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FRACTALKINE INDUCES THE EXPRESSION OF INTERCELLULAR ADHESION MOLECULE-1 ON $CD4^+$ T-LYMPHOCYTES: IMPLICATIONS FOR THE IMMUNOPATHOGENESIS OF MULTIPLE SCLEROSIS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Curriculum of Neurobiology

Chapel Hill
2012

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ABSTRACT

KEVIN RICHARD BLAUTH: Fractalkine Induces the Expression of Intercellular Adhesion Molecule-1 on $CD4^+$ T-lymphocytes: implications for the immunopathogenesis of Multiple Sclerosis
(Under the direction of Silva Markovic-Plese)

Fractalkine (CX3CL1) is a chemokine that has been shown to play roles in lymphocyte chemotaxis, inflammation, and neuroprotection in central nervous system (CNS) diseases. Here we examined roles for CX3CL1 in $CD4^+$ T-cell chemotaxis mediated via their upregulation of adhesion molecule expression as well as secretion of inflammatory cytokines involved in the pathogenesis of relapsing remitting multiple sclerosis (RRMS). We found that CX3CL1 concentrations are elevated in the cerebrospinal fluid (CSF) of RRMS patients, and that CX3CL1 increases mRNA expression of IFN-γ and TNF-α, and protein secretion of IFN-γ by $CD4^+$ T-cells derived from RRMS patients but not those derived from healthy controls (HCs). We also show that blood-derived $CD4^+$T-cells express increased surface levels of CX3CL1 receptor (CX3CR1) and intercellular adhesion molecule (ICAM)-1 in RRMS patients in comparison to HCs. Furthermore, the percentage of $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-cells are increased in the CSF of untreated RRMS patients in comparison to their peripheral blood samples, and $CD4^+$ T-cells which migrate *in-vitro* toward a CX3CL1 gradient express higher levels of ICAM-1 than $CD4^+$ T-cells that do not migrate. Furthermore, we demonstrated that CX3CL1 upregulates ICAM-1

expression on the surface of RRMS patient-derived but not HC derived $CD4^+$ T-cells. Lastly, we show that CX3CL1 stimulates ICAM-1 expression on myelin-antigen-specific $CD4^+$ T-cell lines derived from RRMS and healthy donors. These results indicate that CX3CL1 may preferentially recruit $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-cells into the CNS during RRMS development, and may activate $CD4^+$ T-cells to express higher levels of ICAM-1, as well as the proinflammatory cytokines IFN-γ and TNF-α.

DEDICATION

For my wife, Meg. I could not have done this without you.

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LIST OF ABBREVIATIONS

ADAM10: Disintegrin and metalloproteinase domain-containing protein 10

ADAM17: Disintegrin and metalloproteinase domain-containing protein 17

ANOVA: Analysis of Variance

APCs: antigen presenting cells

AU: arbitrary units

BBB: Blood-Brain Barrier

CIS: clinically isolated syndrome

CMKBRL1: chemokine β receptor-like 1

CNS: Central Nervous System

CSF: cerebrospinal fluid

CX3CL1: Soluble fractalkine

CX3CR1: Fractalkine Receptor

DC: dendritic cell

EAE: experimental autoimmune encephalomyelitis

FBS: Fetal bovine serum

FGF-4: fibroblast growth factor 4

FGF-6: fibroblast growth factor 6

GRO: growth-regulated oncogene

HC: healthy control

HEK293 cells: Human Embryonic Kidney 293 cells

HPAECs: human pulmonary endothelial cells

HUVECs: Human Umbilical Vein Epithelial cells

ICAM-1: Intercellular Adhesion Molecule 1

IFN-γ: Interferon-gamma

IGFBP-4: Insulin-like growth factor binding protein-4

LFA-1: Lymphocyte Function-Associated Antigen 1

LFB: Luxol-Fast blueMAG: Myelin Associated Glycoprotein

MBP: Myelin Basic Protein

MFI: Mean fluorescence intensity

MIF: Macrophage migration inhibitory factor

MMPs: matrix metalloproteinases

MOG: myelin oligodendrocyte glycoprotein

MS: multiple sclerosis

NAWM: normal appearing white matter

NINDs: non-inflammatory neurological diseases

NMO: neuromyelitis optica

PAS: Periodic Acid Schiff

PBMC: Peripheral blood mononuclear cell

pDC: plasmacytoid dendritic cell

PI3K: phosphatidylinositol 3-kinase

PlGF: Placental Growth Factor

PLP: proteolipid protein

PPMS: primary progressive multiple sclerosis

RANKL: Receptor Activator of Nuclear Factor Kappa B Ligand

RRMS: relapsing remitting multiple sclerosis

SNP: single nucleotide polymorphism

SNs: supernatants

SPMS: secondary progressive multiple sclerosis

T_h Cells: T helper cells

TMEV: Theiler's murine encephalomyelitis virus

TNF-α: Tumor Necrosis Factor-α

VEGF: vascular endothelial growth factor

VLA-4: Very Late Antigen 4

WT: wild type

CHAPTER 1

INTRODUCTION

1.1 MS Pathology

The autoimmune response is proposed to play a role in the development of MS, whereby myelin-autoreactive $CD4^+$ T helper (T_H) cells are activated in peripheral circulation (Fig. 1.1 A) and migrate through the permeable blood-brain barrier (BBB) into the CNS [1-3] (Fig. 1.1 B). It is still unclear what triggers $CD4^+$ T-cell activation against CNS-specific myelin autoantigens—however, molecular mimicry by microbial or viral antigens is one of the proposed mechanism [4]. Once inside the CNS, $CD4^+$ T-cells encounter antigen-presenting cells (APCs) such as microglia and dendritic cells (DCs) and are reactivated by abundant myelin-derived antigens [5-7]. This reactivation leads to the release of proinflammatory cytokines which stimulate microglia, increase BBB permeability, and induce leukocyte chemotaxis, allowing for rapid accumulation of proinflammatory $CD4^+$ and $CD8^+$ T-cells, B-cells, and macrophages in the CNS [8] (Fig. 1.1 C). Following this influx of immune cells into the CNS, demyelinating lesions—the hallmark pathological feature of MS—are formed in the brain and spinal cord. Lesions are characterized by myelin loss, axonal damage, and astrocytic proliferation [9] (Fig. 1.1 D). MS typically takes a relapsing remitting

(RR) course, in which episodes of clinical disease activity (relapses) are followed by clinically silent periods (remissions). Most patients (~85%) are initially diagnosed with RRMS, followed years later by secondary progressive (SP)MS, in which disability accumulates over time without periods of remission [10].

1.2 MS Research

Early MS Research

A milestone in the history of MS research was the discovery of myelin in 1854, by Rudolf Virchow [11]. MS was identified in 1868 by Jean-Martin Charcot, who dissected the brain of a patient postmortem, and found lesions that he named *sclerose en plaques*[12]. Charcot's classification of MS as a distinct nosological entity was a landmark achievement in the field of neurology [13-15]. He was the first physician to correlate the clinical symptoms of MS with detailed pathological descriptions of CNS lesions [12, 14, 16]. It was not until 1928, however, that oligodendrocytes were identified by Hortega and Penfield as the cells responsible for myelination in the CNS [17]. A great body of work addressing proper fixation, sectioning, and staining brain tissue preceded this discovery: Hortega and Penfield's work was built upon Golgi and Cajal's efforts to allow for anatomical characterization of the CNS via novel staining and microscopy techniques.

MS disease subsets

The most common subset of MS is RRMS, which is a presenting form of disease in 75-80% of MS patients. RRMS patients experience relapses, characterized by acute onset of neurological symptoms. These periods are often accompanied by the presence of gadolinium-enhancing lesions, which denote BBB disruption. MS relapses are manifested by symptoms including motor weakness, visual symptoms, sensory deficit, coordination difficulties, or vertigo. Relapses can last from days to months, and are abutted by periods of without neurological symptoms called remissions [10].

Most RRMS patients eventually develop SPMS. SPMS is characterized by progressive neurological impairment with no relapses and remissions, CNS atrophy, and less inflammation than is observed in RRMS. Some MS patients exhibit a progressive disease course from the onset of the disease, with no relapses and remissions, and these patients are diagnosed with PPMS. It is unclear at this time whether these “subsets” of MS are truly variations of the same disease, as they are presently classified, or if in fact these subsets represent distinct diseases with unique underlying causes. To this point, neuromyelitis optica (NMO), which was until recently considered to be a subset of MS, is now recognized as a distinct CNS autoimmune disease in which aquaporin-4 water channels expressed on astrocytes are targeted by NMO IgG antibodies, ultimately leading to demyelination of the optic nerve and spinal cord [18].

Environmental Studies

It is broadly accepted that both environmental and genetic factors play roles in the pathogenesis of MS. Epidemiological studies have verified that several environmental factors may trigger MS clinical presentation. These include geographical latitude, vitamin D deficiency [19], smoking [20, 21], and perhaps CNS infection with viruses such as Epstein-Barr virus (EBV) [22, 23] or varicella-zoster virus (VZV) [24]. Environmental studies have broadly given credence to the hypothesis that MS is not only determined by a genetic susceptibility, but is a disease in which individuals of a genetically-susceptible background are exposed to environmental agents that instigate the pathogenesis of MS. The hygiene hypothesis is based on epidemiological evidence that higher incidence of autoimmune diseases in the developed world may be due to lower exposure rates to infection and better sanitation [25]. A reduced exposure to helminthes has been targeted by some researchers as a potential cause of MS development, because helminthes shift the immunological balance toward a more immunosuppressive state. Specifically, helminthes may induce T-cell populations such as $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells that prevent the development of autoreactive pathogenic T-cells such as Th17 cells and Th1 cells, and instead produce a Th2 response [26].

MS Genetics

Genetic factors clearly play a role in MS pathogenesis. Clues of genetic susceptibility to MS include studies which show that people of Caucasian descent are at greater risk to develop MS than other groups [27], and that familial recurrence is common among MS sufferers [28]. More recently, large multi-center collaborative studies revealed that the HLA class II extended haplotype HLA-DRB5*0101-HLADRB11501-HLA-DQA1*0102-HLA-DQB1*0602 confers the majority of genetic susceptibility to MS [29]. Other immunologically-related genetic targets have been identified as potentially related to MS susceptibility as well, including genetic variants of the gene icam1 [30], and immune targets such as IL2RA, IL7R and CD58 [29]. Two single nucleotide polymorphisms (SNPs) in CX3CL1 receptor (CX3CR1) have been shown to alter CX3CR1 expression levels and binding affinity for CX3CL1 [31, 32]. These SNPs are $V_{249}I$ and $T_{280}M$. Combinations of the expression of these SNPs lead to four CX3CR1 haplotypes: $V_{249}T_{280}$, $I_{249}T_{280}$, $I_{249}M_{280}$, and $V_{249}M_{280}$ [33]. While MS susceptibility has not been found to be associated with expression of any of these haplotypes, severity of MS course has been found to be correlated with haplotype expression [34]. $I_{249}T_{280}$ was found to be expressed in a significantly greater number of RRMS patients than in SPMS patients. This suggests that RRMS patients who express this haplotype do not progress to SPMS as quickly as patients who express the other haplotypes, suggesting a potential protective effect for CX3CR1 haplotype $I_{249}T_{280}$ [34]. In summary, human genetic research has proven to be valuable in uncovering specific molecular targets which confer MS susceptibility and may be useful in the future for earlier and more accurate MS diagnosis.

Antigen-specific $CD4^+$ T-cells.

MS was first proposed to be a disease brought on by self-reactive $CD4^+$ T-cells due to experimental autoimmune encephalomyelitis (EAE) studies which showed that CNS demyelination in EAE can be induced by immunization with CNS myelin proteins. Myelin basic protein (MBP) and PLP are the most extensively studied CNS myelin proteins in the context of EAE, and reactivity to these antigens is proposed to be a component of MS immunopathogenesis as well [35, 36]. Autoreactive MBP and PLP-reactive T-cells are present in similar frequencies in patients and controls [1, 37, 38]. However, Zhang and colleagues showed that MBP- and PLP-reactive $CD4^+$ T-cells derived from the CSF and blood of RRMS patients more frequently express IL-2R activation marker, so that their activation state and not their mere presence constitutes a hallmark of the autoimmune response in MS [39]. Taken together, these data suggest that while both HCs and MS patients have circulating myelin-protein autoreactive T-cells, in MS patients they have already been repeatedly activated by myelin protein antigens *in-vivo*.

Immunological Studies

A large body of data derived from human research points to an immunological basis of MS. The immunological etiology of MS is closely tied to MS genetics, as the HLA class II haplotype associated with MS susceptibility codes for Major Histocompatibility Complex II (MHC II), a heterodimer expressed on lymphocytes, and APCs that present antigen to $CD4^+$ T-cells to trigger

immune response [29]. While myelin-reactive T-cells are found in circulation of both MS patients and HCs, those derived from MS patients express activated phenotype, and do not require costimulatory CD28 signaling for recurrent activations [40, 41]. [39]. Importantly, myelin-reactive $CD4^+$ and $CD8^+$ T-cells from MS patients express a distinct inflammatory cytokine profile, expressing increased levels of IFN-γ [42]. Also, myelin oligodendrocyte glycoprotein MOG_{97-109} reactive $CD4^+$ T-cells have been shown to be present in greater frequencies in the blood of MS patients compared to HCs [43].

It is well-established that $CD4^+$, $CD8^+$ T-cells, B-cells and monocyte-derived cells are implicated in the pathogenesis of MS [2, 44-47]. These cell subsets play role in the immunosurveillance of the CNS in health and disease, and in MS lesion formation [48] (Fig. 1.1). In this study, we will evaluate these cell subsets derived from RRMS patients and HCs for differences in surface expression of chemokine receptors and adhesion molecules. The following is a brief overview regarding the role of these cell subsets in MS.

$CD4^+$ T-cells. Adoptive transfer of activated myelin-specific $CD4^+$ T-cells into wild type mice induce EAE, suggesting that $CD4^+$ T-cells are the prime pathogenic cells in EAE and MS [49, 50]. Specifically, T_H1 and T_H17 $CD4^+$ T-cell subsets mediate disease development in multiple EAE models [51-53]. Genetic studies of MS have implicated $CD4^+$ T-cells in the development of the disease: expression of DR2 HLA class II alleles which are involved in antigen presentation to $CD4^+$ cells are associated with MS susceptibility [29, 54]. Therefore, the expression of proteins related to $CD4^+$ T-cell migration into the CNS—chemokine receptors and adhesion molecules—are of great interest.

$CD8^+$ T-cells. $CD8^+$ T-cells' role in MS pathogenesis have not been studied as thoroughly as the role of $CD4^+$ T-cells. However, MS genetic studies implicate $CD8^+$ T-cells as the susceptibility for the disease is associated with the expression of MHC class I allele HLA-A*301, and upregulation of MHC class I molecules has been observed in MS lesions [55]. Clonally-expanded $CD8^+$ T-cells are present in the perivascular regions and at the lesion edge [44, 45]. Furthermore, $CD8^+$ T-cells are present in greater numbers in active MS lesions than $CD4^+$ T-cells [44, 56]. Myelin-specific $CD8^+$ T-cells have been derived from the blood of MS patients and HCs [57], and these cells have been shown to express IFN-γ and TNF-α, and have the ability to lyse oligodendrocytes *in vitro* [58].

$CD19^+$ B-cells. B-cell involvement in MS has been historically understudied, but has recently become a major focus in MS research. As early as 1947, Kabat, et al., have suggested that myelin protein-specific antibodies may play role in human demyelinating disease [59]. Oligoclonal bands representing clonal IgG in the CSF of MS patients are considered a contributing finding for the diagnosis of MS [60], and several studies have demonstrated that antibodies are present in the demyelinated areas of the brain and in the CSF of MS patients [61, 62]. Autoantibodies against MOG, MBP, and proteolipid protein (PLP) have been identified in MS lesions, and proposed to contribute to MS pathogenesis [63-66]. These myelin-reactive autoantibodies are proposed to play a role in MS by several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC) [67], opsonization and phagocytosis[63], complement activation of effector cells, or antibody-induced demyelination [68]. Heightened levels of complement deposition, which support the functions of pathogenic

antibodies, have also been observed in MS lesions. Rituximab, a monoclonal antibody against CD20 that specifically lyses B-cells, has been shown to reduce gadolinium-enhancing lesions and relapse rates at early time point, implicating the role of B-cells in the antigen presentation or the inflammatory response regulation, and not only antibody production [47]. Importantly, in an EAE model, B-cells were required to induce an immune response to CNS myelin proteins [69]. B-cell migration across BBB endothelia has been shown to be dependent upon chemokines, including CCL2 [70].

$CD14^+$ monocytes. Monocytes act as APCs in peripheral circulation and in the lymphoid organs, and also migrate into the CNS where they differentiate into DCs and macrophages [71]. In EAE, monocytes-derived macrophages participate in the disease pathogenesis by migrating across the BBB, and actively participate in demyelination by digesting the myelin sheath surrounding axons [72]. The ablation of monocyte recruitment into the CNS blocks lesion formation in EAE [73]. Notably, CCL2 blockade during EAE has been shown to block pathogenesis of the disease [74]. One feature that characterizes active MS lesions is the presence of macrophages containing myelin degradation products [75]. Monocyte-derived macrophages may also act as APCs in CNS lesions, expressing MHC class II molecules and presenting myelin-Ag products such as α B-crystallin that may reactivate infiltrating $CD4^+$ T-cells [76].

Several other immune cell subsets also play roles in MS. A brief description of two of these subsets is as follows:

Regulatory T cells. In 1977, Adda, et al. showed that “suppressor cells” are increased in number during EAE recovery [77]. Since then, $FoxP3^+$ Regulatory T cells (Tregs) have been shown to play role in MS. Tregs can restrict proliferation and cytokine production of immune cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes, macrophages, and DCs. Tregs play an important role in the control of peripheral tolerance, and the breakdown of tolerance to self-reactive antigens may lead to autoimmune diseases such as MS [78]. Tolerance in MS may be broken by a reduction in total Treg numbers, by the existence of dysfunctional Tregs in individuals susceptible to MS, and inhibition of T-cell suppression by Tregs [79] [80]. Interestingly, Tregs have been found to be increased in numbers due to administration of immunomodulatory therapies IFN-β and glatiramer acetate, further suggesting a protective role for Tregs [81, 82].

Dendritic cells. DCs are APCs that process and present antigens to T cells and secrete regulatory cytokines that are capable of inducing the activation and differentiation of naive and memory T cells. Peripheral blood DCs have been shown to be able to migrate to the CNS [83], and DCs are also found in the CSF and lesions of MS patients [84-86]. In EAE, T cells and DCs interact in the CNS to drive disease pathogenesis [5, 87, 88]. Analyses of MS CNS tissue have shown that perivascular spaces on the border of active lesions contain a substantially greater DC content than NAWM [84]. This suggests that DCs may play a role in antigen presentation at the BBB, and therefore may play a primary role in MS pathogenesis. Recent studies have also shown that DCs play important roles in secretion of proinflammatory cytokines that induce Th17 cell

differentiation [83]; this polarization can be blocked by IFN-β1a [89], as well as simvastatin [90].

Biomarker Identification Studies

Early diagnosis and treatment may improve clinical outcome for MS patients [91]. However, there is currently no single test which can diagnose MS, so in many MS patients the initiation of disease-modifying treatments is delayed [92]. CSF samples are typically available at the time of MS diagnosis, and therefore MS disease activity biomarkers in the CSF would be of great benefit for the improvement of diagnostic accuracy and the timely initiation of the treatment of MS. Several potential biomarkers have been identified which may aid in the diagnosis of MS, or prognosis to determine MS disease course. Potential CSF biomarkers include adhesion molecules such as soluble Vascular Adhesion Molecule-1 (sVCAM-1) [93-95], sICAM-1 [96], and Neural Cell Adhesion Molecule (NCAM) [94, 95], as well as chemokines such as CXCL9, CXCL10, CXCL13, CCL5 [97, 98].

