leveren. De huidige hypothese is dat MS een auto-immuunaandoening is, dit wil zeggen dat het eigen immuunsysteem het myeline gaat beschadigen [8].

Op dit moment is MS niet te genezen. De beschikbare therapieën onderdrukken het immuunsysteem en kunnen zo de frequentie en ernst van klinische opflakkeringen verminderen. Helaas zijn de therapieën niet in staat de neurologische achteruitgang van de patiënten tegen te houden [231]. Bovendien blijken de therapieën weinig efficiënt in de progressieve fase van de ziekte, die vooral gekenmerkt wordt door neuronale schade en minder door inflammatoire reacties [278, 279]. Hierdoor is er een grote nood aan nieuwe therapieën die niet enkel het immuunsysteem moduleren, maar ook beschermen tegen schade aan het CZS en zorgen voor herstel van het CZS. Bepaalde leden van de gp130-cytokinefamilie, namelijk *leukemia inhibitory factor* (LIF) en *oncostatin M* (OSM), hebben gunstige effecten op zowel de cellen van het immuunsysteem als deze van het CZS. Deze cytokines kunnen gelijkaardige effecten uitoefenen aangezien ze beide de LIF-receptor (LIFR) activeren. Daarnaast kan humaan OSM via specifieke activatie van de OSM-receptor (OSMR) additionele effecten uitoefenen [144]. In tegenstelling tot humaan OSM, kan muis OSM alleen de OSMR activeren,

waardoor de muis een ideaal model is om specifiek OSMR-signalering te bestuderen [146]. LIF en OSM beschermen tegen neuronale celdood en stimuleren neuronale regeneratie [170, 174, 216, 217]. Het effect van deze cytokines op de immuunrespons is nog niet duidelijk en werd daarom in deze studie opgehelderd. Verder werd de rol van OSMR-signalering tijden de- en remyelinisatie onderzocht. In de volgende paragrafen worden de verkregen resultaten samengevat.

DEEL 1: De immunomodulerende effecten van LIF en OSM

Hoe wordt de receptor voor LIF en OSM gereguleerd tijdens auto-immuniteit?

Voorgaande studies hebben aangetoond dat LIF en OSM worden geproduceerd in laesies van MS-patiënten [162, 215]. Om te achterhalen hoe ze de auto-immuunrespons moduleren, hebben we in **hoofdstuk 2 en 3** de expressie van de LIFR en OSMR op immuuncellen van MS-patiënten gekarakteriseerd. LIFR- en OSMR-expressie was sterk verhoogd op circulerende T-cellen, B-cellen en macrofagen van MS-patiënten vergeleken met gezonde controles. Activatie van de immuuncellen zorgde voor een upregulatie van de LIFR en OSMR, terwijl de huidige immunosuppressieve therapieën de expressie onderdrukten. In hersenlaesies van MS-patiënten was er een hoge expressie van de LIFR op macrofagen/microglia, en was deze ook aanwezig op perivasculaire T-helpercellen. In de volgende paragrafen wordt aangetoond dat de verhoging van de receptor op immuuncellen tijdens auto-immuniteit een beschermde endogene respons is om inflammatie en schade in het CZS te voorkomen.

Hebben LIF en OSM een effect op de adaptieve immuunrespons?

T-cellen worden beschouwd als de belangrijkste effectorcellen in de pathogenese van MS. Maar de rol van LIF en OSM op de T-celresponsen is nog steeds

onduidelijk. In **hoofdstuk 2** hebben we aangetoond dat LIF geen effect had op de differentiatie van humane $CD4^+$ T-helper(Th)cellen naar Th1-, Th2- en Th17-cellen. Wel verhoogde LIF-behandeling van $CD4^+$ T-cellen het aantal regulatoire T-cellen (Tregs) in een subpopulatie van gezonde controles en MS-patiënten. Deze subpopulatie had een lage serum concentratie van interleukine-6 (IL-6), een ander lid van de gp130-cytokinefamilie. Eerder werd aangetoond dat IL-6 de differentiatie van Tregs verhinderd [33, 197]. Onze studie samen met deze eerdere bevindingen geven aan dat LIF en IL-6 een tegengestelde werking hebben. In een preklinisch dierenmodel van MS, experimentele auto-immune encefalomyelitis (EAE), hebben we LIF therapeutisch tot overexpressie gebracht met behulp van lentivirale vectoren (LV's). Dit resulteerde in een verdubbeling van het aantal Tregs in het CZS en vermindering van de ziektesymptomen. Onze data geven dus aan dat LIF-behandeling van MS-patiënten de auto-immuunreactie zou kunnen limiteren door het aantal Tregs te verhogen.

