MACROPHAGE MIGRATION INHIBITOR FACTOR:
A KEY MEDIATOR OF INFLAMMATORY DISEASE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Aaron P Kithcart, B.S.

The Ohio State University
2009

Disseration Committee:
Professor Caroline Whitacre, Advisor
Professor Phillip Popovich
Professor Michael Racke
Professor Abhay Satoskar
Professor Larry Schlesinger

Approved by

Advisor
Integrated Biomedical Science Graduate Program
ABSTRACT

Multiple sclerosis represents one the most common and complex syndromes of the family of autoimmune diseases. Affecting more than 2.4 million worldwide in the second and third decades of life, it is the primary cause of non-traumatic disability in the United States. Sensory and motor neuron loss follows the demyelination of axons in the brain and spinal cord after autoreactive T lymphocytes gain access to the central nervous system and mediate an inflammatory reaction. Thus, most current therapies in multiple sclerosis seek to suppress the immune system in order to slow progression. Several cytokines have been shown to be involved in the pathogenesis of autoimmune diseases, including multiple sclerosis. We have focused on macrophage migration inhibitory factor, a ubiquitously expressed proinflammatory cytokine that has been described in a number of syndromes. Utilizing an MIF knockout model of EAE in the C57Bl/6 strain of mice, we show that MIF is required for susceptibility to EAE. MIF knockout mice are protected with a decreased incidence of disease and lower clinical scores. Additionally, we show that an inhibitor of MIF is therapeutic during ongoing disease. Administration of an MIF inhibitor reduced clinical scores. We propose several mechanisms of MIF that mediate inflammation. First, MIF inhibits the expansion of
CD4^+CD25^+Foxp3^+ regulatory T lymphocytes. This population of cells is found in higher numbers after immunization in MIF knockout and inhibitor-treated mice. CD4^+CD25^+Foxp^+ regulatory T lymphocytes are protective during EAE through contact inhibition with autoreactive lymphocytes and the production of IL-10. Second, we propose that MIF mediates leukocyte migration into the brain and spinal cord. We show that MIF knockout mice have profoundly reduced inflammation and the administration of an inhibitor of MIF prevents new migration into the brain. Furthermore, MIF knockout recipient mice were universally protected following adoptive transfer of autoreactive lymphocytes. Finally, MIF regulates the synthesis of testosterone. MIF knockout mice have a four fold higher level of testosterone than wild type mice. Testosterone is an immunosuppressive hormone, and gonadectomy of MIF knockout mice greatly increased the incidence of EAE. We propose that MIF mediates inflammation via different mechanisms depending on the timing of disease. Inhibition of testosterone in naïve mice enhances susceptibility to disease. Later changes during inflammation, including inhibition of regulatory T cell differentiation and facilitation of migration into peripheral tissues allows the progression of disease. We propose that targeting MIF during multiple sclerosis could be therapeutic through novel regulation of multiple aspects of inflammation.
Dedicated to my father, Keith Earl Kithcart.
"From the smallest necessity to the highest religious abstraction, from the wheel
to the skyscraper, everything we are and everything we have comes from one
attribute of man – the function of his reasoning mind." – Ayn Rand
ACKNOWLEDGMENTS

This project represents a collaborative effort upon which a number of individuals are due a great deal of gratitude. First and foremost, I need to thank my advisor, Dr. Caroline Whitacre, for her unyielding guidance and mentorship. I also need to acknowledge the support my committee members, Drs. Phillip Popovich, Michael Racke, and Abhay Satoskar; as well as Dr. Larry Schlesinger, who many years ago planted the seed for joining an MD/PhD program.

I would also like to thank Cytokine PharmaSciences, for the use of their line of inhibitors, as well as past members of the lab, including Tracey Papenfuss, for supporting the project as a young faculty member, Ingrid Gienapp, for her technical expertise and camaraderie, and Nicole Domico-Powell, who started the MIF studies. Also, current members of the lab, including Jessica Williams, Gina Mavrikis-Cox, Kristen Smith, and Todd Shawler for their invaluable assistance, and Abby Short, who was integral to many of the experiments described herein.