CSF-derived Cell Analysis in MS

T-cells are the predominant lymphocyte subset derived from MS CSF [99-101]. A greater percentage of CSF-derived T-cells are activated in comparison to the blood-derived T-cells, particularly during relapses of the disease [102]. The analysis of chemokine receptor surface levels on the CSF-derived $CD4^+$ T-

cells has revealed that several chemokine receptors (CCR5, CXCR3) are upregulated significantly on these cells, correlating with upregulation of these receptors on lesion-derived $CD4^+$ T-cells [98].

Animal Models of MS

Animal models of MS have served as tools to understand the genetic and molecular mechanisms of MS pathology and have been instrumental in developing several effective MS therapies. Three animal models have been widely used to study MS: EAE, chemically-induced demyelination such as cuprizone-induced demyelination, and virally-induced chronic demyelination. In the following subsections, we will briefly discuss each MS animal model and its contribution to our understanding of MS.

EAE

Thomas Rivers characterized EAE in 1933 [103]. It was not until a decade later, however, that it was observed that the neurological damage induced in EAE is pathologically similar to human CNS demyelinating disorders [104]. In classic EAE, a mouse or other mammalian recipient is immunized with CNS myelin antigen, or disease is elicited via passive transfer of encephalitogenic T-cells [59, 105]. In these models, a T_H1 cell-mediated immune response at the spinal cord and brain leads to CNS demyelination [104]. This myelin damage results in clinical manifestations similar to those seen in MS, such as sensory deficits, motor weakness, visual loss or coordination deficits [35]. Research in EAE has

led to a wealth of data pertaining to MS, and insights attained from these models have led to the development of disease-modifying therapies for MS [106, 107]. Largely due to research in EAE animal models, the immune-initiated disease hypothesis of MS has been favored by the majority of MS researchers. In this model, myelin autoreactive T-cells enter the CNS and induce focal inflammatory demyelination of the brain and spinal cord, causing acute and chronic disability in MS patients. EAE has several drawbacks, however. First, in B6 mice, EAE affects the lumbar spinal cord, while the brain is predominantly affected in MS [108]. Second, EAE does not recreate the MS pathological profile in its entirety; for instance, classic EAE does not mirror B-cell autoantibody production adequately [109]. Third, Lastly, therapies that have been shown to be effective for the treatment of EAE have often not been shown to be effective for the treatment of MS [110].

Additional findings from the EAE model show that adhesion molecules such as ICAM-1 are upregulated on the luminal side of endothelial cells of the BBB [111, 112], and facilitate autoreactive cell entry into the CNS (Fig. 1.1 B). ICAM-1 expression on T-cells is also critical for EAE development [111], and CX3CR1 has been shown to be critical for the transmigration of NK-cells into the CNS during EAE [113].

The Cuprizone intoxication Model of Demyelination and Remyelination

Several models of demyelination utilize a drug or toxin to induce demyelination. These models include lysolecithin injections, which induce demyelination via activation of phospholipase A_2 [114], ethidium bromide

injections into the spinal cord [115], and introduction of cuprizone in the diet [116]. Of these models, cuprizone-induced demyelination is the most widely used toxin-induced demyelination model in MS research. In the late 1960s, studies by William Carlton established that ingestion of the copper chelator cuprizone has neurotoxic effects [117, 118]. It was later found that mature oligodendrocytes are particularly susceptible for apoptosis in the presence of cuprizone, while other cell types are not affected [119]. Samuel Ludwin then characterized cuprizone-induced demyelination in the superior cerebellar peduncle, finding that axons remained undamaged while demyelination and later oligodendrocyte death occurred [120]. After removal of cuprizone from the diet, remyelination was found to progress quickly, remyelinating axons to roughly half the thickness of undamaged myelinated fibers [120]. Ludwin then studied the source of remyelinating oligodendrocytes during remyelination, finding that immature, proliferating oligodendrocytes differentiated into mature oligodendrocytes, which were ultimately responsible for remyelination of cuprizone-demyelinated axons [121].

Demyelination by cuprizone represents an attractive model to study demyelination and remyelination because cuprizone-induced demyelination holds to a predictable time course in which complete demyelination of several brain regions, including the corpus callosum and cerebellar peduncle, occurs over several weeks of cuprizone ingestion [122]. If this period of damage is followed by removal of cuprizone from the diet, subsequent robust remyelination is consistently observed [119, 122]. The extent of demyelination, remyelination, and infiltration of damaged areas by macrophages and migroglia can be scored by Luxol-Fast blue (LFB) staining for myelin and Periodic Acid Schiff (PAS)

staining for macrophages and microglia [123]. One drawback of the cuprizone model is that it does not feature infiltration of peripheral immune cells that is a part of MS pathogenesis. However, the cuprizone model of demyelination has been optimized in several strains of mice, including C57/B6, as this background features an abundance of transgenic and knockout lines pertinent to MS pathogenesis [116]. Because of the reproducibility of this model, it is useful tool to study the effects of therapies to suppress demyelination, and is especially valuable in evaluating therapies that may enhance remyelination [124]. In recent years, the cuprizone model of demyelination has been utilized to uncover the importance of insulin-like growth factor-1 (IGF-1) in protection of mature oligodendrocytes from apoptosis during demyelination [125], whose upregulation may be induced in microglia, macrophages, and astrocytes by IL-1β in to promote remyelination [126]. This model has also been utilized to uncover roles for chemokines such as MIP-1α in recruitment of microglia to areas of damage and upregulation of TNF-α during demyelination [124], as well as a previously unknown role for TNF-α interactions with TNFR2 in oligodendroctye regeneration after a demyelinating event [127].

Virus-induced Demyelination

Potential roles for viruses in MS have historically been studied and intensely debated. Many researchers currently believe that MS may be virally-induced, perhaps via molecular mimicry, leading to an autoimmune response to CNS myelin proteins in genetically-susceptible individuals. Others believe that a single, yet-unidentified virus may be responsible for MS pathology, evidenced by

the persistent presence of oligoclonal banding in CSF derived from MS patients [128]. Several viral models of demyelination have been developed; the most commonly used is Theiler's murine encephalomyelitis virus (TMEV). TMEV presents a model which recapitulates several features of MS: (1) presence of CNS lesions, (2) the triggering of an autoimmune response via the CNS viral infection (3) the presence of inflammatory cells in and around lesions during myelin destruction, and (4) several features of MRI results in mice that are similar to human MS MRIs, including the presence of brain, brainstem, and spinal cord lesions, and T2 hyperintense spinal cord lesions [129].

Mechanisms of Myelin destruction and lesion formation

Four distinct patterns of lesions have been identified in CNS tissue derived from MS patients biopsies by Lucchinetti, et al. The four distinct patterns of lesions are as follows [130]:

Pattern I: The lesion infiltrate is composed predominately of $CD3^+$ T-lymphocytes and macrophages. Demyelination is found in a perivenous pattern, and there is a sharp distinction between the lesion edge and neighboring tissue. Oligodendrocytes are present in higher numbers than in lesion patterns III and IV.

Pattern II: Similar to pattern I, pattern II lesions are largely composed of $CD3^+$ T-lymphocytes and macrophages. However, pattern II lesions also feature high levels of IgG, and complement C9neo antigen. Ig reactivity in pattern II lesions was associated with degenerating myelin at the active plaque edge and with degenerating myelin products within lesional macrophages. A perivenous pattern of demyelination is noted, and there is a sharp distinction between the lesion edge and neighboring tissue. Oligodendrocytes are present in similar

numbers to pattern I lesions, and higher numbers than in lesion patterns III and IV. The presence of shadow plaques indicates that some remyelination has occurred.

Pattern III: The infiltrate in these lesions is composed of $CD3^+$ T-lymphocytes and macrophages. The edge between the lesion and surrounding tissue is not sharply defined, but rather is ill-defined, and in about one-third of pattern III lesions, a concentric shape is observed. Importantly, oligodendrocyte dystrophy was observed throughout this lesion type as measured by loss of myelin-associated glycoprotein (MAG) and by oligodendrocyte apoptosis. The oligodendrocyte destruction in this lesion pattern was similar to that seen in toxin- or virus-induced animal models of MS.

Pattern IV: Macrophages compose a larger proportion of total infiltrate in this subset, while a significant $CD3^+$ T-lymphocyte presence is also observed. Oligodendrocyte cell death was observed in a small ring of periplaque white matter as measured by DNA fragmentation: no shadow plaques were observed, and remyelination appeared to be absent. Similar to pattern III, the oligodendrocyte destruction in this lesion pattern was similar to that seen in toxin- or virus-induced animal models of MS. This lesion subset was found exclusively in patients with PPMS.

Interestingly, these four lesion pattern subsets were found to be homogeneous within an individual patient [130]. However, these findings are still somewhat controversial because another group has found that all acute MS lesions display complement activation and oligodendrocyte apoptosis, while subacute lesions derived from the same patients display complement activation

but no signs of oligodendrocyte apoptosis [131]. This was interpreted as evidence that oligodendrocyte apoptosis is a hallmark of the earliest stage of MS lesion formation [131].

Henderson, et al., attempted to address mechanisms of myelin destruction in MS by analyzing immune cell infiltrate in active lesions derived from patients with early MS [84]. This group found that the normal appearing white matter (NAWM) surrounding newly formed lesions contained an abundance of activated microglia that expressed CD45 and MHC Class II compared with lower numbers of microglia in NAWM that was not near lesion tissue [84]. NAWM also contained astrocytes, neurons, and oligodendrocytes that expressed IgG. Prephagocytic tissue (defined in this study as still-intact tissue exhibiting signs of impending myelin damage) directly adjacent to actively demyelinating lesions was found to contain damaged and apoptotic oligodendrocytes, and only a slight increase in T cells compared to NAWM [84]. In phagocytic lesion tissue, IgG+ phagocytic MHC Class II-negative microglia containing myelin products were found in abundance compared to in NAWM. This group speculated that microglia in phagocytic lesion tissue may expand active MS lesions by secreting molecules toxic to oligodendrocytes, or that active lesions may expand due to hypoxic stress in oligodendrocytes. Interestingly, CD4- and $CD8^+$ T-cells were not found at greatly heightened levels in phagocytic tissue, but were found in high levels in perivascular prephagocytic tissue [84]. In recently demyelinated tissue, demyelinated axons were found in close proximity to IgG+ activated microglia and macrophages. CD4- and $CD8^+$ T-cells, B-cells, and IgG-producing plasma cells were found in this tissue in greater numbers than in phagocytic tissue, and regenerating oligodendrocytes were also observed [84].

1.3 The Role of CX3CL1 and CX3CR1 in CNS Inflammatory Diseases

Chemokines facilitate the chemotaxis of proinflammatory leukocytes toward areas of inflammation and activate adhesion molecules to bind their receptors, enabling cell migration and transmigration [132, 133]. In experiments relevant to the study of MS, several groups have characterized various chemokine and chemokine receptor knockout mice, allowing analysis of chemokine function *in vivo* [113, 134, 135]. A broad conclusion drawn from these studies is that chemokines direct migration and extravasation of leukocytes into the CNS during inflammation. Experiments utilizing EAE have shown that robust upregulation of a variety of chemokines and chemokine receptors occurs in concert with disability accumulation and demyelination [72], and chemokines and chemokine receptors have been shown to be critical for EAE development [136]. Because of convincing temporal and spatial correlations between chemokine expression and CNS leukocyte infiltrate in EAE, chemokines have become a major focus of MS research [72, 137, 138].

Chemokines and chemokine receptors play multiple roles MS. Levels of several chemokines, including CXCL9, CXCL10, CXCL13, CCL5, and CX3CL1 are enhanced in the CSF of MS patients during acute attacks of inflammatory demyelination in comparison to the CSF derived from healthy controls (HCs) or neurological disease controls [97, 98, 139]. A higher percentage of CSF-derived leukocytes express chemokine receptors, including CCR5, CXCR3, and CCR7 during acute MS exacerbations, suggesting functional significance of elevated chemokine levels observed in the CSF [98, 100, 140]. Robust expression of multiple chemokine receptors has been observed on the surface of leukocytes in the demyelinating lesions of RRMS patients, suggesting that chemokine

receptors may allow inflammatory cells to hone to lesions [139, 141, 142]. Expression of CX3CR1 has been observed on the surface of leukocytes in the demyelinating lesions of relapsing remitting (RR) MS patients, indicating that CX3CR1 may guide inflammatory cells to lesions [139].

In 1997, Bazan and colleagues identified CX3CL1, noting that unlike all other known chemokines, the sequence for CX3CL1 does not terminate at the end of the chemokine domain (76 aa). Rather, the CX3CL1 chemokine domain is connected to a mucin stalk (241 aa), followed by a transmembrane domain (18 aa) and an intracellular domain (37 aa) [143]. This group found that, when expressed in HEK293 cells, soluble CX3CL1 is shed as a 95 KDa glycoprotein [143]. Later, Pan and colleagues noted that CX3CL1 mRNA is expressed predominantly in the healthy murine brain [144]. Later in 1997, Imai and colleagues identified CX3CR1, a high affinity functional CX3CL1 receptor that was shown to mediate cell adhesion and transendothelial migration of T-cells and monocytes [145]. CX3CR1 had been previously identified as “orphan” receptor chemokine β receptor-like 1 (CMKBRL1) by Combadiere and colleagues [146]. CX3CR1 is a seven-transmembrane, G-Protein-coupled receptor [145].

CX3CL1 is the only member of the δ-chemokine family, distinguished from other members of the chemokine superfamily by 1) a distinct structural motif in the N-terminus [143] and 2) duality as a membrane-bound form, in which it acts as an adhesion molecule, and a soluble form, in which it mediates immune cell migration. Membrane-bound CX3CL1 is cleaved constitutively by disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) to produce baseline levels of soluble CX3CL1 (Table 1.1). During inflammation, activated cells

produce disintegrin and metallopeptidase domain-containing protein 17 (ADAM17) to enhance the production of soluble CX3CL1 [147-149] (Table 1.1). The cleavage of CX3CL1 by these two matrix metallaproteinases (MMPs) is intriguing for several reasons. First, the ADAM 10—ADAM 17 system of cleavage of membrane-bound CX3CL1 allows for immediate soluble CX3CL1 production in response to an inflammatory extracellular microenvironment. Second, MMPs have recently come into focus as potential therapeutic targets in neuroinflammatory diseases such as MS [150], and ADAM17 and ADAM10 levels have specifically been shown to be upregulated in chronic active MS lesions [151]. Last, ADAM17 is known to cleave several cytokine receptors and membrane-bound adhesion molecules, which are intricately involved in neuroinflammation, such as IL-6R, TNF-α, and ICAM-1 [152-155].

CX3CL1 and its Downstream Signaling Pathways

Molecular pathways downstream of CX3CL1 vary between different cell subsets. In $CD14^+$ monocytes, co-culture with CX3CL1-expressing human umbilical vein epithelial cells (HUVECs) leads to up-regulation of IL-6, CCL2, and MMP-9 [156]. In murine microglia, however, CX3CL1 suppresses production of IL-6, NO, and TNF-α, [157] (Table 1.1). Application of CX3CL1 *in-vitro* suppresses neuronal cell death by activated microglia [157] (Table 1.1). This discrepancy between molecular pathways induced by membrane-bound and soluble CX3CL1 may underlie distinct molecular actions by these two isoforms, or may indicate cell subtype specific actions of CX3CL1.

CX3CR1 Surface Expression is Modulated by Several Cytokines and Chemokines

CX3CR1 expression is upregulated by several cytokines and chemokines, and it is dependent upon cell type. IL-2 upregulates expression of CX3CR1 via NFAT2, while IL-15 down-regulates CX3CR1 expression via NFAT1 in peripheral blood mononuclear cells (PBMCs) [158] (Table 1.1). In human monocytes, CCL2-CCR2 interactions were shown to stimulate surface expression of CX3CR1 [159] (Table 1.1). In a recent study involving human monocytes, IL-10 was shown to upregulate surface expression of CX3CR1 via phosphatidylinositol 3-kinase (PI3K); conversely, IFN-γ decreased surface expression of CX3CR1 [160] (Table 1.1). In contrast, in HUVECs, the effect of IFN-γ incubation was an increase in CX3CR1 expression [161] (Table 1.1). Furthermore, sIL-6R signals through gp130 in HUVECs to inhibit the IL-1 or IFN-γ-induced CX3CR1 expression [162] (Table 1.1). These findings indicate that regulation of CX3CR1 surface expression involves several cytokines and is dependent upon cell type. This specificity of CX3CR1 surface expression modulation per cell subset may provide a therapeutic opportunity to modify the expression of CX3CR1 on specific cell types.

CX3CR1 and its Downstream Signaling Pathways

CX3CR1 is a G-protein-coupled receptor that is responsible for intracellular signaling via several pathways. Expression of CX3CR1 mRNA is down-regulated in response to Receptor Activator of Nuclear Factor Kappa B

Ligand (RANKL) in a monocyte-like cell line, inhibiting chemotaxis [163] (Table 1.1). In human pulmonary artery endothelial cells (HPAECs) and HUVECs, CX3CL1 signals through CX3CR1 to increase surface ICAM-1 via the JAK2-STAT5 pathway, thereby promoting adhesion of neutrophils [164] (Table 1.1). In rat hippocampal neurons expressing CX3CR1, application of CX3CL1 induces antiapoptotic pathways via PI3K-Akt pathway, resulting in neuroprotection [165].

Potential Roles for CX3CL1-CX3CR1 in CNS Inflammatory Diseases

In humans, membrane-bound CX3CL1 is highly expressed on neurons and at lower levels on microglia, macrophages, astrocytes, endothelial cells, and lymphocytes. Within the CNS, neurons produce far more CX3CL1 than any other cell subset. Furthermore, CX3CL1 has been shown to function as a neuroprotectant by inhibition of Fas-mediated microglial apoptosis [166]. In several mouse models of CNS disease, CX3CL1-CX3CR1 interaction has been shown to be critical for effective microglial responses to inflammatory and neurotoxic stimuli, and in the absence of CX3CR1, microglia undergo cell-autonomous neurotoxicity [167]. In human mononuclear cells, CX3CR1 is expressed on monocytes, macrophages, microglia, $CD4^+$ T-cells, CD8+ T-cells, DCs, and NK-cells [145, 168]. In monocytes, surface expression of CX3CR1 is increased by CCL2 via p38 MAPK, and this up-regulation enhances the binding of fractalkine to monocytes [159].

Neuroprotective Effects of CX3CL1-CX3CR1 Interaction may Contribute to the Attenuation of CNS Lesion Formation in MS

CX3CR1 is expressed in astrocytes and microglia in MS lesions, as well as in astrocytes of the NAWM surrounding MS lesions [169]. CX3CR1 has been shown previously to be expressed on 8% of $CD4^+$ T-cells derived from HCs [170], and Foussat and colleagues have reported that CX3CR1 expression is significantly higher in $CD4^+CD45RO^+$ memory T-cells in comparison to the naïve T-cells[170]. CX3CR1 surface expression is stimulated by CCL2 which induces surface expression of CX3CR1 in monocytes [159]. Chemotaxis assays performed *in-vitro* have demonstrated that a significantly greater percentage of $CD4^+CD45RO^+$ cells migrate toward CX3CL1 in comparison to the naïve T-cells[170].