OSM zou identieke gunstige effecten kunnen uitoefenen als LIF door de LIFR te activeren. Maar OSM induceert ook additionele effecten door het activeren van de OSMR. In **hoofdstuk 3** hebben we voor OSM dezelfde set-up als voor LIF behandeling tijdens EAE uitgevoerd. Therapeutische OSM-behandeling met behulp van LV's had geen effect op de EAE symptomen. Deze data suggereren dat OSMR-signalering, in tegenstelling tot LIFR-signalering, tijdens auto-immuniteit geen effect heeft op het aantal Tregs in het CZS.

Verder hebben we in **hoofdstuk 3** aangetoond dat OSM geen effect had op de cytotoxiciteit en cytokineproductie van $CD8^+$ T-cellen, een ander belangrijk celtype in de pathogenese van MS. Het effect van LIF op de $CD8^+$ T-cellen is nog onduidelijk. Samengevat, hebben we aangetoond dat LIF de auto-immuunrespons kan moduleren door het aantal Tregs te verhogen, het effect van OSM op de T-cellen verreist verder onderzoek.

Hebben LIF en OSM een effect op de aangeboren immuunrespons?

Macrofagen en microglia zijn het meest voorkomende celtype in laesies van MS-patiënten [62]. Bovendien hebben we aangetoond dat een hoog percentage van deze cellen de LIFR en OSMR tot expressie brachten (**hoofdstuk 2 en 3**). Verschillende studies bewijzen dat LIF een anti-inflammatoir fenotype in macrofagen stimuleert [191, 192]. In **hoofdstuk 4** tonen we aan dat OSM-behandeling de cuprizone-geïnduceerde demyelinisatie verminderde door een anti-inflammatoir M2-fenotype te induceren. Dit is in overeenstemming met een recente studie in onze groep, waarin OSM-behandeling in EAE M2-polarisatie stimuleert en ziekteontwikkeling voorkomt (Slaets, in voorbereiding). In tegenstelling tot deze studies uitgevoerd in muizen, vonden we geen effect van OSM op de differentiatie van humane macrofagen naar een M1- of M2-fenotype, of op antigenpresentatie (**hoofdstuk 2**). Dit kan mogelijk verklaard worden doordat humaan OSM, in tegenstelling tot muis OSM, ook de LIFR activeert. Het is ook mogelijk dat de M2-polarisatie in cuprizone-geïnduceerde demyelinisatie en EAE het resultaat is van een indirect effect op de microglia/macrofagen. Als conclusie, LIF en OSM zorgen beide voor anti-inflammatoir fenotype in macrofagen.

DEEL 2: De rol van LIF en OSM in de- en remyelinisatie

Heeft LIFR- en OSMR-signalering een effect op de celdood van oligodendrocyten?

Schade aan het myeline en de oligodendrocyten is het belangrijkste pathologisch kenmerk van MS. *In vitro* en *in vivo* studies in EAE en cuprizone-geïnduceerde demyelinisatie hebben bewezen dat LIF beschermt tegen oligodendrocytceldood en demyelinisatie [176, 179, 181, 182]. In **hoofdstuk 4** hebben we een beschermde rol van OSMR-signalering in demyelinisatie aangetoond. In muizen

deficiënt voor de OSMR was dit proces verergert, terwijl OSM-behandeling met behulp van LV's depletie van oligodendrocyten en demyelinisatie sterk verhinderde. In tegenstelling tot LIF en andere leden van de gp130-cytokinefamilie zoals IL-11 en *ciliary neurotrophic factor* (CNTF) [176, 255, 256], had OSMR-signalering geen direct beschermend effect op de oligodendrocyten. In de OSM-behandelde muizen, werd wel een verhoogde expressie van IL-4 en polarisatie van microglia/macrofagen naar een M2-fenotype waargenomen wat de verminderde depletie van oligodendrocyten en demyelinisatie kan verklaren. Samengevat, LIFR- en OSMR-signalering beschermen beide, via verschillende mechanismen, tegen oligodendrocytceldood en demyelinisatie.

Heeft LIFR- en OSMR-signalering een effect op OPC-proliferatie, differentiatie en myelinisatie?