In EAE, CX3CL1 has been demonstrated to selectively recruit NK-cells into the CNS. CX3CR1 KO mice, in which NK-cell migration is impaired, demonstrate increased EAE severity, lesion volume, and higher mortality [113], likely due to a regulatory role of NK-cells in CNS demyelinating disorders. While a role for CX3CL1 in ICAM-1 up-regulation has not been studied in leukocytes, CX3CL1 has been shown to increase expression of ICAM-1 in vascular endothelial cells via CX3CR1 and Jak2-Stat5 signaling [164]. Levels of phosphorylated Jak2 and Stat5 were increased in sequential order within 30 minutes of exposure to CX3CL1; an effect which was blocked by transfection of CX3CR1 siRNA [164]. This increase of ICAM-1 expression via CX3CL1-CX3CR1 interactions resulted in firmer adhesion of neutrophils to endothelial cells [164].

CX3CL1-CX3CR1 interactions are well-studied in regard to their functions in transendothelial migration. For transendothelial migration to occur, a leukocyte follows a series of steps: first, it tethers to the endothelial cell surface; then it rolls, and firmly adheres to the surface; lastly, it migrates between the tight junctions of the endothelial barrier [171]. CX3CL1 cleaved from the surface of BBB endothelial cells contributes to this process by attracting CX3CR1-expressing leukocytes [143, 145], and CX3CL1 expressed on the BBB cell surface may help mediate firm adhesion of leukocytes to endothelial cells [172]. However, it is unclear whether CX3CL1-CX3CR1 interactions are critical for the migration of immune cells into the CNS during MS lesion development.

Recently Broux, et al., found that CX3CR1 is a marker for $CD4^{+}CD28^{-}$ T-cells, and that this cell subset is expanded in a subgroup of MS patients [139]. Interestingly, this subset of cells expressed high levels of adhesion molecules such as LFA-1, ICAM-1, and VLA-4. MS patients exhibited higher levels of ICAM-1 expression on $CD4^{+}CD28^{-}CX3CR1^{+}$ T-cells than did HCs [139]. These cells were found to degranulate after anti-CD3 stimulation, and importantly, a small number of these cells degranulated after stimulation with MBP and MOG peptides [139]. Increased levels of CX3CL1 were found in CSF derived from MS patients compared to HCs, and $CD4^{+}CD28^{-}$ cells were found to preferentially migrate toward CX3CL1 *in-vitro*. Lastly, $CD4^{+}CX3CR1^{+}$ T-cells were accumulated in active MS lesions, and the apoptotic maker cleaved caspase-3 was expressed by oligodendrocytes in close proximity to $CD4^{+}CX3CR1^{+}$ T-cells, indicating that these cells may play a role in MS pathology by destroying myelin-forming oligodendrocytes[139].

CX3CL1-CX3CR1 interactions mediate leukocyte-endothelial cell adhesion [145], have roles in both inflammation and neuroprotection [157], are found to be expressed in neurons, astrocytes, macrophages, and endothelia in MS lesions [169], are responsible for immune cell trafficking into the CNS in EAE [113], and correlate with the disease activity in MS patients [173]. Therefore, CX3CL1-CX3CR1 interactions should be considered important to our understanding of MS pathology. Our studies address roles for CX3CL1 in the induction of the adhesion molecule ICAM-1 expression and of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-17A secretion, and migration of $CD4^+$ T-cells.

1.4 ICAM-1 is an Adhesion Molecule Critical for Transmigration through BBB

Adhesion molecules coordinate with chemokines to direct lymphocytes towards the sites of inflammation [174, 175]. Adhesion molecules are critical for lymphocyte adhesion to BBB endothelia, and transmigration of lymphocytes into the CNS during EAE [106, 111]. Expression of adhesion molecules on circulating lymphocytes has been correlated with T2 lesion load [176], suggesting that elevated expression of adhesion molecules may affect CNS tissue damage in MS.

ICAM-1 is a transmembrane adhesion molecule that is expressed on endothelia and leukocytes [177, 178]. ICAM-1 expression is regulated by several cytokines. IFN-γ, IL-1, IL-4, IL-6, IFN-γ, and TNF-α, lymphotoxin and lipopolysaccharide (LPS) stimulate its expression [177, 179], while TGF-β and IL-10 inhibit its surface expression in several cell subsets [180]. CX3CL1 has been

shown to stimulate ICAM-1 surface expression in vascular endothelia [164]; however, its role in the regulation of ICAM-1 expression in leukocytes has not been studied. In this study, we will address a role for CX3CL1 in ICAM-1 induction.

Under inflammatory conditions, expression of ICAM-1 is increased on BBB endothelial cells (ECs) and is associated with the disruption of tight junctions, increased EC permeability, and increased migration of T-lymphocytes across the BBB [181, 182]. Data from EAE, pathological examination of MS CNS tissue, and *in-vitro* transendothelial migration assays suggest that the expression of ICAM-1 on endothelial cells may contribute to the pathogenesis of MS by controlling inflammatory cell migration across the BBB via interaction with leukocyte-expressed lymphocyte function-associated protein 1 (LFA-1) [112, 183-186]. In EAE, BBB endothelial ICAM-1 expression is increased during relapses and decreased during remission, consistent with the hypothesis that ICAM-1 surface expression on BBB endothelia is critical for EAE lesion formation [187]. $CD4^+$ T-lymphocyte expression of ICAM-1 is also required for the development of EAE [111]. Interestingly, roles for T-lymphocytes' ICAM-1 expression in MS is less well understood than the one of endothelial ICAM-1. CX3CL1 has been shown to stimulate ICAM-1 surface expression in vascular endothelia; however, its role in the regulation of leukocyte ICAM-1 expression has not been studied [164].

A recent study has highlighted the requirement of ICAM-1 expression on T-cells for the development of EAE [111]. ICAM-1 null mice, induced with EAE, had attenuated clinical symptoms accompanied by a reduction of $CD4^+$ and $CD8^+$

T-cell infiltration into the CNS, decreased axonal degeneration, and demyelination throughout the spinal cord [111]. Furthermore, transfer of ICAM-1 null MOG-sensitized T-cells into the wild type (WT) mice failed to induce EAE or facilitate the transmigration of T-cells into the CNS, suggesting that ICAM-1 expression on T-cells is required for EAE CNS lesion development [111]. Finally, ICAM-1 expression on T-cells, but not APCs, was found to be critical for T-cell proliferation [111]. The absence of ICAM-1 also reduced splenic T-cell production of IFN-γ, TNF-α, lL-4, IL-10 and IL-12 following MOG restimulation, and reduced CSF-derived $CD4^+$ and $CD8^+$ T-cell production of IFN-γ [111].

sICAM-1

sICAM-1 lacks the transmembrane and cytoplasmic regions of ICAM-1 and is produced by many cell types, including endothelia and leukocytes. Astrocyte- and endothelia-derived ADAM-17 cleave ICAM-1 to produce sICAM-1 [152, 153]. sICAM-1 binds competitively to ICAM-1 ligands including LFA-1, and therefore has a therapeutic potential in MS. sICAM-1 may therefore play a role in reducing T-cell transmigration via competitive inhibition of LFA-1-surface ICAM-1 interactions [188, 189]. Indeed, serum levels of sICAM-1 have been shown to be increased in response to rIFNβ-1 treatment, and this increase is correlated with decreased contrast-enhancing brain MS lesion load [190]. Interestingly, *in-vitro* application of sICAM-1 to activated lymphocytes blocks their adhesion to CNS-derived endothelial cells, and this effect is abrogated in a dose-dependent manner upon application of anti-ICAM antibody [189]. These data imply that

sICAM-1 may inhibit neurological damage in RRMS by blocking lymphocyte BBB transmigration via interference with adhesion molecule-mediated cell adhesion.

Data pertaining to roles for sICAM-1 in MS are mixed, but sICAM-1 may act by competitively binding LFA-1 to block LFA-1-ICAM-1 interactions, and therefore transmigration across the BBB, in MS. sICAM-1 has been shown to interfere with the adhesion of PBMCs to cerebral endothelial cells *in-vitro*, suggesting that it competes with ICAM-1 for interaction with LFA-1 [189]. Furthermore, sICAM-1 serum levels in MS patients have been shown to be increased by treatment with rIFNβ-1b, paralleling a period of improvement as measured by decreased frequency of relapses, disability levels, and gadolinium-enhancing lesion load [190]. In this context, suppressed CSF sICAM-1 expression in MS patients in our study may be interpreted as an indicator of an inflammatory response, therefore enabling greater migration of inflammatory leukocytes across the BBB.

1.5 Proinflammatory Cytokines IFN-γ and TNF-α are Potential Therapeutic Targets in MS

Cytokines are critically involved in immune responses, and can be divided into proinflammatory cytokines and antiinflammatory cytokines. Many of the proinflammatory cytokines are thought to play roles in MS pathogenesis, including in peripheral activation of circulating immune cells, as well as direct damage to myelin. Proinflammatory cytokines include IFN-γ, TNF-α, IL-17A, IL-17F, IL-12, and IL-23. Antiinflammatory cytokines are generally thought to play

disease-attenuating roles in MS, and include IL-4, IL-10, and TGF-β. Here we will focus on the proinflammatory cytokines IFN-γ and TNF-α.

Administration of IFN-γ, once proposed as a potential MS therapeutic, is now known to cause exacerbations in MS patients [110], perhaps due to its roles in the generation of cytotoxic T-cells and prevention of remyelination [191, 192]. TNF-α is also known to play role in MS pathology. TNF-α expression is increased in the CSF and MS lesions [193, 194], and is critical for the initiation of tissue damage in EAE [195, 196]. These effects are blocked by anti-TNF-α antibodies [197]. However, TNF-α has also been shown to have immunosuppressive effects at later EAE time points [198]. IFN-γ and TNF-α have been shown to stimulate CX3CL1 expression and release by endothelial cells, and CX3CL1 expression is increased in the IFN-γ-mediated Th1 disease psoriasis [199]. Immobilized CX3CL1 and CX3CL1-transfected 293E cells induce IFN-γ expression in NK-cells [200]. These data suggest that CX3CL1, IFN-γ, and TNF-α may act in an inflammatory positive feedback loop during Th1-mediated diseases.

Results from studies regarding involvement of IFN-γ and TNF-α in MS pathology have been mixed; while several studies have shown IFN-γ mRNA levels to be increased in PBMCs derived from MS patients, and IFN-γ protein levels in serum derived from MS patients is enriched, several other studies report no differential IFN-γ expression between patients and controls [201, 202]. TNF-α has also been studied with conflicting results. TNF- αhas been shown to induce oligodendrocyte cell death [203]. Several groups have found PBMC TNF-α mRNA levels, serum TNF-α, and secretion of TNF-α by PBMCs to be increased in MS patients [201, 204, 205]. Unfortunately, though, TNF-α-blocking therapies

such as sTNF-α receptor Ig fusion protein or anti-TNF-α mAb were found to worsen MS exacerbations [206].

Chemokine receptors that we analyzed on leukocyte subsets

In recent years, several chemokine receptors and adhesion molecules such as CCR1, CCR4, CCR6, ICAM-1, and VLA-4 have been shown to play a role in the development of EAE, and blockade of these adhesion molecules and chemokine receptors via humanized monoclonal antibodies has led to the development of therapies that may decrease disease progression [111, 136, 138, 207-209]. Here is a brief synopsis of what is known about each chemokine receptor and its relation to RRMS:

CCR1. CCR1$^{-/-}$ mice exhibit reduced EAE symptoms, suggesting that CCR1 is critical for the development of neuroinflammation [209]. In humans, CCR1 is expressed on blood-derived T-cells and monocytes, and on CSF- and lesion-derived monocytes [46, 140, 210]. Its ligands are chemokines MIP-1α (CCL3), RANTES (CCL5), and MCP-3 (CCL7), CCL14 (HCC-1), CCL16 (HCC-4), and CCL23 (MPIF-1, CKβ8) (Table 1.2). One study found that CCR1^{+} phagocytic cells accumulated in the perivascular areas of MS lesions [46], and that the CCR1 ligand CCL5's concentration is elevated in CSF derived from RRMS patients [98]. Several groups have begun clinical trials using CCR1 small molecule antagonists as a treatment of RRMS and other inflammatory CNS disorders [211-213], but the data so far have been disappointing. Oral CCR1 antagonist BX471 was tested in clinical trials, with the primary endpoint of a number and volume of active Gd enhancing lesions on T1-weighted scans at 16

weeks after treatment onset in 105 MS patients [214]. No decrease in lesion formation was observed [214].

CCR2. CCR2 is expressed on monocytes, T-cells, B-cells, and DCs [215]. Its ligands include MCP-1 (CCL2), MCP-3 (CCL7), MCP-2 (CCL8), and HCC-4 (CCL16) [216] (Table 1.2). Studies in EAE implicated that CCR2 plays a role in EAE induction and lesion formation [217]. CCR2 expression is increased in RRMS lesion-derived macrophages [142, 218], but T-cells and monocytes from RRMS patients have not shown differential expression of CCR2 compared to controls [219]. Migration of B-cells across the brain endothelial cells is dependent upon CCL2 [70].

CCR3. CCR3 is expressed on T-cells, B-cells, monocytes, eosinophils, and basophils. CCR3 is a promiscuous chemokine receptor, which binds to RANTES (CCL5), MCP-3 (CCL7), MCP-2 (CCL8), Eotaxin-1 (CCL11), MCP-4 (CCL13), HCC-2 (CCL15), Eotaxin-2 (CCL24), Eotaxin-3 (CCL26), and MEC (CCL28) (Table 1.2). CCR3 is expressed on macrophages in MS lesions, but has not been extensively studied in MS [218].

CCR5. CCR5 is expressed on multiple cell subsets, including T-cells, B-cells, and monocytes. Its ligands include MIP-1α (CCL3), LD78β (CCL3L1), RANTES (CCL5), and CCL14 (HCC-1) (Table 1.2). Multiple lines of evidence point to the CCR5 involvement in the development of the inflammatory response in MS. First, genetic evidence points to a pathological effect for CCR5. A cohort of individuals expresses the “Δ32” CCR5 gene mutation, leading to the loss of functional expression of CCR5. Interestingly, while Δ32 CCR5 homozygotes are not protected from MS [220], Δ32 CCR5 heterozygotes with

RRMS experience significantly longer periods of remission than patients expressing functional CCR5 [221]. Second, several groups have shown increased CCR5 expression on blood-derived T-cells [98, 222], particularly during relapse [210], and $CCR5^+$ T-cells were found to express higher levels of IFN-γ and TNF-α[223]. Last, $CCR5^+CD4^+$ T-cells and $CCR5^+CD8^+$ T-cells are also enriched in MS CSF compared to matched blood-derived cells, and cells derived from non-inflammatory neurological diseases (NINDs) controls and HCs. CCR5 is also expressed on macrophages and microglia [98]. Lastly, CCR5 is expressed on microglia and CD4+ T-cells in MS lesion tissue [218].

CCR6. CCR6 is expressed on T-cells, B-cells, and monocytes, and is the only receptor for CCL20 [224] (Table 1.2). With the recent discovery that CCR6 is a lineage marker for the T_H17 $CD4^+$ T-cell subset, CCR6 expression has become an attractive focus of research in EAE and MS for their potential roles in pathology, as well as the search for Th17-targeting therapeutics [135]. CCR6 is expressed at similar levels on T-cells derived from blood and CSF of MS patients, and is expressed at similar levels during inflammation and in non-inflammatory controls [140]. In our study we did not find CCL20 to be differentially expressed in the CSF derived from RRMS patients and HCs

CX3CR1. CX3CR1 is the lone receptor that interacts with the chemokine CX3CR1 (Table 1.2). Because we found increased CX3CL1 concentrations in the CSF from untreated RRMS patients in comparison to HCs, we chose to evaluate the differential expression of CX3CR1 on MS-related immune cell subsets derived from untreated RRMS patients compared to HCs.

Adhesion molecules that we analyzed on leukocyte subsets

Adhesion molecules are involved in the inflammatory cell migration across the endothelial barriers, and the blockade of interaction between adhesion molecules and their ligands have been used as a treatment approach in MS. Particularly, blockade of VLA-4 interaction with VCAM-1—involved in BBB transmigration by several leukocyte subsets—by the humanized anti-VLA-4 monoclonal antibody natalizumab, has demonstrated clinical efficacy in reducing the number of exacerbations and decreasing CNS lesion load in RRMS patients [208, 225]. Because of the importance of adhesion molecules in MS pathogenesis, we measured the expression of three adhesion molecules, ICAM-1, VLA-4 and LFA-1 on $CD4^+$ T-cells, $CD8^+$ T-cells, $CD19^+$ B-cells, and $CD14^+$ monocytes in RRMS patients and HCs to address changes in adhesion molecule expression that may occur during MS and reflect changes in chemokines, cytokines, and chemokine receptor expression. Following is a brief synopsis of what is known about each adhesion molecule and its relation to RRMS:

VLA-4. VLA-4 is a ligand for VCAM-1, and its blockade via the monoclonal antibody natalizumab has proven to be an effective MS therapy due to attenuation of inflammatory cells migration through BBB [208, 225]. Because VLA-4 is proven to be critical for BBB transmigration and lesion formation, we decided to measure its expression levels on T-cells, B-cells, and monocytes.

ICAM-1. ICAM-1 (a ligand for LFA-1) has been implicated in EAE and MS pathology, and became a major focus of our research when we found sICAM-1 decreased levels in the CSF of untreated RRMS patients in comparison to HCs. We also noted that ICAM-1 expression on $CD4^+$ and $CD8^+$ T-cells is critical for

EAE induction [111], and that increased levels of CX3CL1 upregulate ICAM-1 via CX3CR1 [164]. We therefore hypothesized that in MS patients, in the presence of increased levels of CX3CL1, ICAM-1 would have increased expression on immune cell subsets in comparison to HCs.

LFA-1. LFA-1 is an integrin that binds in *trans* with endothelial-expressed ICAM-1 to allow migration of lymphocytes into the CNS for normal immunosurveillence and during neuroinflammation [225]. LFA-1 has recently been shown to require one of several bound chemokine receptors (CXCR4, CCR7, or CXCR3) so that it may bind ICAM-1 under shear stress conditions [175]. We hypothesized that LFA-1 may be expressed at increased levels on T-cells, B-cells, or monocytes derived from untreated RRMS patients when compared to HCs.

1.6 Rationale

Initially, we focused on the expression of CX3CL1 in the CSF of MS patients. Using an inflammatory protein array approach, CX3CL1 was the only chemokine with increased expression in the CSF of MS patients in comparison to HCs. These results were confirmed and quantified by ELISA. An increase in CX3CL1 concentrations has been shown to correlate with increased expression of CX3CR1 on circulating lymphocytes and monocytes during inflammatory disorders [226, 227]. Therefore, we asked, to what extent do increased CSF concentrations of CX3CL1 induce increased levels of CX3CR1 expression on lymphocytes and monocytes? We found that blood-derived $CD4^+$ T-cells

selectively express increased surface levels of CX3CR1 in RRMS patients in comparison to HCs.

Chemokines and chemokine receptors play a role in the regulation of surface expression of adhesion molecules in the inflammatory cells. Therefore, we reasoned that since CX3CL1 levels are increased in MS CSF, and since CX3CR1 is up-regulated on $CD4^+$ T-cells derived from MS blood, then the expression of adhesion molecules on $CD4^+$ T-cells may be increased. We found that ICAM-1 surface expression is increased on $CD4^+$ T-cells derived from MS patients in comparison to HCs, suggesting that CX3CL1-CX3CR1 signaling may lead to increased ICAM-1 expression on $CD4^+$ T-cells.

Next, we asked to what extent CX3CL1 increases gene expression and protein secretion of proinflammatory cytokines. We found that IFN-γ and TNF-α levels were significantly increased in $CD4^+$ T-cells derived from RRMS patients cultured in the presence of CX3CL1. However, this increase was not observed in $CD4^+$ T-cells derived from HCs. We also found that IL-17A gene expression was increased in $CD4^+$ T-cells incubated in the presence of CX3CL1 when derived from RRMS patients compared to HCs. However, the increase was not statistically significant. To address whether increases in IFN-γ gene expression resulted in greater protein secretion of IFN-γ, we incubated $CD4^+$ T-cells in the absence or presence of CX3CL1. $CD4^+$ T-cells derived from RRMS patients but not those derived from HCs secreted higher levels of IFN-γ compared to control cell cultures. However, we found no changes in the secretion of IL-4, TNF-α, IL-17A, or IL-17F.

Lymphocyte extravasation from the bloodstream into the CNS is a critical component of RRMS pathogenesis. The molecular profile of lymphocytes found in the CSF of MS patients has been shown to mirror the molecular profile of lymphocytes which populate MS lesions [98]. Therefore, we reasoned that if CX3CR1 and ICAM-1 upregulation on $CD4^+$ T-cells is critical during cell transmigration across the BBB, then CSF-derived $CD4^+$ T-cells will express higher surface levels of CX3CR1 and ICAM-1 than matched blood-derived $CD4^+$ T-cells. We found that $CD4^+$ T-cells derived from the CSF of MS patients express significantly higher levels of CX3CR1 and ICAM-1 than the matched blood-derived $CD4^+$ T-cells.

To further investigate the functional role of CX3CL1 in $CD4^+$ T-cell migration, we performed $CD4^+$ T-cell migration *in-vitro* assays directed toward CX3CL1, and found that $CD4^+$ T-cells preferentially migrate toward CX3CL1 in a concentration-dependent manner, with 1 ng/mL concentration inducing a maximal migration. We then examined if $CD4^+$ T-cells that migrated toward CX3CL1 gradient express higher levels of CX3CR1 and ICAM-1 than $CD4^+$ T-cells which do not migrate toward CX3CL1. We found that CX3CR1 and ICAM-1 expression were higher on cells that migrated toward CX3CL1 compared to those which did not migrate against this chemokine gradient.

These results suggested that CX3CL1-CX3CR1 interactions might play a role in the increased expression of ICAM-1 on $CD4^+$ T-cells. To determine if increased CX3CL1 levels led to the upregulation of ICAM-1, we incubated PBMCs in the presence of CX3CL1, and found that the percentage of ICAM-1^+ $CD4^+$ cells increased in MS patients but not in HCs.

In order to address the above findings in the context of myelin-specific immune response, relevant for this demyelinating disease, we co-incubated myelin-antigen specific $CD4^+$ T-cell lines with APCs in the presence or absence of native myelin peptide and CX3CL1. We found that ICAM-1 was selectively increased in the presence of both CX3CL1 and myelin peptide in myelin-reactive $CD4^+$ T-cell lines. This indicated that CX3CL1 in the presence of antigen increased ICAM-1 expression on the surface of activated myelin-reactive $CD4^+$T-cells to a greater degree than antigen or CX3CL1 alone. Taken together, these data indicate that CX3CL1, CX3CR1, ICAM-1, IFN-γ and TNF-α interact to play previously undefined roles in the development of the inflammatory response in MS.

Figure 1.1 MS Pathogenesis

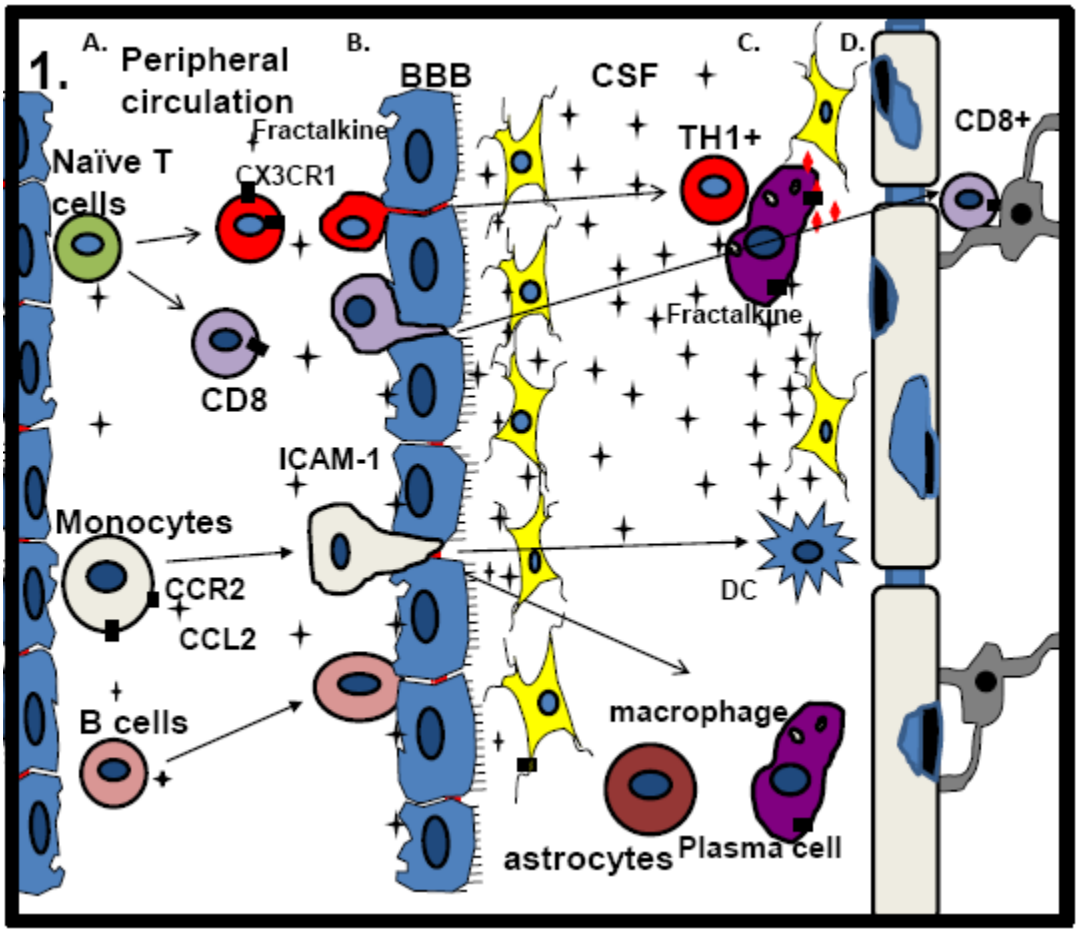

Figure 1.1 Four steps of MS immunopathogenesis:

A. Myelin-autoreactive $CD4^+$Th cells are activated in peripheral circulation

B. Activated $CD4^+$T-cells migrate through permeable BBB

C. $CD4^+$T-cells encounter APCs (i.e. microglia and DCs), and are reactivated by myelin-derived antigens. Cytokine production increases, influx of inflammatory cells.

D. Myelin destruction and axonal transection

Table 1.1. Cytokines and other proteins that interact with CX3CL1 and CX3CR1

Proteins	Cell subset	Interaction	Function	Ref.
IL-1/TNF-α	HPAEC/ HUVEC	IL-1 and TNF-α increase expression of fractalkine mRNA	Fractalkine induction by proinflammatory cytokines	[143]
IL-2/NFAT2	PBMCs(h)	IL-2 upregulates CX3CR1 via recruitment of NFAT2 to CX3CR1 promoter	CX3CR1 induction by proinflammatory cytokines	[158]
sIL-6Rα	HUVEC	sIL-6R inhibits IL-1 or IFN-γ-induced expression of CX3CR1 via signaling through gp130.	Decreases adhesion of mononuclear cells to endothelia	[162]
IL-10	Monocytes (h)	IL-10 increases surface expression of CX3CR1 via PI3K	Increased surface expression of CD11b, indicating monocyte activation	[160]
CXCL8/IL-12/ IL-15	$CD16^-$ NK cells	Incubation with CXCL8, IL-12, and IL-15 decrease expression of CX3CR1	Production of fractalkine	[228]
IL-15 / NFAT1	PBMCs(h)	IL-15 downregulates CX3CR1 expression via recruitment of NFAT1 to CX3CR1 promoter	Regulation of CX3CR1 expression by cytokines	[158]
IFN-γ	Monocytes (h)	IFN-γ increases surface expression of CX3CR1 via PI3K.	Regulation of CX3CR1 expression by cytokines	[160]
IFN-γ	HUVEC	IFN-γ increases expression of CX3CR1.	Increases adhesion of PBMCs to HUVECs	[161]
CCL2	Monocytes (h)	CCL2-CCR2 interactions stimulate surface expression of CX3CR1 expression via p38 MAPK	Increases adhesion of monocytes to fractalkine	[159]
IL-6/CCL2/ MMP-9	Monocytes	$CD16^+$ monocytes produce IL-6, CCL2, and MMP-9 after interacting with fractalkine-expressing HUVECs	The supernatants from this culture induce neuronal cell death *in-vitro*	[156]
IL-6/NO/ TNF-α	Murine microglia	Fractalkine suppressed production of IL-6, NO, and TNF-α	Fractalkine suppressed neuronal cell death induced by activated microglia	[157]
ICAM-1	HPAEC /HUVEC	Fractalkine-CX3CR1 binding upregulates ICAM-1 expression via JAK2-STAT5 pathway	Promotion of neutrophils adhesion	[164]
Akt/PI3K/ NF-κB	Hippocampal neurons (r)	Upon exposure to fractalkine, CX3CR1-expressing hippocampal neurons upregulate the expression of pro-survival proteins	neuroprotection	[165]
CCL26	L1.2 cells, HUVECs	CCL26 binds to CX3CR1	CCL26 induces Ca^{++} mobilization, chemotaxis, and adhesion to L1.2 cells	[229]
RANKL/PI3K/ Akt	RAW264.7 (macrophage cell line, m)	RANKL induces downregulation of CX3CR1 mRNA via PI3K/Akt pathway.	Inhibition of chemotaxis	[163]
ADAM10	ECV-304 (urinary bladder carcinoma, h), fibroblasts (m)	ADAM10 is required for the constitutive cleavage of fractalkine from the cell surface, producing fractalkine	Production of fractalkine	[230]
ADAM17	ECV-304, CHO, HUVEC	ADAM17 is responsible for the inducible cleavage of fractalkine	Production of fractalkine	[147, 149]

Table 1.2. Chemokines and chemokine receptors

Receptor	Chemokine	Old nomenclature
CC family		
CCR1	CCL3	MIP-1α
	CCL3L1	LD78β
	CCL5	RANTES
	CCL7	MCP-3
	CCL14	HCC-1
	CCL15	HCC-2, Lkn-1, MIP-1α
	CCL16	HCC-4
	CCL23	MPIF-1, CKβ8
CCR2	CCL2	MCP-1
	CCL7	MCP-3
	CCL8	MCP-2
	CCL13	MCP-4
CCR3	CCL5	RANTES
	CCL7	MCP-3
	CCL8	MCP-2
	CCL11	Eotaxin-1
	CCL13	MCP-4
	CCL15	HCC-2, Lkn-1, MIP-1Δ
	CCL24	Eotaxin-2, MPIF-2
	CCL28	MEC
CCR4	CCL17	TARC
	CCL22	MDC, STCP-1
CCR5	CCL3	MIP-1α
	CCL3L1	LD78β
	CCL4	MIP-1β
	CCL5	RANTES
	CCL14	HCC-1
CCR6	CCL20	MIP-3α, exodus-1
CCR7	CCL19	MIP-3β, exodus-3
	CCL21	SLC, exodus-2
CCR8	CCL1	I-309
CCR9	CCL25	TECK
CCR10	CCL27	CTACK, ILC
	CCL28	MEC
Unknown	CCL18	PARC, DC-CK1, AMAC1

Receptor	Chemokine	Old nomenclature
CXC family		
CXCR1	CXCL1	GRO-α
	CXCL6	GCP-2
	CXCL8	IL-8
CXCR2	CXCL1	GRO-α
	CXCL2	GRO-β
	CXCL3	GRO-γ
	CXCL5	ENA-78
	CXCL6	GCP-2
	CXCL7	NAP-2
	CXCL8	IL-8
CXCR3	CXCL4	PF4
	CXCL9	Mig
	CXCL10	IP-10
	CXCL11	I-TAC
CXCR4	CXCL12	SDF-1
CXCR5	CXCL13	BLC/BCA-1
CXCR6	CXCL16	
Unknown	CXCL14	BRAK
CX3C family		
CX3CR1	CX3CL1	Fractalkine
C family		
XCR1	XCL1	Lymphotactin
XCR2	XCL2	SCM-1β

Adapted from [231].

CHAPTER 2

CX3CL1 increases Intercellular Adhesion Molecule-1 on $CD4^+$ T Lymphocytes: Implications for the Immunopathogenesis of MS

2.1 INTRODUCTION

MS is an inflammatory, demyelinating CNS disease that is characterized by chemokine- and adhesion molecule-dependent infiltration of the CNS by activated, self-reactive $CD4^+$ T-cells [1, 39, 139]. Membrane-bound CX3CL1 functions as an adhesion molecule, and is expressed on CNS neurons and endothelium [144]. It is comprised of an N-terminal chemokine domain, attached to a mucin-like stalk, followed by transmembrane and intercellular domains [143]. Inflammation and neuronal damage cause the chemokine domain to be cleaved by the metalloproteinase ADAM-17 [149, 232], which may contribute to increased CSF and serum levels of CX3CL1 in MS and other inflammatory diseases [226, 227, 233]. Proinflammatory cytokines IFN-γ and TNF-α induce CX3CL1 expression and release by endothelium, while anti-inflammatory cytokines IL-4 and IL-13 inhibit CX3CL1 expression [199]. CX3CL1 expression is increased in the IFN-γ-mediated Th1 disease psoriasis, but not in Th2-dominated atopic dermatitis [199]. Immobilized CX3CL1 and CX3CL1-transfected 293E cells increase the IFN-γ expression in NK-cells [200]. Together, these data suggest

that CX3CL1 and IFN-γ may act in an inflammatory positive feedback loop during Th1-mediated diseases [199, 200]. CX3CR1 is a G-protein coupled receptor expressed by microglia, monocytes, NK-cells and T-cells [145, 170]. $CX3CR1^{+}CD4^{+}$ T-cells are present in lesions derived from RRMS patients [139], indicating that CX3CL1 may recruit $CX3CR1^{+}CD4^{+}$ T-cells into CNS demyelinating lesions

CX3CL1 stimulates ICAM-1 surface expression in vascular endothelia [164]; however, its role in the regulation of ICAM-1 expression in leukocytes is unknown. ICAM-1 is an Ig-like, transmembrane cell adhesion molecule expressed on vascular endothelial cells, T-cells, B-cells and monocytes [177, 178]. $CD4^{+}$ T-cell expression of ICAM-1 is critical for the induction of the MS disease model EAE [111]. ICAM-1 is regulated by several cytokines: IFN-γ, TNF-α, IL-1, IL-4, IL-6, lymphotoxin and LPS stimulate its expression [177, 179], while TGF-β and IL-10 inhibit it in several cell subsets [180]. Similar to membrane-bound CX3CL1, ICAM-1 is cleaved by ADAM-17 during inflammation to produce sICAM-1 [153]. sICAM-1 binds competitively to the ICAM-1 ligand LFA-1, whose expression on lymphocytes is critical for their transmigration across the BBB [178]. Serum levels of sICAM-1 are increased during rIFNβ-1b treatment, and this increase correlates with a decrease in contrast-enhancing MS brain lesion load [190]. Therefore, circulating sICAM-1 may play a role in reducing T-cell BBB transmigration via competitive inhibition of LFA-1-ICAM-1 interactions [189].

We hypothesized that increased CX3CL1-CX3CR1 interactions induce ICAM-1 and IFN-γ expression in $CD4^{+}$ T-cells, and that $CX3CR1^{+}ICAM\text{-}1^{+}CD4^{+}$ T-cells are enriched in the CSF during RRMS. The present study has identified

an increase in CX3CL1, and a decrease in sICAM-1 in the CSF of RRMS patients in comparison to HCs, and has shown an increase in the percentage of $CX3CR1^{+}ICAM\text{-}1^{+}CD4^{+}$ T-cells derived from CSF compared to blood of early untreated RRMS patients. CX3CL1 induced an increased IFN-γ gene expression and protein secretion in RRMS-derived but not in HC-derived $CD4^{+}$ T-cells. CX3CL1 was also found to increase ICAM-1 expression on the surface of RRMS-patient-derived but not HC-derived $CD4^{+}$ T-cells, and on stimulated myelin-antigen-specific $CD4^{+}$ T-cell lines. These results indicate that CX3CL1 may play a pathological role in RRMS by enhancing migration of IFN-γ-secreting $CX3CR1^{+}ICAM\text{-}1^{+}CD4^{+}$ T-cells into the CNS.

2.2 MATERIALS AND METHODS

2.2.1 Study Subjects

48 RRMS patients (average age = 44 yrs. 5 mos., sex = 34 F / 14 M, race = 35 Caucasian, 13 African American, disease duration 3 yrs. 11 mo. and 38 HC patients (average age = 43 yrs. 4 mos., sex = 25 F / 13 M, race = 31 Caucausian, 7 African American) were enrolled in this study after signing an institutional review board-approved consent form. None of the RRMS patients had received immunomodulatory or immunosuppressive treatments prior to the CSF or blood sample collection.

2.2.2 CSF samples

CSF samples were collected for the purposes of diagnosis and/or this study. CSF sample was centrifuged for 6 minutes; supernatant was removed and stored at -80°C until protein measurements.

2.2.3 $CD4^+$ T-lymphocyte isolation

PBMCs were obtained from blood samples by Ficoll-Paque gradient centrifugation. Further isolation of $CD4^+$ T-lymphocytes was performed by magnetic bead separation (Miltenyi biotech, Auburn, CA). The negatively-selected $CD4^+$ T-lymphocytes were > 95% pure as demonstrated by FACS analysis.

2.2.4 Protein Array

Cytokine array C Series 1000 (RayBiotech, Inc., Norcross, GA) was used as described previously [234]. Briefly, membranes were blocked for 30 min. in blocking buffer. They were then incubated in 1 mL CSF at room temperature for two hours. After incubation, membranes were washed and incubated with the appropriate Biotin-conjugated antibodies for two hours. Wash steps were repeated, 1:1000-diluted HRP-conjugated streptavidin was applied for two hours followed by wash steps. Detection buffers were applied for two minutes, and membranes were exposed to HyBlot CL autoradiography film (Metuchen, NJ) for 1 – 30 s. Membranes were then scanned with an Epson America Expression 1680 Scanner (Epson America Inc., Long Beach, CA). Meta Imaging Series 5.0

software (Molecular Devices, Downington, PA) was used to quantify signal intensities as previously described [89]. The lowest value on the membrane was considered the background, and was subtracted from all measured proteins. For the purpose of comparisons between membranes, results were normalized as follows: the background measurement was subtracted from all measured proteins. The results were normalized by dividing each protein value by the sum of optical density values for all proteins on the membrane. The relative protein level results were expressed in arbitrary units (AUs) of the untreated RRMS CSF in comparison to HC CSF.Proportion of total signal.

1. corrected density = average of background - raw density

2. normalized density = LOG (100 / (highest corrected density - corrected density) / highest corrected density x 100)

3. Relative protein expression in arbitrary units (AU) = average of normalized density of duplicates / sum of normalized density of all protein spots in single membrane

2.2.5 ELISA

CSF and $CD4^+$ T-lymphocyte supernatants (SNs) were collected as described above, and ELISAs for CX3CL1, sICAM-1 (R&D), IFN-γ (BD Biosciences), and fibroblast growth factor 6 (FGF-6) (RayBiotech, Inc.) were performed as per manufacturer's instructions.