Remyelinisatie vindt plaats als oligodendrocytprecursorcellen (OPC's) migreren naar de gedemyeliniseerde laesies, differentiëren naar mature oligodendrocyten en de myelineschedes herstellen [99]. In MS-patiënten is remyelinisatie meestal gelimiteerd ondanks de aanwezigheid van OPC's in de laesies [259]. Aangezien het een zeer complex proces is, zijn de moleculaire signalen die OPC-migratie, differentiatie en myelinisatie reguleren nog steeds niet helemaal gekend. Een recente studie heeft bewezen dat LIF de proliferatie van OPC's rechtstreeks stimuleert [187]. Bovendien, zorgt LIF-behandeling na cuprizone-geïnduceerde demyelinisatie voor differentiatie van oligodendrocyten en remyelinisatie [187]. In **hoofdstuk 5** hebben we aangetoond dat OSMR-signalering een cruciale rol speelt tijdens remyelinisatie. In OSMR-deficiënte muizen was OPC-proliferatie verminderd en faalde remyelinisatie. OSM-behandeling met behulp van LV's, stimuleerde OPC-differentiatie en induceerde remyelinisatie. OSM reguleerde de astrogliale respons en *tissue inhibitor of metalloproteinase 1* (TIMP-1), geproduceerd door astrocyten, werd geïdentificeerd als een belangrijke factor

verantwoordelijke voor de OSM-geïnduceerde remyelinisatie. TIMP-1 is een inhibitor van matrix metalloproteinases, enzymen die betrokken zijn in behoud en afbraak van de extracellulaire matrix. Recente studies tonen aan dat TIMP-1 differentiatie van oligodendrocyten en myelinisatie stimuleert [264, 266]. Tijdens demyelinisatie is TIMP-1 productie sterk verhoogd [265, 267, 268]. In muizen deficiënt voor de OSMR, detecteerde we geen upregulatie van TIMP-1, dus OSMR-signalering is essentieel voor de verhoging van TIMP-1 in gedemyeliniseerde laesies. Deze studie toont dus een essentiële rol aan voor OSMR-signalering bij remyelinisatie. In tegenstelling tot LIFR-signalering, heeft OSMR-signalering indirect gunstige effecten op de OPC's en remyelinisatie.

Heeft LIFR- en OSMR-signalering een effect op de gliale respons tijdens CZS-schade en -herstel?

Microglia en astrocyten spelen een cruciale rol in CZS-schade en -herstel. Ze kunnen bijdrage aan schade in het CZS door de productie van pro-inflammatoire mediatoren, chemokines, reactieve zuurstofradicalen en inhibitoire moleculen [81, 243]. Maar ze kunnen ook herstel stimuleren door het produceren van trofische factoren en anti-inflammatoire cytokines [78, 244]. LIF kan een anti-inflammatoire fenotype in macrofagen induceren [191, 192]. In **hoofdstuk 4** hebben we aangetoond dat de OSMR sterk verhoogd was op microglia/macrofagen en astrocyten tijdens demyelinisatie. Bovendien had OSMR-signalering gunstige effecten op de- en remyelinisatie door het moduleren van de microgliale en astrogliale respons. De microgliale reactie in het gedemyeliniseerd corpus callosum was verminderd in de OSM-behandelde muizen. In tegenstelling werd er op de plaats van OSM-overexpressie een accumulatie van microglia/macrofagen gevonden, die een anti-inflammatoir M2-fenotype hadden. Verder werd er een verhoogde expressie van IL-4 in de hersenen van de OSM-behandelde muizen waargenomen. IL-4 is een anti-inflammatoir cytokine dat

zorgt voor M2-polarisatie. Dus door het induceren van IL-4-expressie en M2-polarisatie, beschermt OSMR-signalering mogelijk tegen demyelinisatie.

Tijdens remyelinisatie verminderde de microgliale respons sterk, terwijl de astrogliale respons aanhield. TIMP-1 werd geïdentificeerd als een belangrijke factor verantwoordelijk voor de OSM-gemedieerde remyelinisatie. Astrocyten produceerden TIMP-1 in response op de- en remyelinisatie, en in primaire astrocyten werd TIMP-1 productie geïnduceerd door OSM. Onze studies bevestigen dat astrocyten en microglia een belangrijke rol spelen tijdens de- en remyelinisatie en tonen aan dat OSMR-signalering betrokken is in het reguleren van hun gunstige effecten.

DEEL 3: Zijn LIF en OSM de ideale kandidaten voor MS-therapieën?