2.2.6 Chemotaxis Assays

Separated $CD4^+$ and $CD8^+$ T-cells were independently assessed via flow cytometry to assure a purity of >95%. A transwell system with pore size 5 μm (Neuroprobe, Gaithersburg, MD, USA) was utilized for the chemotaxis assay. In the lower compartment, the CX3CL1 chemokine domain (R&D) was added to the media in increasing concentrations (0, 0.1, 1.0, 10.0 ng/ml). Each upper chamber was seeded with $5X10^5$ $CD4^+$ T-cells, and incubated for four hours at 37°C/5% CO_2. The total number of cells that migrated into the lower compartment was counted, and chemotactic index was determined as described [139]: (number of cells migrated toward CX3CL1 / number of cells migrated in the absence of CX3CL1)

For analysis of migrated cells by flow cytometry, a longer migration protocol was used to increase total numbers migrated. In this assay, isolated CD4+ T-cells migrated toward the optimal concentration of CX3CL1 (1 ng/mL) for 18 hours, and then were immediately immunostained and analyzed via flow cytometry.

2.2.7 Flow cytometry

Four-color flow cytometry acquisition was performed using BD FACScalibur hardware and CellQuest Pro software (both BD Biosciences, San Jose, CA). Cells were gated on lymphocyte or monocyte population and cell subset using the following antibodies: CD4 PE-Cy5, CD8 PE-Cy5 or CD8 FITC, CD19 PE-Cy5, and CD14 PerCP-Cy5.5 (BD Biosciences). At least 40,000 blood-

derived lymphocytes or monocytes and up to 10000 CSF-derived mononuclear cells were analyzed per experiment. Chemokine receptors were analyzed by staining with the following antibodies: APC-CCR1 (R&D, Minneapolis, MN), APC-CCR2, PE-CCR3, FITC-CCR5, FITC-CCR6 (all BD Biosciences), and PE-CX3CR1 (US Biologicals, Swampscott, MA). Adhesion molecules were analyzed using the following antibodies: APC-VLA-4 (R&D), FITC-LFA-1, PE-ICAM-1, and APC-ICAM-1 (all BD Biosciences).

Where indicated, *in vitro*-expanded myelin peptide-specific $CD4^+$ T-cell lines were co-cultured with EBV-immortalized B-cells and stimulated with antigenic peptide (10 µg/ml MBP_{83-99}, $PLP_{180-199}$, or control influenza virus hemagglutinin $(FluHA)_{306-318}$ peptide) in the absence or presence of CX3CL1 (1 ng/ml) for 48 h prior to ICAM-1 cell surface expression measurement using flow cytometry.

2.2.8 Quantitative RT-PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a High Capacity cDNA Archive Kit (Applied Biosystems, Branchburg, NJ). The primers for IFN-γ, IL-17A and TNF-α were purchased from Applied Biosystems. TaqMan Gene Expression Assay (Applied Biosystems) was used to measure gene expression via quantitative RT-PCR in duplicate. All results are expressed as relative gene expression normalized against 18S mRNA expression.

2.2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Graphpad software, San Diego, CA). Student's t-tests were utilized to analyze comparisons between two groups. Significance was achieved when p values were less than 0.05. A repeated measures ANOVA was used for comparison analysis of multiple groups, followed by an appropriate posttests.

2.3 RESULTS

2.3.1 CX3CL1 Levels are Increased and sICAM are Decreased in CSF of RRMS Patients in Comparison to HCs

An inflammatory protein profile of CSF derived from RRMS patients may provide clues that will lead to more accurate early diagnosis and treatments for RRMS. The protein array analysis that contained 120 cytokines, chemokines, neurotrophic factors, and adhesion molecules, was performed using the CSF of 10 RRMS patients and 19 HCs.

CX3CL1 was the only chemokine found to be increased in RRMS CSF vs. HC CSF (2.0-fold increase, $p = 0.011$) (Fig. 2.1A). The complete set of array data are presented in Supplementary Fig. 1. To confirm and quantify differences in CX3CL1 levels found in the cytokine array, CX3CL1 concentrations in the CSF from an independent cohort of 18 RRMS patients and 15 HCs were evaluated by ELISA. This confirmed that CX3CL1 levels were higher in RRMS patients' than HC CSF (Fig. 2.1B, 2.9-fold increase, $p = 0.017$).

sICAM-1 was found to be significantly decreased in the CSF derived from RRMS patients in comparison to HCs (Fig. 2.1C, 5.6 -fold decrease, p = 0.0046). sICAM-1 levels in the CSF were further quantified by ELISA. sICAM-1 levels were confirmed to be lower in the CSF derived from 18 untreated RRMS patients in comparison to 15 HCs (Fig. 2.1D, 5.9-fold decrease, p = 0.0027).

2.3.2 CX3CL1 Increases Gene Expression and Protein Secretion of Proinflammatory Cytokines in $CD4^+$ T-cells Derived from RRMS Patients but not from HCs

Because CX3CL1 has been shown to induce IFN-γ expression in NK-cells [200], we wondered if IFN-γ gene expression is also increased in $CD4^+$ T-cells in the presence of CX3CL1. The levels of IFN-γ gene expression in $CD4^+$ cells from 7 RRMS patients and 6 HCs was analyzed after 6 h incubation in the absence or presence of CX3CL1. IFN-γ gene expression was significantly increased in $CD4^+$ T-cells derived from RRMS patients in comparison to HCs (Fig. 2.2A, 2.2-fold). CX3CL1 also induced higher gene expression of IFN-γ in CD4+ T-cells derived from RRMS patients compared to $CD4^+$ T-cells derived from HCs(Fig. 2.2A, 4.7-fold).No significant difference of IFN-γ gene expression was found between CD4+ T-cells from HCs, cultured in the absence or presence of CX3CL1 (Fig. 2.2A).

CX3CL1 has been shown to induce TNF-α expression during Th1-mediated inflammation [199]. Therefore, we examined to what extent CX3CL1 may upregulate TNF-α gene expression. In $CD4^+$ T-cells derived from 6 RRMS patients and 6 HCs, CX3CL1 significantly increased TNF-α gene expression in $CD4^+$ T-cells from RRMS patients, but not from HCs (Fig. 2.2A**,** 2.1-fold). $CD4^+$

T-cells from 6 RRMS patients cultured with CX3CL1also showed higher IL-17A gene expression than $CD4^+$ T-cells from 6 HCs, but this change did not reach significance (Fig. 2.2A, 2.7-fold).

We asked whether the CX3CL1-induced increase in IFN-γ gene expression in RRMS patient-derived $CD4^+$ T-cells led to an increased secretion of IFN-γ. $CD4^+$ T-cells derived from the blood of 6 RRMS patients and 6 HCS were incubated in 1 ng/ml CX3CL1 for 48 h, and supernatants were harvested to measure cytokine secretion using ELISA. CX3CL1 increased IFN-γ secretion by $CD4^+$ T-cells derived from RRMS patients but not from HCs (Fig. 2.2B, 12.5-fold). CX3CL1 also induced a significantly greater increase in IFN-γ secretion in RRMS $CD4^+$ T-cells compared to HCs (Fig. 2.2B, 5.1-fold).

2.3.3 $CX3CR1^+CD4^+$ T-cells are Enriched in Blood Samples Derived from Untreated RRMS Patients in Comparison to HCs

Cell surface expression of chemokine receptors is determined in part by the extracellular molecular environment [199]. Thus, we proposed that the expression of CX3CR1 and other chemokine receptors may be increased on the cell surfaces of circulating mononuclear cells in MS. To evaluate differences in chemokine receptor expression on T-cells, B-cells, and monocytes that may correspond to increased levels of CX3CL1 in RRMS-derived CSF, flow cytometry analysis of the aforementioned cell subsets was performed to identify the percentage of each cell subset that had a surface expression of CX3CR1. Other chemokine receptors that have been implicated in neuroinflammation were also analyzed (CCR1 [235], CCR2 [218], CCR3 [222], CCR5 [218], CCR6 [135]).

Among the six chemokine receptors analyzed on $CD4^+$ T-cells derived from 8 RRMS patients and 10 HCs, CX3CR1 was the only chemokine receptor that was expressed on a significantly higher percentage of $CD4^+$ T-cells from RRMS patients in comparison to HCs (Fig 2.3A, 3.3-fold, $p = 0.034$).

The same panel of chemokine receptors was analyzed on $CD8^+$ T-cells derived from 8 RRMS patients and 10 HCs. The only chemokine receptor that was expressed on a significantly higher (percentage of $CD8^+$ T-cells from RRMS patients was CCR5 (Fig 2.3B, 2.0-fold, ($p = 0.043$). Expression of these chemokine receptors was also evaluated on $CD19^+$ B-cells from 8 RRMS patients and 8 HCs. We found that the percentage of $CCR2^+$ B-cells derived from RRMS patients was significantly lower than in HCs (Fig 2.3C, 3.0-fold, $p = 0.037$). No differences in chemokine receptor expression were observed on $CD14^+$ monocytes derived from 7 RRMS patients and 7 HCs (Fig. 2.3D).

2.3.4 The Percentages of ICAM-1^+CD4^+ and ICAM-1^+CD8+T-cells are increased in Blood Samples Derived from RRMS Patients in comparison to HCs

Chemokines signal through chemokine receptors and upregulate surface expression of adhesion molecules [164]. Thus, we measured surface expression of three adhesion molecules that may be regulated by the interaction of chemokines and corresponding receptors, which play a role in the development of the inflammatory response in MS—ICAM-1 [164], VLA-4 [236], and LFA-1 [175]—on $CD4^+$, $CD8^+$ T-cells, $CD19^+$ B-cells, and $CD14^+$ monocytes. The percentages of ICAM-1^+CD4^+ T-cells (Fig. 2.4A) and ICAM-1^+CD8^+ T-cells (Fig.

2.4B) were increased in blood samples derived from 8 RRMS patients when compared to 10 HCs. The percentage of $CD4^+$ T-cells expressing ICAM-1 was 2.5-fold higher ($p = 0.0004$) on $CD4^+$ T-cells from RRMS patients in comparison to HCs (Fig. 2.4A). The percentage of ICAM-1^+CD8^+T-cells was 1.5-fold higher in RRMS patients in comparison to HCs (Fig 2.4B. $p = 0.016$). No differences in adhesion molecule expression between RRMS patients and HCs were observed on $CD19^+$ B-cells (8 RRMS patients, 8 HCs) (Fig. 2.4C) or $CD14^+$ monocytes (7 RRMS patients, 7 HCs) (Fig. 2.4D).

2.3.5 The Percentages of $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-lymphocytes are significantly increased in RRMS-derived CSF Compared to the Corresponding Blood Samples

In order to determine whether $CD4^+$ and $CD8^+$ T-cells that have migrated into the CSF during RRMS have elevated levels of surface CX3CR1 and ICAM-1, flow cytometry studies were performed on PBMCs and corresponding CSF-derived mononuclear cells from 9 RRMS patients at the time of establishing diagnosis.

The percentage of $CX3CR1^+CD4^+$ T-cells was increased in the CSF derived from 9 RRMS patients in comparison to the corresponding blood samples (Fig. 2.5A, 4.0-fold, $p = 0.00028$). CX3CR1 surface expression was measured also via mean fluorescence intensity (MFI) readings, and found that CX3CR1 MFI was significantly higher on 7 CSF sample-derived $CD4^+$ T-cells than on 7 blood sample-derived $CD4^+$ T-cells (Fig. 2.5A., 4.2-fold, $p = 0.00082$).

The percentage of ICAM-1-positive $CD4^+$ T-cells derived from CSF was also significantly higher than ICAM-1^+CD4^+ T-cells derived from corresponding blood samples (Fig. 2.5A, 1.5-fold, p = 0.013). MFI of ICAM-1 expression on $CD4^+$ T-cells was significantly higher in CSF-derived $CD4^+$ T-cells compared to blood (Fig. 2.5A, 2.1-fold, p = 0.006) indicating that ICAM-1 is up-regulated on $CD4^+$ T-cells that have migrated into the CSF during RRMS. Finally, we analyzed percentages of $CX3CR1^+CD4^+$ T-cells derived from CSF vs. blood that co-express ICAM-1, and found that a significantly greater proportion of $CX3CR1^+CD4^+$ T-cells express ICAM-1 in CSF-derived cells than on blood-derived $CX3CR1^+CD4^+$ T-cells (Fig. 2.5A, p = 0.013).

$CD8^+$ T-cells derived from the CSF of RRMS patients did not exhibit significantly higher percentages of CX3CR1 or ICAM-1 than blood-derived $CD8^+$ T-cells (Fig. 2.5B). However, CX3CR1 MFI (5.4-fold, p = 0.021) on $CD8^+$T-cells was significantly higher on CSF-derived $CD8^+$ T-cells (Fig. 2.5B). CSF-derived $CX3CR1^+$ICAM-1^+CD8^+ T-cells were also not found to be present in higher percentages in CSF compared to blood samples (Fig. 2.5B).

2.3.6 $CD4^+$ T-cells Migrate Toward CX3CL1 in a Dose-dependent Manner

We next utilized an *in-vitro* migration assay to test the hypothesis that $CD4^+$ T-cells migrate toward CX3CL1 gradient. In chemotaxis assays involving $CD4^+$ and $CD8^+$ T-cells derived from RRMS patients, we found that $CD4^+$ but not $CD8^+$ T-cells migrated in a concentration-dependent manner toward CX3CL1 gradient with 1 ng/ml concentration inducing maximal cell migration (Fig. 2.6A).

Because $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-cells are enriched in RRMS CSF compared to corresponding blood samples, we hypothesized that $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-cells may preferentially migrate toward CX3CL1 *in-vitro*. $CD4^+$ T-cells derived from 4 RRMS patients and 4 HCs were used in the migration experiments toward optimal CX3CL1 concentration (1 ng/ml) for 18 h. After migration, CX3CR1 and ICAM-1 surface expression was determined in migrated and non-migrated cells using flow cytometry. A significantly higher percentage of migrated $CD4^+$ T-cells derived from RRMS patients (Fig. 2.6B, $p = 0.01$) and HCs (Fig. 2.6B, $p = 0.01$) expressed ICAM-1 than non-migrated $CD4^+$ T-cells. A significantly higher percentage of migrated $CX3CR1^+CD4^+$T-cells derived from RRMS patients (Fig. 2.6B, $p = 0.03$) and HCs ($p =0.03$) expressed ICAM-1 than did non-migrated cells. In addition, a significantly higher percentage of migrated $CD4^+$ T-cells derived from RRMS patients expressed ICAM-1 than migrated $CD4^+$ T-cells from HCs ($p = 0.0078$). Combined data from 4 RRMS and 4 HC assays indicated that a higher percentage of migrated $CD4^+$ T-cells expressed ICAM-1 than non-migrated $CD4^+$ T-cells ($p= 0.000019$).

2.3.7 CX3CL1 Induces the Surface Expression of ICAM-1 on $CD4^+$ T-lymphocytes Derived from Blood of RRMS Patients but not of HCs

High concentrations of CX3CL1 in the CSF and blood may be responsible for the increase in the percentage of $CX3CR1^+$ and $ICAM\text{-}1^+$ $CD4^+$ T-cells found in RRMS. To test if CX3CL1 up-regulates CX3CR1 and ICAM-1 *in-vitro*, we incubated freshly isolated PBMCs from 7 RRMS patients and 7 HCs in the absence or presence of 1 ng/ml CX3CL1. A significantly higher percentage of

$CD4^+$ T-cells derived from RRMS patients incubated with 1 ng/ml CX3CL1 expressed ICAM-1 than in chemokine-free cultures, (Fig. 2.7, 1.8-fold). CX3CL1 did not induce up-regulation of ICAM-1 in $CD4^+$ T-cells derived from HCs (Fig. 2.7). $CD4^+$ T-cells derived from RRMS patients cultured in the presence of CX3CL1 expressed significantly higher levels of ICAM-1 than $CD4^+$ T-cells derived from HCs. (Fig. 2.7, 2.5-fold). CX3CL1 was not found to induce CX3CR1 expression on $CD4^+$ T-cells from RRMS patients and HCs.

2.3.8 CX3CL1 Induces Expression of ICAM-1 on myelin-Ag-specific $CD4^+$ T-cells

Myelin-autoreactive $CD4^+$ T-cells play a role in the initiation and perpetuation of the inflammatory response in MS [3, 37]. To explore whether myelin-reactive $CD4^+$ T-cells increase the expression of chemokine receptors and adhesion molecules in response to myelin peptide stimulation and CX3CL1, 3 myelin-Ag specific $CD4^+$ T-cell lines (1 $PLP_{180-199}$-specific, 2 MBP_{83-99}-specific lines) were activated in the absence or presence of CX3CL1, along with native antigenic peptide for 48 h, and surface chemokine receptors and adhesion molecules expression was measured by flow cytometry (Fig. 2.8A).In each myelin Ag-specific line, ICAM-1 expression as measured by MFI was found to be higher in Ag- and CX3CL1-stimulated $CD4^+$ lymphocytes than in baseline conditions (Fig. 2.8A. representative histogram Fig. 8B). The results demonstrate that CX3CL1 induces ICAM-1 surface expression on $CD4^+$ T-cell in the presence Ag to a greater degree than in the presence of CX3CL1 or myelin Ag alone.

2.3.9 Supplementary text and figure description

Supplementary Figure 2.1. Protein Arrays Demonstrate Differential Expression of Inflammatory Proteins in the CSF of RRMS Patients and HCs

The prevailing approach to the simultaneous analysis of multiple protein expression levels is the two-dimensional polyacrylamide SDS page coupled with mass spectrometry [237]. However, the lack of quantitative measurements by this method limits its usefulness and broader application. We approached the problem of biomarker identification by utilizing the RayBio Human Cytokine Antibody Array, a novel technology which was used to simultaneously analyze multiple protein levels in the CSF of RRMS patients and HCs. 120 cytokines, chemokines, growth factors and adhesion molecules were analyzed in the CSF of RRMS patients and HCs (Table S1).

Of the 120 measured proteins, 13 were differentially expressed in CSF from RRMS patients in comparison to HCs. These proteins included four chemokines (CX3CL1, HCC-4, Eotaxin-2, GRO), 4 growth factors (FGF-6, PIGF, FGF-4, IGFBP-4) and 1 receptor tyrosine kinase that binds growth factors (Axl), 2 cytokines (IL-3 and MIF), and 2 adhesion molecules (sICAM-1 and sICAM-3) (Fig. S1).

Supplementary Figure 2.2. FGF-6 Levels are elevated in CSF of RRMS Patients in Comparison to HCs

FGF-6 was the only growth factor found in the protein array to be increased in RRMS CSF vs. HC CSF (2.3-fold increase, $p = 0.044$) (Fig S2).

FGF-6 also showed the greatest fold change of the three proteins found to be increased in the CSF. To confirm and quantify increased FGF-6 levels found in the cytokine array, FGF-6 concentrations were measured in the CSF from an independent cohort of 11 RRMS patients and 16 HCs using ELISA. This confirmed that FGF-6 levels were higher in RRMS CSF than HC CSF (Fig. S2, 3.7-fold increase, $p = 0.0001$).

2.4 DISCUSSION

In the present study we used protein array technology [234, 238, 239] to identify in an unbiased fashion a differential expression of cytokines, chemokines, and growth factors in the CSF from untreated RRMS patients in comparison to HCs (Fig. S1). We found significantly elevated FGF-6, CX3CL1, and IL-3 levels in the CSF of untreated RRMS patients in comparison to HCs. CX3CL1 was the only chemokine identified by protein array and confirmed by ELISA to be significantly increased in the CSF of RRMS patients in comparison to HCs, confirming previous studies [139, 233]. Elevated CX3CL1 levels in the CSF have recently been shown to attract$CX3CR1^{+}CD4^{+}$ T-cells into the CNS lesions of MS patients [139]. sICAM-1 was found by protein arrays and confirmed by ELISA to be decreased in the CSF of RRMS patients in comparison to HCs (Fig. 2.1C-D). sICAM-1 has been shown to interfere with the ICAM-1-LFA-1-mediated adhesion of PBMCs to cerebral endothelial cells *in-vitro* [189]. In this context, suppressed CSF sICAM-1 expression in MS patients may be interpreted as an indicator of an inflammatory mechanism in MS, by which decreased sICAM-1 enables transmigration of inflammatory leukocytes into the CNS.

The CX3CL1-induced increase in TNF-α and IFN-γ gene expression, and increased IFN-γ secretion in $CD4^+$ T-cells derived from untreated RRMS patients but not HCs, suggests that increased CX3CL1 concentrations may play a role in the induction of proinflammatory cytokines in RRMS. Fraticelli et al., has identified that CX3CL1 contributes to the Th1-cell migration, and amplification of polarized T-cell responses in Th1 mediated but not Th2 mediated disease [199].

Bullard et al., have recently found that $CD4^+$ T-cells adoptively transferred from myelin MOG-immunized ICAM-1 null mice into wild type (WT) mice failed to induce EAE, providing evidence that ICAM-1 expression on T-cells is critical for EAE induction [111]. These result suggest that ICAM-1 expression on T-cells may be more critical for the development of MOG-induced EAE than ICAM-1 expression on BBB endothelia and other ICAM-1-expressing cells [111]. The same group also reported that ICAM-1 expression on T-cells, but not on APCs, was critical for proliferation of MOG-sensitized T-cells. This group has demonstrated that splenic ICAM-1 null T-cells secrete lower levels of pro-inflammatory cytokines IFN-γ, IL-17, and TNF-α. Furthermore, they found that spinal cord T-cells from ICAM-1 null EAE mice produced lower levels of IFN-γ than from WT mice, indicating that ICAM-1 expression has a direct effect on proinflammatory cytokine production [111].

Recently, Broux et al. have found that CX3CR1 is a marker for $CD4^+CD28^-$ T-cells, a cell subset that is expanded in MS patients [139]. This cell subset was characterized by increased expression of LFA-1, ICAM-1, and VLA-4 adhesion molecules in comparison to $CD4^+CD28^+$ T-cells. In this study, MS patients exhibited higher levels of ICAM-1 on $CD4^+CD28^-CX3CR1^+$ T-cells than healthy controls. These cells were found to degranulate after anti-CD3 mAb stimulation,

and a small number of these cells degranulated after stimulation with myelin peptides MBP and MOG. $CD4^+CD28^-$ cells preferentially migrated toward CX3CL1 *in-vitro* [139]. Lastly, $CD4^+CX3CR1^+$ T-cells were found in active MS lesions, and the apoptotic maker cleaved caspase-3 was expressed by oligodendrocytes in close proximity to $CD4^+CX3CR1^+$ T-cells, indicating that these cells may contribute to the MS lesion formation by causing oligodendrocyte apoptosis [139]. Our finding that $CX3CR1^+CD4^+$ T-cells are enriched in the CSF derived from RRMS patients supports the above findings.

Our finding that $CX3CR1^+ICAM\text{-}1^+CD4^+$ T-cells are enriched in the CSF compared to the blood samples of RRMS patients, together with upregulation of ICAM-1 on $CD4^+$ T-cells following their *in-vitro* migration against CX3CL1, indicates that CX3CR1 and ICAM-1 may play a role in the migration of $CD4^+$ T-cells through the BBB. Therefore, blockade of CX3CL1-CX3CR1 interaction may be a therapeutic approach in MS.

While a role for CX3CL1 in ICAM-1 upregulation had not been previously studied in lymphocytes, CX3CL1 was reported to induce ICAM-1 expression in vascular endothelial cells via CX3CR1 and Jak2-Stat5 signaling[164]. We have demonstrated here for the first time that CX3CL1 increases ICAM-1 expression on $CD4^+$ T-cells derived from RRMS patients. Furthermore, in myelin peptide-specific $CD4^+$ T-cell lines, CX3CL1 in addition to myelin peptide increased ICAM-1 expression in comparison to the stimulation with myelin Ag or CX3CL1 alone. Future studies will focus on characterization of signaling pathways involved in the CX3CL1-induced increase in ICAM-1 expression in lymphocytes.

Targeting CX3CL1-CX3CR1 interactions is currently a potential therapeutic approach for several diseases [167, 240, 241]. In RRMS, it may be the case that the overarching role for CX3CL1-CX3CR1 interactions is that of recruitment of inflammatory cells into the CNS —however, it would still be important to demonstrate whether this recruitment mostly involves inflammatory lymphocyte subsets that exacerbate disease, or rather lymphocytes and NK-cells that have immunoregulatory effects. Secondly, given the different roles of membrane bound and soluble CX3CL1, it will be important to uncover whether modulation of these distinct CX3CL1 molecules differentially affects the immunopathogenesis of MS.

Figure 2.1. CX3CL1 is increased in CSF and sICAM-1 is decreased in the CSF of RRMS patients in comparison to HCs.

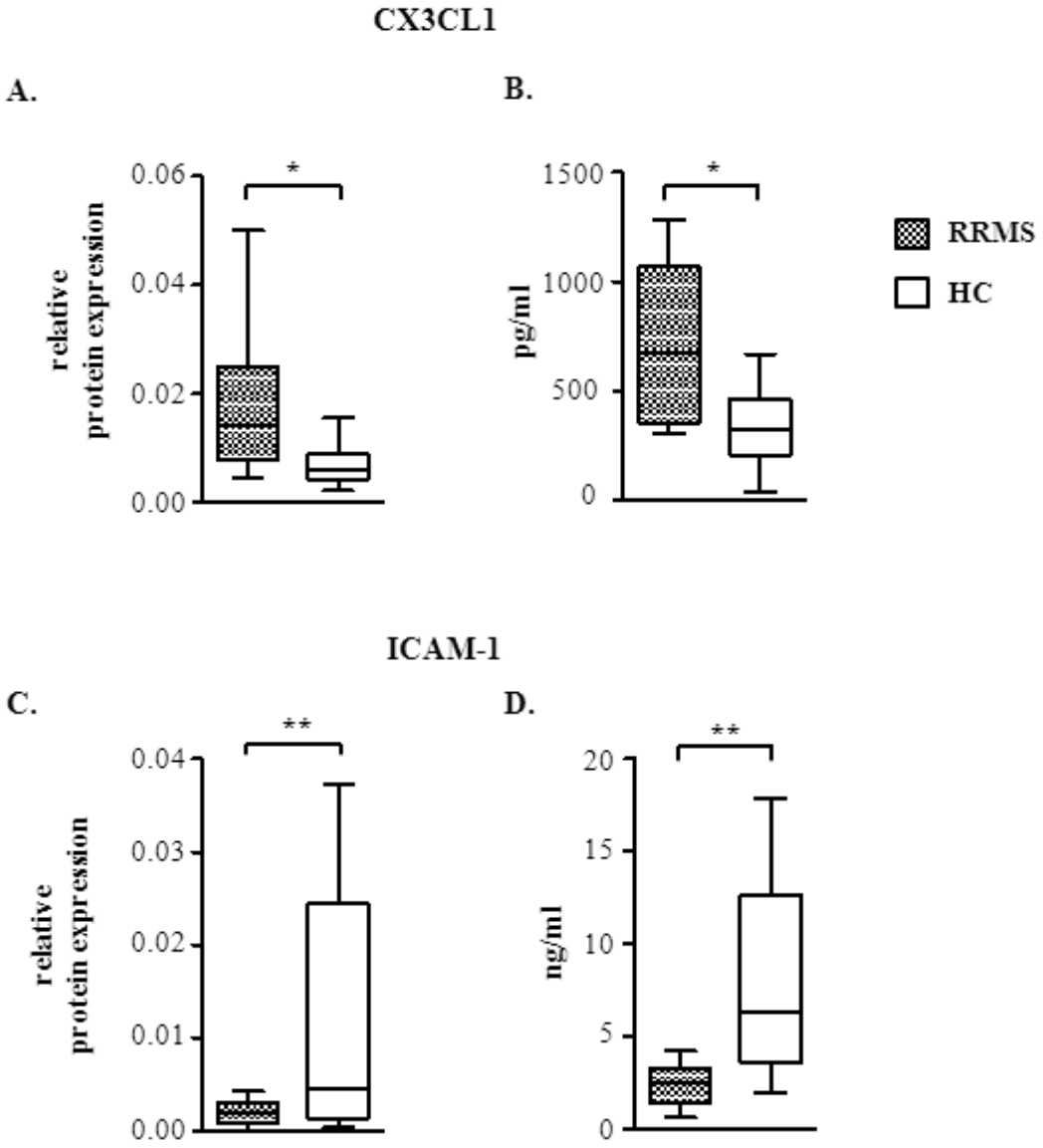

Figure 2.1. CX3CL1 is increased and sICAM-1 is decreased in the CSF of RRMS patients in comparison to HCs. A. 10 RRMS and 19 HC CSF samples were tested for the expression of 120 cytokine, chemokine, and growth factor proteins using protein array. **B**. CX3CL1 protein levels were measured in 18 RRMS and 15 HC CSF samples, using ELISA. **C.** Relative protein expression of sICAM-1 was determined in 10 RRMS and 19 HC CSF samples using protein array. **D.** sICAM-1 protein concentration was determined in 18 RRMS and 15 HC CSF samples using ELISA. For the cytokine array, relative protein expression is expressed in arbitrary units (AU). AU = Average of Normalized Density of Duplicates / Sum of normalized density of all protein spots on a single membrane. Statistical analysis was performed using unpaired t-tests. *$p < 0.05$ ** $p < 0.01$.

Figure 2.2.CX3CL1 induces gene expression of IFN-γ and TNF-α, and protein secretion of IFN-γ by CD4$^+$T-cells derived from RRMS patients.

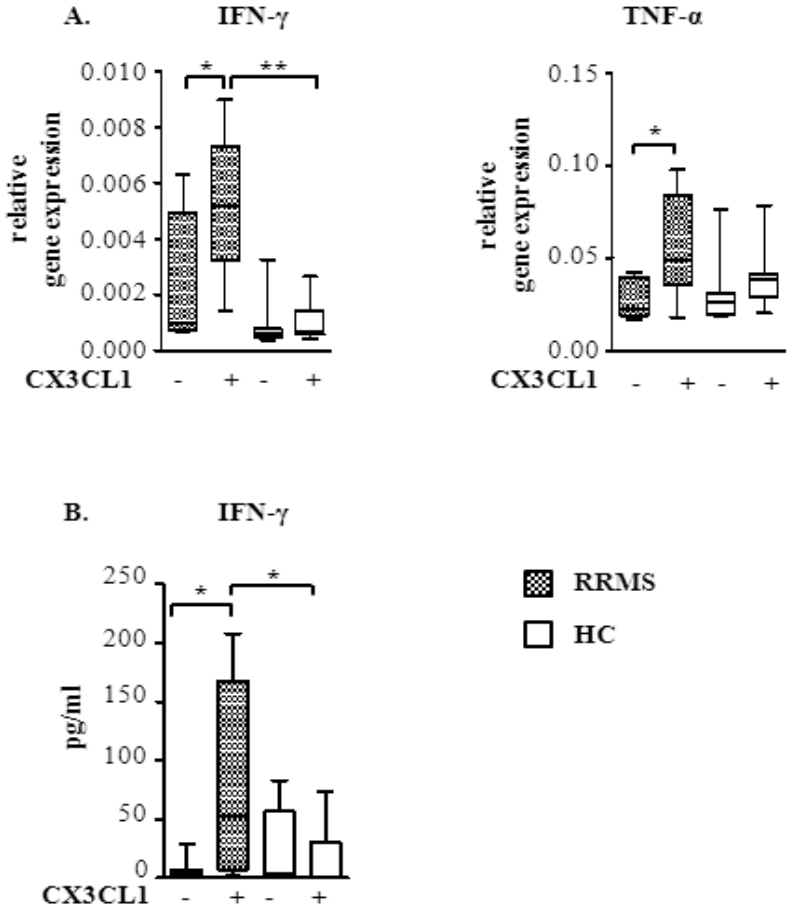

Figure 2.2. CX3CL1 induces gene expression of IFN-γ and TNF-□, and protein secretion of IFN-γ by CD4$^+$T-cells derived from RRMS patients. A. 1 x 10^6CD4$^+$ T cells isolated from 6 RRMS patients and 7 HCs were cultured in the absence or presence of 1 ng/ml CX3CL1 for 6 h. RNA was isolated and cDNA was synthesized. qRT-PCR was performed for indicated cytokines **B**. 1 x 10^6CD4$^+$ T-cells isolated from 6 untreated RRMS patients and 6 HCs were cultured in the absence or presence of 1 ng/ml CX3CL1 for 48 h. Supernatants were collected and ELISAs were performed for measurements of IFN-γ secretion. Statistical analysis was performed using ANOVA. * = $p < 0.05$. ** $p< 0.01$.

Figure 2.3. The percentage of CX3CR1^{+}CD4^{+}T-cells is increased in blood-derived CD4^{+} cells from RRMS patients in comparison to HCs.

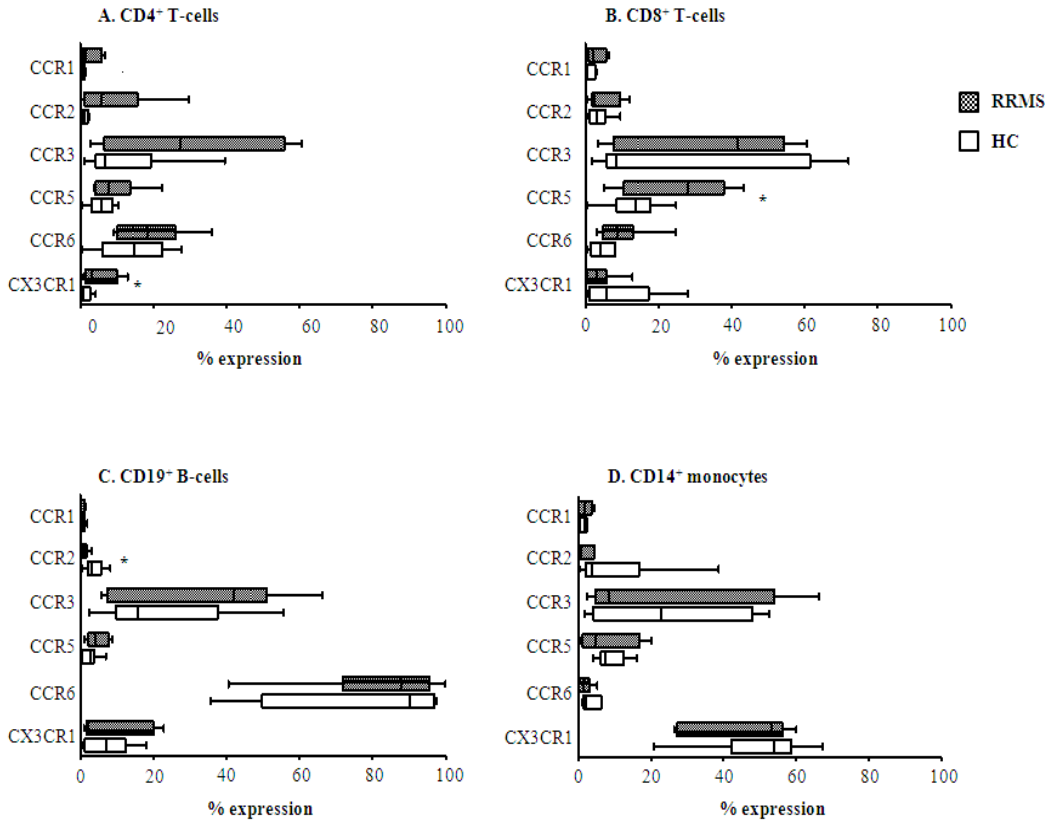

Figure 2.3.The percentage of CX3CR1^{+}CD4^{+} T cells is increased in blood samples from RRMS patients in comparison to HCs. PBMCs derived from 9 RRMS patients and 10 HCs were stained with fluorescently-labeled **A.**CD4 (8 RRMS, 10 HC), **B.**CD8 (8 RRMS, 10 HC), **C.**CD19 (8 RRMS, 8 HC) mAbs, and **D.**CD14 (7 RRMS and 7 HC) mAb for gating, and co-stained for CCR1, CCR2, CCR3, CCR5, CCR6, and CX3CR1. The results are expressed as a percentage of gated cells expressing the chemokine receptor of interest. Statistical analysis was performed using unpaired t-tests. * $p < 0.05$.

Figure 2.4. ICAM-1^{+}CD4^{+} T and ICAM-1^{+}CD8^{+}T-cells are increased in blood derived from untreated RRMS patients compared with HC blood.

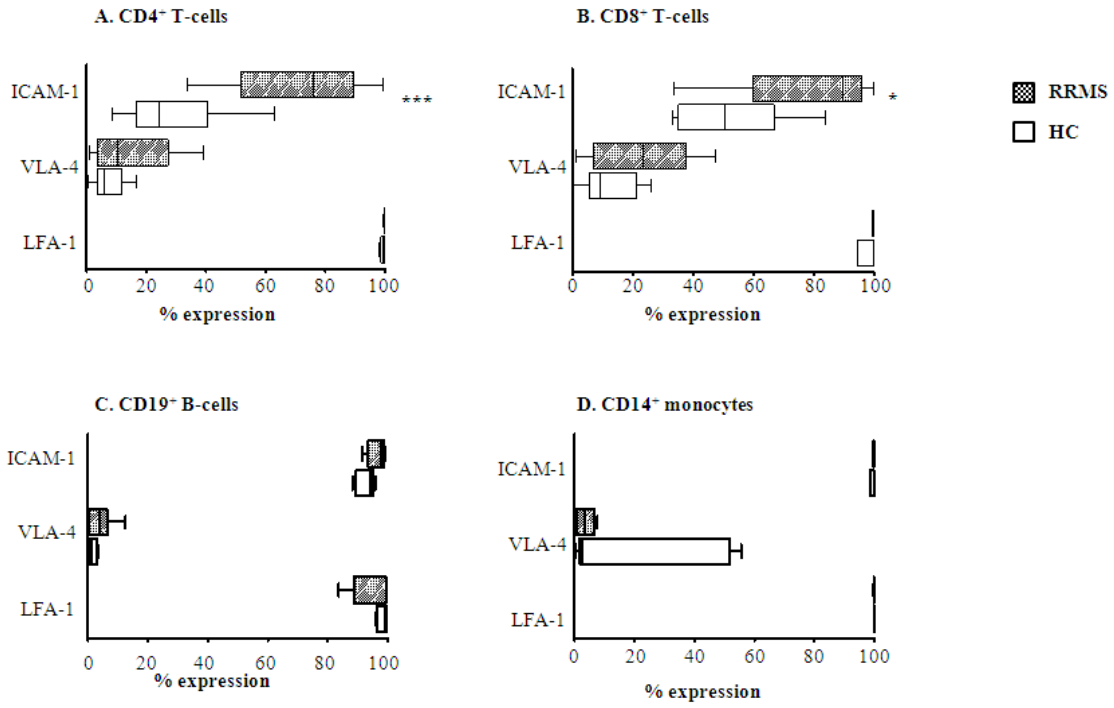

Figure 2.4. The percentage of ICAM-1^{+}CD4^{+} T and ICAM-1^{+}CD8^{+} T cells is increased in blood samples from RRMS patients in comparison to HCs. PBMCs derived from 9 RRMS patients and 10 HCs were stained with fluorescently-labeled **A.**CD4 (8 RRMS, 10 HC), **B.**CD8 (8 RRMS, 10 HC), **C.**CD19 (8 RRMS, 8 HC) mAbs, and **D.**CD14 (7 RRMS and 7 HC) mAb for gating, and co-stained for ICAM, VLA-4, and LFA-1. The PBMCs were then analyzed for expression of these adhesion molecules via flow cytometry. Statistical analysis was performed using unpaired t-tests. * $p < 0.05$, *** $p < 0.001$.