MS is een zeer complexe ziekte waarin verschillende cellen van het immuunsysteem en CZS betrokken zijn. De huidige therapieën onderdrukken de immuunreactie, maar kunnen de neurologische achteruitgang niet voorkomen. LIF moduleert het immuunsysteem door het aantal Tregs te verhogen (**hoofdstuk 2**) en door een anti-inflammatoire fenotype in macrofagen te induceren [191, 192]. Bovendien, voorkomt LIF celdood van neuronen en oligodendrocyten, en stimuleert het axonale regeneratie en remyelinisatie (Fig. 6.1) [170, 174, 179, 187]. Door dus het immuunsysteem alsook de cellen van het CZS te moduleren is LIF een veelbelovende therapeutische kandidaat voor MS-patiënten in zowel de vroege als progressieve fase van de ziekte. OSM is mogelijk nog een betere therapeutische kandidaat omdat het gelijkaardig gunstige effecten als LIF kan uitoefenen door activatie van de LIFR en additionele effecten via OSMR-activatie. De effecten van OSMR-signalering zijn wel in veel mindere mate bestudeerd in vergelijking met LIFR-signalering. OSMR-signalering beschermt tegen neuronale celdood en stimuleert neuronale regeneratie (Fig. 6.2) [216, 217]. In **hoofdstuk 4**

hebben we aangetoond dat OSMR-signalering demyelinisatie voorkomt door IL-4-expressie en M2-polarisatie te promoten. Bovendien, ontdekte we een cruciale rol voor OSMR-signalering tijdens remyelinisatie (**hoofdstuk 5**). OSMR-signalering heeft dus ook veelbelovende effecten de cellen van het CZS en het immuunsysteem. Additionele studies met culturen van humane cellen zijn nodig om de effecten van humaan OSM, die zowel de LIFR als OSMR activeert, op te helderen. Bovendien zijn er nog enkele bezorgdheden voor de klinische toepasbaarheid van beide cytokines. Ze hebben een korte halfwaardetijd wat betekent dat herhaaldelijke toediening noodzakelijk is. Daarnaast is hun transport doorheen de bloed-hersenbarrière gelimiteerd, waardoor hoge dosissen nodig zijn om effecten in het CZS te bekomen, wat het risico op bijwerkingen vergroot. Alternatieve methoden zoals virale vectoren, liposomen of nanopartikels kunnen hiervoor een oplossing bieden. Dus aanvullende studies zijn nodig om de ideale leveringsmethode, alsook de dosering en tijdspunt van behandeling te bepalen. Samengevat, hoewel er nog meer onderzoek vereist is, tonen we in deze thesis nieuwe gunstige effecten aan van zowel LIF als OSM in de pathogenese van MS.

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Curriculum vitae

Kris Janssens werd geboren op 29 december 1987 te Bree. In 2005 behaalde ze haar diploma Technisch Secundair Onderwijs (TSO) in de afstudeerrichting Techniek Wetenschappen aan de WICO campus Sint-Maria te Neerpelt. Vervolgens startte ze haar opleiding Biomedische Wetenschappen aan de Universiteit Hasselt, waar ze in 2008 haar diploma bachelor in de Biomedische Wetenschappen met onderscheiding behaalde. Aansluitend behaalde ze in 2010 haar diploma master in de Biomedische Wetenschappen met grote onderscheiding. Haar eindwerk, getiteld “Neural stem cells and interleukin-13 as combination therapy for spinal cord injury“ voerde ze uit aan het Biomedisch Onderzoeksinstituut van de Universiteit Hasselt in groep Morfologie onder leiding van Prof. dr. Sven Hendrix. In 2010 startte ze haar doctoraat in de Immunologie/Biochemie groep onder het promotorschap van Niels Hellings. Tijdens deze periode van 4 jaar deed ze onderzoek naar de rol van gp130 cytokines in de pathogenese van MS. Daarnaast, was ze lid van het onderwijsteam in de opleidingen Biomedische Wetenschappen en Geneeskunde, en volgde ze de cursussen proefdierkunde (FELISA C), project management, bioveiligheid, *good scientific conduct and lab book taking*, *scientific writing and oral presentations*, flowcytometrie, patent as bron van informatie, en parametrische/niet-parametrische methode voor levenswetenschappen. Bovendien heeft ze actief deelgenomen aan het schrijven van een FWO krediet voor een onderzoeksproject en aan de organisatie van het “International Life Sciences Master Student Research Conference” in 2012.

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Kris Janssens, februari 2015

"The only way to do great work is to love what you do."

–Steve Jobs