Figure 2.5.The percentage of CX3CR1^{+}ICAM-1^{+}CD4^{+} T lymphocytes is increased in RRMS patients-derived CSF in comparison to corresponding blood samples.

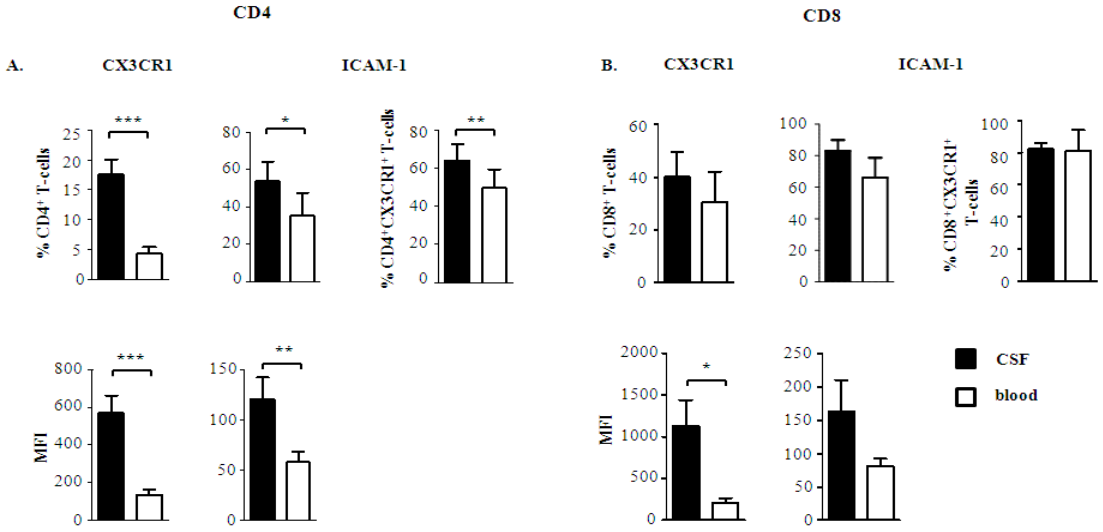

Figure 2.5. The expression of CX3CR1 and ICAM-1 is increased in both CD4^{+} and CD8^{+} T cells from CSF in comparison to the corresponding blood samples from RRMS patients. A. Surface expression of CX3CR1 and ICAM-1 was measured on CD4^{+} T cells derived from the CSF and corresponding blood samples of 8 RRMS patients. **B.** Surface expression of CX3CR1 and ICAM-1 was measured on CD8^{+} T cells derived from the CSF and corresponding blood samples of 7 RRMS patients. Samples in (A) and (B) were evaluated for percentage of cells expressing CX3CR1 and ICAM-1, and mean fluorescence intensity (MFI) was used to quantify the mean level of CX3CR1 and ICAM-1 surface expression per cell. Statistical analysis was performed using paired t-tests. $p< 0.05$, ** $p < 0.01$, ***$p < 0.001$.

Figure 2.6. ICAM-1 is expressed on a higher percentage of CD4$^+$T-cells which have migrated toward CX3CL1 than on the non-migrated cells.

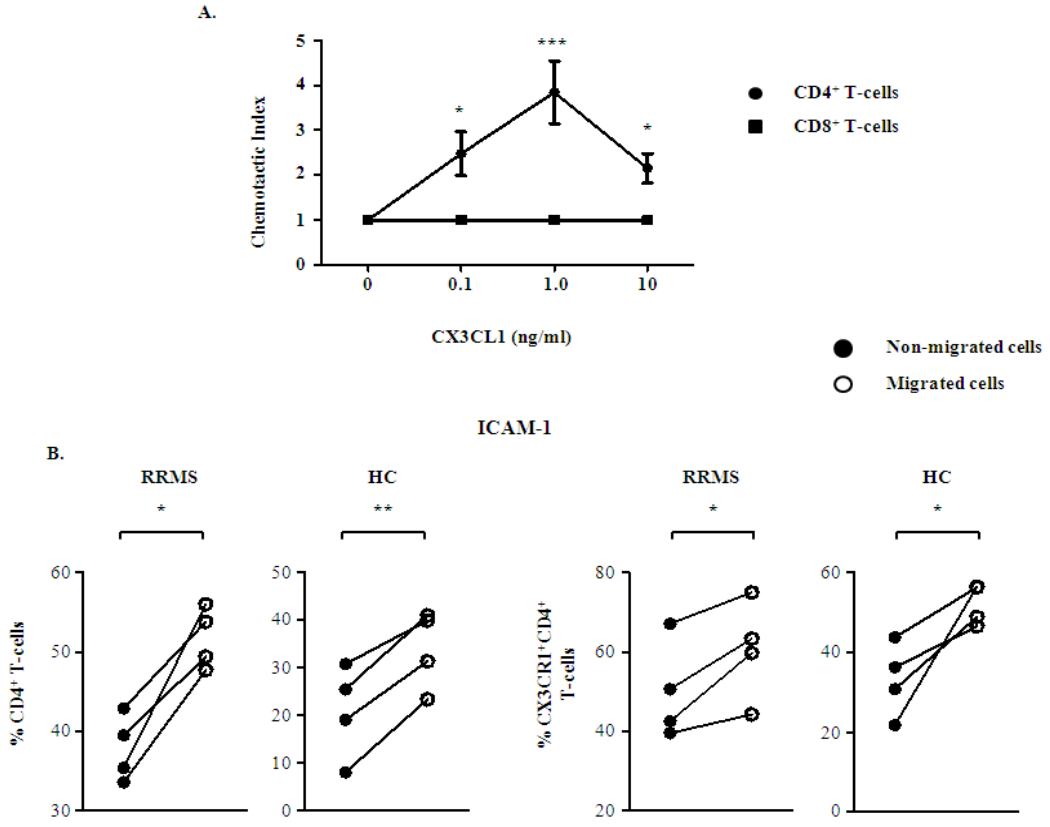

Figure 2.6. ICAM-1 expression increases on CD4$^+$ T-cells that migrate toward CX3CL1. A. CD4$^+$ T-cells migrate in a dose-dependent fashion against the CX3CL1 gradient.A.CD4$^+$ and CD8$^+$ T-cells (5x10^5 per condition) derived from 4 RRMS patients were used for a migration experiments against ascending concentrations of CX3CL1 over 4 h in a chemotactic chamber. Data are presented as chemotactic index (CI), calculated as total cells migrated toward CX3CL1 / total cells migrated in the absence of CX3CL1. **B.** A higher percentage of migrated CD4$^+$T-cells express ICAM-1 in comparison to non-migrated cells from both RRMS patients and HCs. 5x10^5 separated CD4$^+$T-cells per experiment from 4 MS patients and 4 HCs were used in migration experiments against 1 ng/ml of CX3CL1 for 18 h in a chemotactic chamber. ICAM-1 surface levels were assessed on CD4$^+$ T-cells and CD4$^+$CX3CR1$^+$ T-cells via flow cytometry. Statistical analysis was performed using paired t-tests. * p< 0.05, ** p < 0.01, ***p < 0.001.

Figure 2.7.CX3CL1 induces ICAM-1 expression on $CD4^+$ T-cells derived from RRMS patients.

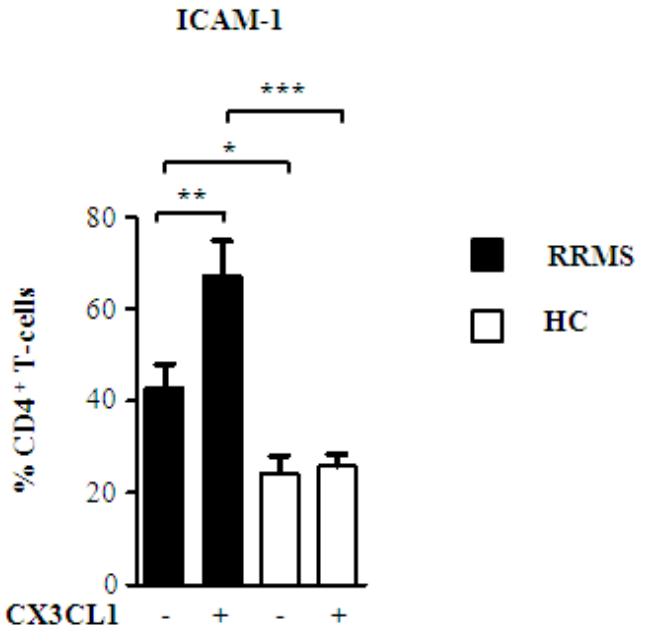

Figure 2.7. CX3CL1 induces ICAM-1 expression on a higher percentage of $CD4^+$T-lymphocytes derived from RRMS patients in comparison to HCs. $1x10^6$/ml PBMCs derived from 6 RRMS patients and 7 HCs were cultured in the absence or presence of 1 ng/ml CX3CL1 for 48 h, stained and analyzed via flow cytometry to determine the percentages of $CD4^+$T-cells that express ICAM-1. Statistical analysis was performed using ANOVA. * = $p < 0.05$. ** = $p < 0.01$. *** = $p < 0.001$.

Figure 2.8.CX3CL1 upregulates ICAM-1 on myelin-antigen-specific CD4$^+$ T-cells.

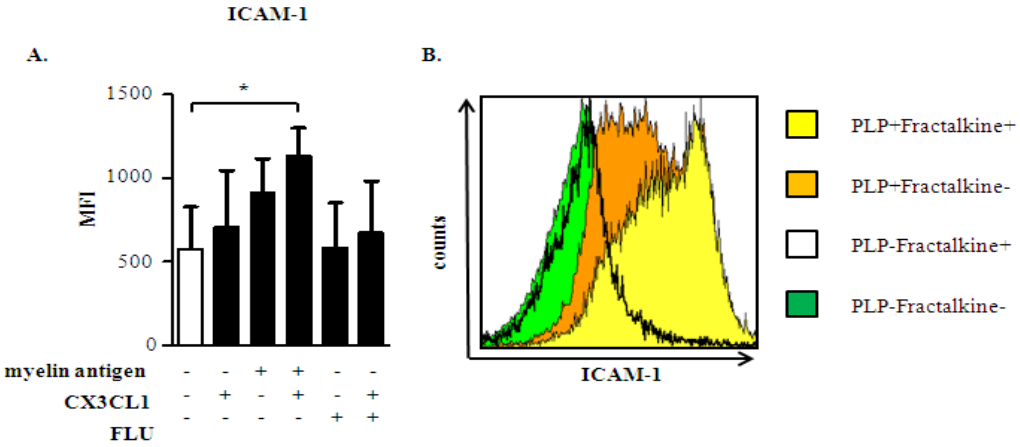

Figure 2.8.CX3CL1 upregulates ICAM-1 on myelin-antigen-specific CD4$^+$ T cells. Three myelin-peptide specific CD4$^+$ T cell lines were co-cultured with EBV-transformed B cells in the absence or presence of CX3CL1, myelin peptide antigen (PLP $_{180-199}$ or MBP $_{83-99}$) or control FLU-HA $_{306-318}$ antigen (48 h). Cells were stained and analyzed via flow cytometry to determine the intensity of ICAM-1 expression on CD4$^+$T cells. CD4$^+$ T cells were co-incubated 1:1 with irradiated EBV-transformed B cells (48 h), immunostained and analyzed by flow cytometry. **B.** Representative histogram of one out of three experiments. Statistical analysis was performed using ANOVA, * = $p < 0.05$.

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTION

3.1 CONCLUSIONS

Two of the most important goals of MS research are (1) the development of a diagnostic biomarker, and (2) the development of new therapeutics to more effectively treat MS.

The founding goal of this study was to identify proteins in the CSF during the initial acute stage of RRMS that might serve as biomarkers of early inflammatory response in MS. To meet this goal, we utilized recently-developed protein array technology to perform an unbiased screen of cytokine, chemokine, and neurotrophic factors in the CSF derived from patients with RRMS compared to HCs. This allowed us to profile CSF inflammatory proteins in RRMS. CX3CL1 was the only chemokine found by protein array—and subsequently confirmed by ELISA in a separate cohort—to be significantly increased in the CSF of RRMS patients in comparison to HCs. This indicated to us that CX3CL1 is constitutively produced in the CSF of healthy individuals, but this production increases during RRMS.

While we found the increase of CX3CL1 in CSF to be interesting for the purposes of studying RRMS immunopathogenesis, it would be difficult to validate

CX3CL1 as a biomarker for RRMS for two reasons. First, given that CX3CL1 is upregulated in other CNS inflammatory disorders [233], a finding of increased levels of CX3CL1 in the CSF of a single patient would not rule out the possibility of another inflammatory disease. Second, while we found CX3CL1 to be increased significantly in the CSF of RRMS patients compared to HCs, a subset of RRMS patients expressed CX3CL1 at comparable levels to HCs. Therefore, these constraints reflecting suboptimal specificity and sensitivity of CX3CL1, would make analysis of CSF levels of CX3CL1 alone an imperfect biomarker for RRMS. However, it should be noted that CX3CL1 may have the potential to be analyzed for the purposes of diagnosis in a panel with other chemokines, cytokines, and adhesion molecules.

Another relevant finding from the CSF cytokine array was that sICAM-1 levels are diminished in the CSF derived from untreated RRMS patients compared to HCs. This finding was confirmed by ELISA in a separate cohort of patients. Because sICAM-1 competes with ICAM-1 to bind LFA-1, it has been speculated that it might play disease-attenuating role in MS [188-190]. sICAM-1 binds LFA-1 on inflammatory lymphocytes, thus ablating LFA-1 interactions with BBB endothelial ICAM-1, and therefore may reduce lymphocyte migration into the CNS during MS [190].

We found that CX3CR1 upregulates IFN-γ and TNF-α gene expression. We also found that CX3CL1 increases secretion of IFN-γ in $CD4^+$ T-cells derived from RRMS patients but not HCs. This suggests that increased CX3CL1 concentrations play a role in the production of proinflammatory cytokines during RRMS. This data is especially relevant in light of a study by Fraticelli and colleagues that has identified CX3CL1 as a contributor to Th1 polarization and

migration, and amplification of polarized T-cell responses in Th1-mediated but not Th2-mediated diseases [199]. Taken together, these data suggest that CX3CL1 may be part of an inflammatory positive feedback loop with IFN-γ and TNF-αthat is prevalent during Th1-mediated diseases such as MS.

We found that CX3CR1 is expressed on a greater percentage of $CD4^+$ T-cells derived from RRMS patients in comparison to HCs. This led us to speculate that CX3CR1 may be upregulated due to increased concentrations of CX3CL1, which may represent an immunopathological aspect of the disease. An upregulation of CX3CR1 on $CD4^+$ T-cells during RRMS may increase migratory ability of these cells, and their BBB transmigration.

We found that CCR5 is expressed on a greater percentage of $CD8^+$ T-cells derived from RRMS patients compared to HCs. CCR5 is a receptor for CCL3, CCL4, CCL5, CCL8, and CCL14, and has been previously implicated in MS pathogenesis [98]. Interestingly, Kivisakk, et al., recently showed that CCR5 is increased on blood-derived $CD8^+$ T-cells after natalizumab treatment compared to untreated patients [107]. The authors conclude that the increase in percentage of $CD8^+$ T-cells expressing CCR5 may be due to sequestration of activated cells in the blood due to blockade of BBB transmigration via natalizumab[107]. Interestingly, Eikelenboom, et al., found that CCR5 expression on $CD8^+$ T-cells is correlated with T2, but not T1 lesion load, in MS patients [176]. Our findings suggest that upregulation of CCR5 on $CD8^+$T-cells may be one mechanism by which $CD8^+$T-cells increase their ability to migrate toward the CNS during RRMS pathogenesis.

We found that a greater percentage of $CD4^+$T-cells and $CD8^+$T-cells derived from untreated RRMS patients express ICAM-1 compared to $CD4^+$T-cells derived from HCs. This increase in the percentage of T-cells that express ICAM-1 during RRMS suggests that ICAM-1 may play a role in RRMS pathogenesis, perhaps by increasing the chemotactic ability of $CD4^+$ and $CD8^+$T-cells that migrate into the CNS to cause lesion formation and tissue damage. In Bullard, et al., 2007, T lymphocyte adoptive transfer experiments from myelin oligodendrocyte glycoprotein (MOG)-sensitized ICAM-1 null mice into wild type (WT) mice failed to induce EAE, providing evidence that ICAM-1 expression on T-cells is critical for EAE pathogenesis. The authors observed lower levels of clinical symptoms as well as a lower percentage of mice exhibiting any clinical signs of disease in ICAM-1 null T cell transfers > WT than in WT T cell transfers > ICAM-1 null mice or the WT > WT internal controls. This implies that the expression of ICAM-1 on T-cells is more critical for the development of MOG-induced EAE than the expression of ICAM-1 on BBB endothelia and all other ICAM-1-expressing cells [111].

We find that $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells make up a significantly greater proportion of $CD4^+$T-cells derived from the CSF than do $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells derived from the blood of RRMS patients. This suggests that either this subset of $CD4^+$T-cells migrates into the CNS more effectively during RRMS, or that CX3CR1 and ICAM-1 are upregulated on $CD4^+$T-cells after migration into the CNS during RRMS pathogenesis. *In vivo* migration assays uncovered that $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells preferentially migrate toward CX3CL1.

Our study uncovers that upregulation of ICAM-1 via CX3CL1 occurs in $CD4^+$ T lymphocytes, and is magnified in $CD4^+$ T lymphocytes derived from RRMS patients or stimulated with either PLP or MBP peptide. These data indicate that CX3CL1 induced upregulation of ICAM-1 on $CD4^+$ T lymphocytes may represent a previously unrecognized aspect of RRMS pathology. While a role for CX3CL1 in ICAM-1 upregulation had not previously been studied in leukocytes, CX3CL1 was shown to upregulate ICAM-1 in vascular endothelial cells via CX3CR1 and Jak2-Stat5 signaling[164]. Levels of phosphorylated Jak2 and Stat5 were increased in sequential order within 30 minutes of exposure to CX3CL1; this effect was blocked by transfection of CX3CR1 siRNA [164].

CX3CL1-CX3CR1 interactions play roles in several inflammatory diseases and animal disease models and are therefore currently the focus of attention as a potential therapeutic target for a number of illnesses [167, 240]. As roles for CX3CL1-CX3CR1 interactions in MS have not been comprehensively studied, it is difficult to predict how various types of modulation of this chemokine-receptor duo and the interacting network of related molecules may affect MS pathology. It may be the case that the overarching role for CX3CL1-CX3CR1 interactions is simply that of recruitment of immune cells into the CNS during MS—however, if this is the case, it would still be important to uncover whether this recruitment mostly involves inflammatory leukocyte subsets that exacerbate disease condition, or rather leukocytes that function in immunosuppressive and neuroprotective roles. Secondly, given the different role of membrane bound and soluble CX3CL1, it will be important to uncover whether upregulation and downregulation of these distinct chemokines have synergistic or opposing effects

on disease state. Overall, a great deal of work is left unfinished regarding roles for CX3CL1-CX3CR1 in MS pathology.

3.2 Future Directions

Several results generated by our findings in this dissertation compel us to further investigate roles for CX3CL1, CX3CR1, ICAM-1, and related molecules in MS immunopathogenesis. The finding that CX3CL1 upregulates ICAM-1 on $CD4^+$T-cells in the presence of PBMCs derived from untreated RRMS patients, paired with the finding that CX3CL1 upregulates ICAM-1 on myelin-Ag-specific $CD4^+$T-cells in the presence of EBV-transformed B cells, presents the possibility that CX3CL1 upregulation of ICAM-1 requires other cell subsets for upregulation of ICAM-1 in $CD4^+$ cells. Co-culture of isolated $CD4^+$T-cells with candidate APCs such as monocytes and B cells in the absence or presence of CX3CL1, compared to isolated $CD4^+$ T cell culture, followed by quantitation of surface ICAM-1 on $CD4^+$T-cells via flow cytometry, will answer which cell subsets or secreted soluble factors are critical for CX3CL1-induced expression of ICAM-1 on $CD4^+$T-cells.

It would be important to elucidate which intracellular pathway or pathways are responsible for the upregulation of ICAM-1 via CX3CL1. CX3CL1 has been shown to upregulate ICAM-1 via signaling through CX3CR1 in HUVECs, which increases phosphorylation of JAK2 and STAT5 [164]. An intracellular pathway mediating ICAM-1 upregulation via CX3CL1 has not yet been identified in $CD4^+$T-cells. If CX3CR1 is directly involved in the upregulation of ICAM-1 in CX3CL1-stimulated $CD4^+$T-cells, we would expect that transfection with CX3CR1-interfering RNA, would decrease levels of ICAM-1 as measured by Western blot

or flow cytometry analysis. To establish that JAK2 and STAT5 phosphorylation are necessary for fractalkine-induced ICAM-1 upregulation, we would expect that incubation with CX3CL1, followed by analysis of phosphorylated JAK2 and STAT5 via Western blot or flow cytometry, would show an increase in JAK2 and STAT5 phosphorylation, and that this increase would be ablated by JAK2 and STAT5 siRNA.

Our findings that IFN-γ and TNF-α transcripts, and IFN-γ protein secretion are increased in the presence of CX3CL1 in $CD4^+$T-cells derived from RRMS patients but not HCs indicates that CX3CL1 may upregulate proinflammatory cytokines in $CD4^+$T-cells during inflammation. This data is interesting in the context of study by Fraticelli, et al., who showed that IFN-γ and TNF-α induce CX3CL1 expression and release by endothelium, and CX3CL1 expression is increased in the IFN- γ-mediated Th1 disease psoriasis, but is not upregulated in Th2-dominated atopic dermatitis. In this context, upregulation of IFN-γ and TNF-α via CX3CL1 in MS may represent an amplified Th1 response. It would be interesting to test this hypothesis by incubating PBMC cultures with IFN-γ and TNF-α, followed by quantitation of CX3CL1 levels in the supernatant via ELISA.

Our finding that $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells are both increased in percentage of cells and in per-cell expression of CX3CR1 and ICAM-1 in RRMS-derived CSF compared to blood suggests that these cells may preferentially migrate into the CNS during RRMS. To address this issue further, it would be informative to immunostain acute MS lesions for evidence that $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells are present at the site of tissue damage in MS.

We hypothesized that increased CX3CL1-CX3CR1 interactions upregulate ICAM-1 and IFN-γ in $CD4^+$T-cells, and that $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells are enriched in the CSF during RRMS. The present study has identified an increase in CX3CL1, and a decrease in sICAM-1 in the CSF of RRMS patients compared to HCs, and has shown an increase in the percentage of $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells derived from CSF compared to blood of early untreated RRMS patients. CX3CL1 increased IFN-γ gene expression and protein secretion in RRMS-derived but not HC-derived $CD4^+$T-cells. CX3CL1 was found to upregulate ICAM-1 on the surface of RRMS-patient-derived but not HC-derived $CD4^+$T-cells, and on stimulated myelin-antigen-specific $CD4^+$ T cell lines. These results indicate that CX3CL1 may play a pathological role in RRMS by enhancing migration of IFN-γ-secreting $CX3CR1^+ICAM\text{-}1^+CD4^+$T-cells into the CNS.

Supplementary Text: Protein array analysis

In our initial CSF screen, we identified 13 proteins which were differentially expressed between RRMS and HC CSF. Interestingly, only three of these 13 proteins were found to be expressed at higher levels in RRMS CSF; the remaining 10 were decreased. The following is a brief description of each protein found to be increased or decreased in the CSF of untreated RRMS patients compared to HCs.

Proteins increased in the CSF of untreated RRMS patients

Trophic factor: FGF-6

We found FGF-6 expression to be 2.3-fold higher in the CSF derived from RRMS patients compared to the CSF from HCs ($p = 0.44$ please check in figure $p<0.05$). To our knowledge, FGF-6 has not been previously found to be expressed at elevated levels in the CSF of MS patients, nor has it been found to play a role in MS pathogenesis. However, elevated levels of the closely-related growth factor FGF-2 have been found in the CSF of RRMS patients [242], and FGF-2 has been shown to have a potential neuroprotective role in active MS lesions [243]. Studies in mouse models of demyelination have suggested that FGF-2 may play a role in the remyelination that occurs in active lesions by directing oligodendrocyte stimulating[244].

Cytokine: IL-3

We found IL-3 expression to be 1.8 fold higher in the CSF derived from RRMS patients when compared to the CSF derived from HCs ($p = 0.005$). IL-3

has previously been found to be upregulated in MS lesions [235]. Furthermore, IL-3 has been shown to induce activation of the JAK2-STAT5 pathway in microglia, leading to microglial proliferation, expression of CD40, and expression of MHCII [245]. Therefore, increased levels of IL-3 in the CSF may reflect microglial activation and antigen presentation during early MS. Recently, a role for IL-3-stimulated plasmacytoid (p)DCs derived from RRMS patients were shown to exhibit delayed maturation and diminished secretion of IFN-α/β compared to pDCs derived from HCs. This diminished pDC function in MS may have immunopathological consequences, as pDCs have immunoregulatory properties that may limit MS pathology by stimulation of regulatory T-cells[246]. As CX3CL1 has been found to upregulate ICAM-1 via the JAK2-STAT5 pathway [164], it would be interesting to investigate whether upregulation of IL-3 can also be induced by CX3CL1 in microglia, pDCs, or other immune cell subsets.

Chemokine: CX3CL1

In this study, CX3CL1 was found to be expressed at significantly higher levels in the CSF of RRMS patients compared to HCs (2.0 fold increase, $p = 0.01$). These data confirm an earlier report that CX3CL1 expression is increased in CSF derived from patients with RRMS and other neuroinflammatory disorders [233].

Proteins decreased in the CSF of untreated RRMS patients

Proinflammatory cytokine: MIF

We found Macrophage migration inhibitory factor (MIF) to be downregulated 1.7 fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.05$). MIF is a proinflammatory cytokine and inhibitor of macrophage trafficking that is secreted by macrophages and activated T-cells. It has previously been shown to be expressed at elevated levels in the CSF of relapsing MS patients [208]. Recently, a small molecule inhibitor of MIF has been shown to reduce the clinical manifestations of EAE, as well as the total number of relapses, potentially by inhibiting lymphocyte migration into the CNS [247]. In this context, our finding that MIF is significantly decreased in early MS can be interpreted as an indication that that ability of MIF to inhibit macrophage migration is diminished in early RRMS, potentially suggesting that blockade of MIF early in RRMS could be one mechanism of pathogenesis by which macrophages are able to migrate more freely toward active lesions.

Chemokines: HCC-4, Eotaxin-2

HCC-4 is downregulated 1.9 -old in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.020$). HCC-4 is a chemokine which binds to CCR1 and CCR2 on lymphocytes and monocytes. HCC-4 has not previously been shown to play a role in MS. However, CCR1 and CCR2 have both been implicated in EAE and MS.

Eotaxin-2 is downregulated 2.1-fold in the CSF of RRMS patients compared to the CSF of HCs ($p = 0.043$). Eotaxin-2 binds to CCR3 and has

traditionally been characterized as an eosinophil chemoattractant. CCR3 is expressed on macrophages in MS lesions, but otherwise has not been studied in MS [218]. Eotaxin-2 was recently found to be expressed at significantly higher levels in NMO CSF compared to RRMS or PPMS CSF [248].

We found growth-regulated oncogene (GRO) to be downregulated 4.4-fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.016$). GRO is a chemoattractive protein produced by astrocytes that binds CXCR2 on oligodendrocytes. Diminished GRO concentration indicates a suppression of oligodendrocyte progenitor cell (OPC) migration into demyelinated areas in EAE [249]. It is especially interesting that GRO is downregulated in early RRMS, because in the cuprizone model of demyelination and remyelination, $NG2^+$ OPCs migrate into demyelinated corpus callosum as early as two weeks after first toxic insult [250]. It is plausible that in early RRMS, downregulation of GRO may represent a mechanism of damage, or suppression of repair, as OPCs may be prohibited from localizing to lesions to remyelinate damaged axons.

Trophic factors: PIGF, FGF-4, and IGFBP-4

We found Placental Growth Factor (PIGF) to be downregulated 1.8 fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.048$). PIGF is a trophic factor and a member of the vascular endothelial growth factor (VEGF) family that is a ligand for two receptors: VEGFR-1 and neuropilins. PIGF has generally been studied in the context of its roles in angiogenesis. PIGF can be considered proinflammatory because it is a known chemoattractant of monocytes and a stimulator of proinflammatory cytokine secretion [251], but it also has known neurotrophic properties in several animal models of the central

nervous system disease [252]. Thus a reduction in PIGF expression in the CSF in RRMS can be interpreted as an inhibition of monocyte chemotaxis as well as inhibition of neuroprotection.

We found fibroblast growth factor 4 (FGF-4) to be downregulated 3.0 fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.039$). While no information is currently available regarding potential roles for FGF-4 in MS, CSF derived from RRMS patients has been reported to contain increased levels of FGF-2 [242]. Furthermore, FGF-2 plays a potential neuroprotective role in active MS lesions, leading us to speculate that FGF-4 may play a similar role [243]. FGF-2 has also been shown in mice to direct oligodendrocyte differentiation in active lesions, thus playing a potential role in remyelination. [244]. Taken together, if FGF-4 functions in a similar manner as FGF-2, then the decrease in FGF-4 in CSF may be interpreted as contributing to a diminished capacity for remyelination.

Insulin-like growth factor-4 (IGFBP-4) is downregulated 6.8-fold in RRMS CSF compared to HC CSF ($p = 0.02$). IGFBP-4 binds IGF-I to inhibit cellular growth, differentiation, and survival of brain-derived endothelia and other cell subsets. To our knowledge, IGFBP-4 has not been studied in MS. It is primarily known as an anti-angiogenic factor in glioblastomas and a promising therapy for glioblastoma[253]. In the context of IGFBP-4 primarily acting as an inhibitor of endothelial growth and survival, downregulation of IGFBP-4 in early MS CSF may allow the survival in endothelia of the BBB, and may therefore be interpreted as a part of a potential MS BBB repair mechanism.

Adhesion molecules: sICAM-1 and sICAM-3

sICAM-1 is downregulated 5.6-fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.005$). sICAM-1 lacks the transmembrane and cytoplasmic regions of ICAM-1 and is produced by many cell types, including endothelia and leukocytes. sICAM-1 binds competitively to ICAM-1 ligands including LFA-1, and therefore has a therapeutic potential. Interestingly, *in vitro* application of sICAM-1 to activated lymphocytes blocks their adhesion to CNS-derived endothelial cells, and this effect is abrogated in a dose-dependent manner upon application of anti-ICAM antibody [189].

Soluble (s)ICAM-3 is downregulated 1.9-fold in the CSF of RRMS patients compared to the CSF of HCs ($p = 0.018$). ICAM-3 is a ligand for LFA-1 that competes with ICAM-1 and ICAM-2. ICAM-3 has been previously been shown to be upregulated in MS serum [254]. Kraus and colleagues found that the numbers of $CD45RA^{+}ICAM\text{-}3^{+}$ cells in the CSF of relapsing patients was higher than in patients in remission [255]. Since sICAM-3 most likely acts to competitively inhibit interactions between membrane bound ICAMs and LFA-1, sICAM-3 may be considered as an inhibitor of immune cell transmigration in MS. Therefore, depressed levels of sICAM-3 may be interpreted as having a proinflammatory effect in MS and may therefore be considered a potential therapeutic target.

Soluble Receptor Tyrosine Kinase: Axl

Axl is downregulated 4.2 fold in the CSF of untreated RRMS patients compared to the CSF of HCs ($p = 0.047$). Axl is one of the three receptor tyrosine kinases (Mertk and Tyro3) that constitute the TAM family of Tyrosine

kinases. Axl binds to Growth arrest-specific protein 6 (Gas-6). Weinger et al (2011) recently found that when $Axl^{-/-}$ mice were subjected to MOG-induced EAE, disease severity was greater than in WT mice, suggesting that Axl alleviates EAE progression [256]. We find Axl to be diminished in early MS, during the inflammatory stage of the disease, thus suggesting that decreased Axl expression may contribute to MS pathology. In MS lesions, however, Axl has been shown to be upregulated, which seems to contradict our data [151]. However, because we analyzed CSF in early MS, while Weinger and colleagues analyzed Axl expression in lesion tissue derived from chronic MS patients, it may be that while Axl expression is suppressed in the early, inflammatory stage of MS, it is upregulated at later time point in lesions as part of a repair mechanism. This discrepancy could be resolved by (a) immunostaining lesion tissue from MS patients near the time of diagnosis and (b) evaluating the CSF of MS patients later in disease.

Supplementary Figure S1. Protein array determined the differential expression of inflammatory proteins in the CSF of RRMS patients and HCs

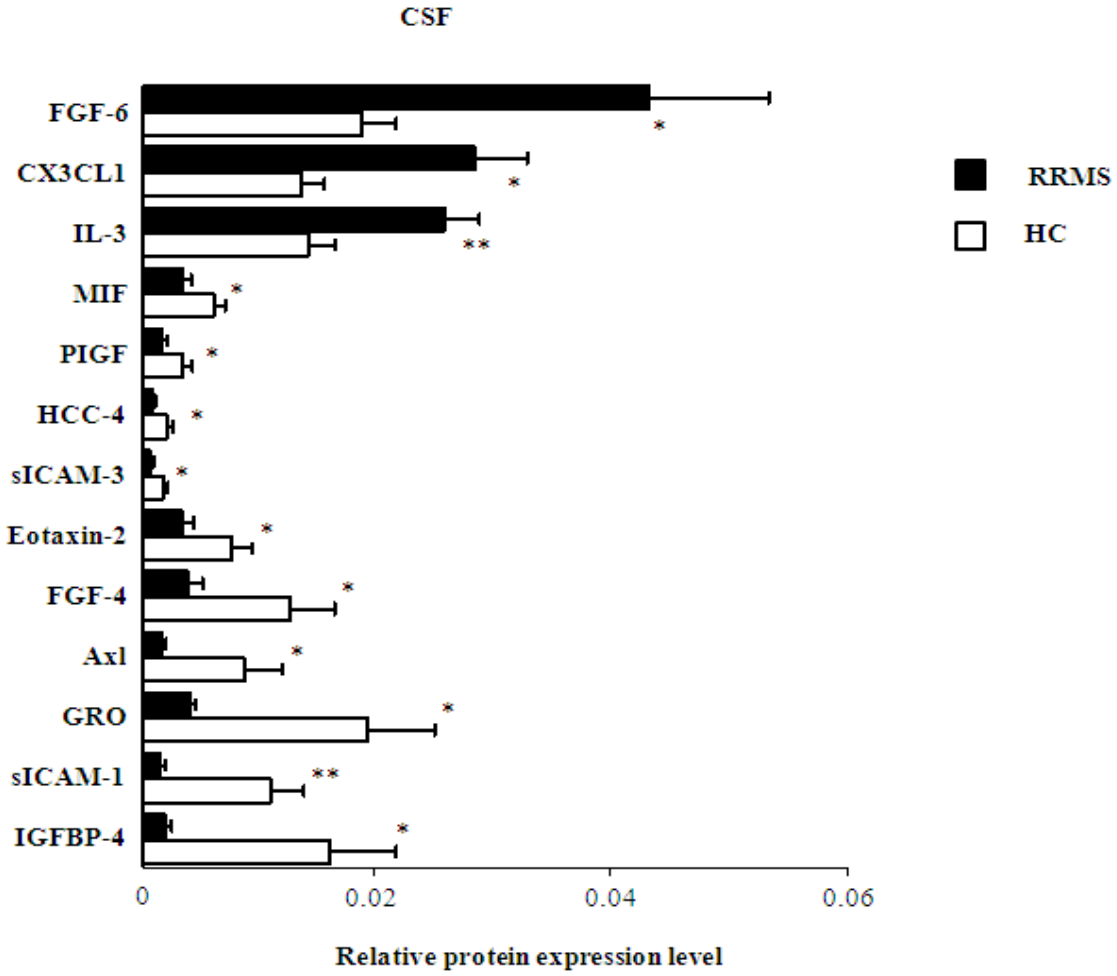

Figure S1. Differential expression of cytokines, chemokines, and growth factors in the CSF derived from RRMS patients compared to HCs. Ray Biotech cytokine array was utilized to reveal changes in CSF protein levels in RRMS patients compared to HCs. 10 RRMS CSF and 19 HC CSF samples were tested for expression of 120 cytokines, chemokines, and growth factors. Graph shows differential protein expression in the CSF from RRMS patients and HCs. Arbitrary units (AU) = Average of Normalized Density of Duplicates / Sum of normalized density of all protein spots in single membrane * $p < 0.05$. ** $p < 0.01$.

Supplementary figure 2. FGF-6 is elevated in CSF of RRMS patients in comparison to HCs.

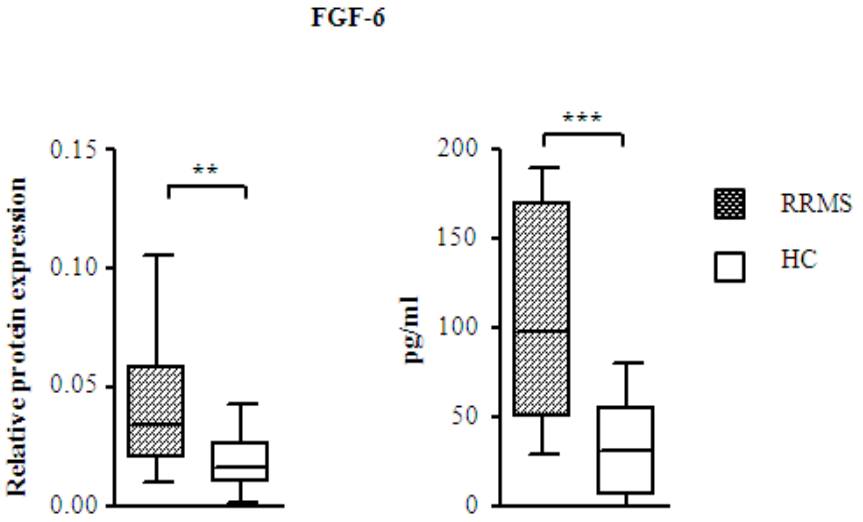

Figure S2. FGF-6 is elevated in CSF of RRMS patients in comparison to HCs. A. 10 RRMS and 19 HC CSF samples were tested for the expression of 120 cytokine, chemokine, and growth factor proteins using protein array. **B.**FGF-6 protein levels were measured in 11 RRMS and 16 HC CSF samples, using ELISA. For the cytokine array, relative protein expression is expressed in arbitrary units (AU). AU = Average of Normalized Density of Duplicates / Sum of normalized density of all protein spots on a single membrane. Statistical analysis was performed using unpaired t-tests. ** = $p < 0.01$. *** = $p < 0.001$.

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