Lastly, I would like to thank my family for their support throughout the length of this journey, especially my mother, Luann, and my father, Keith, as well as my extended family – friends back home and especially new friends here in Columbus, who were crucial to my success at Ohio State and remind me daily what is most important in life. Thank you.
VITA

May 4, 1981.................................................................Born, Chelsea, Iowa, USA

2003.................................................................B.S. Microbiology
Department of
Microbiology
University of Iowa
Iowa City, Iowa, USA

PUBLICATIONS


FIELD OF STUDY

Major Field: Integrated Biomedical Sciences

Areas of Research Emphasis: Immunology, Translational Research
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abstract</strong></td>
<td>ii</td>
</tr>
<tr>
<td><strong>Dedication</strong></td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>vi</td>
</tr>
<tr>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiv</td>
</tr>
</tbody>
</table>

**Chapters:**

1. **Introduction**
   - 1.1 Multiple Sclerosis | 1
   - 1.2 Experimental Autoimmune Encephalomyelitis | 2
   - 1.3 Hormone Regulation of the Immune System | 9
   - 1.4 Current Multiple Sclerosis Therapies | 11
   - 1.5 The Blood Brain Barrier | 14
   - 1.6 Chemokines and Cytokines | 16
   - 1.7 Macrophage Migration Inhibitory Factor | 21
   - 1.8 Objectives | 24

2. **Materials and Methods**
   - 2.1 Mice | 25
   - 2.2 Antigens | 25
   - 2.3 Induction and Transfer of Experimental Autoimmune Encephalomyelitis | 26
   - 2.4 Gonadectomy | 28
   - 2.5 Bone Marrow Chimeras | 29
   - 2.6 Drug Administration | 29
   - 2.7 Inhibitor Assays | 31
   - 2.8 Hormone and IgE Assays | 32
   - 2.9 Flow Cytometry | 33
   - 2.10 Proliferation and Suppression Assays | 35
   - 2.11 Cytokine Assays | 36
3. MIF is a Key Mediator of EAE

3.1 MIF Does Not Preferentially Expand Populations of Leukocytes

3.2 MIF is Required for Susceptibility to EAE

3.3 Functional Activity of Regulatory T Cells in MIF Deficient Mice

3.4 A Small Molecule Inhibitor of MIF Reduces Ongoing EAE

3.5 MIF Functions Through Its Ligand-Binding Activity

3.6 Conclusions

4. Myeloid Cells are a Necessary Source of MIF

4.1 Expression of MIF from Bone Marrow-Derived Cells is Required for EAE

4.2 Lymphocytes Alone Lacking MIF are not Sufficient to Transfer EAE

4.3 Conclusions

5. MIF Regulates Hormone Homeostasis

5.1 MIF Reduces Serum Levels of Corticosterone and Testosterone

5.2 Inhibition of Corticosterone Does Not Increase the Severity of EAE

5.3 MIF Antagonizes Testosterone and Reduces Incidence of EAE

5.4 An MIF Inhibitor Did Not Increase Corticosterone or Testosterone

5.5 Conclusions

6. MIF Facilitates Leukocyte Migration

6.1 MIF Knockout Mice Have Reduced Mononuclear Infiltration

6.2 An Inhibitor of MIF Prevents Ongoing Migration

6.3 MIF is Not Required for the Induction of EAE

6.4 Conclusions

7. Discussion

Bibliography
LIST OF TABLES

Table ........................................................................................................................................Page

3.1 MIF knockout mice have reduced incidence and severity of EAE...........54
3.2 Lymphocytes from MIF knockout mice respond to antigen stimulation.................................................................55
3.3 MIF inhibitor-treated mice have reduced severity of EAE...............58
3.4 Lymphocytes from inhibitor-treated mice are able to respond to antigen..........................................................................59
4.1 The expression of MIF from myeloid and lymphoid cells was sufficient for the induction of EAE.................................70
4.2 MIF is required in recipient mice for the induction of EAE by adoptive transfer..............................................................72
5.1 Gonadectomy increased the incidence of EAE in MIF knockout mice .....86
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>The genetic deletion of MIF does not cause a shift in the populations of T lymphocytes</td>
<td>51</td>
</tr>
<tr>
<td>3.2</td>
<td>Mice lacking MIF have higher serum levels of IgE</td>
<td>52</td>
</tr>
<tr>
<td>3.3</td>
<td>The genetic deletion of MIF is protective against EAE</td>
<td>53</td>
</tr>
<tr>
<td>3.4</td>
<td>Mice lacking MIF have a larger population of regulatory cells that are functional</td>
<td>56</td>
</tr>
<tr>
<td>3.5</td>
<td>A small molecule inhibitor of MIF reduces disease severity in two animal models of EAE</td>
<td>57</td>
</tr>
<tr>
<td>3.6</td>
<td>A small molecule inhibitor of MIF expands a regulatory population of T lymphocytes</td>
<td>60</td>
</tr>
<tr>
<td>3.7</td>
<td>The ligand-binding function of MIF is critical for disease progression</td>
<td>61</td>
</tr>
<tr>
<td>4.1</td>
<td>The expression of MIF from bone marrow-derived cells is required for progression of EAE</td>
<td>69</td>
</tr>
<tr>
<td>4.2</td>
<td>Lymphocytes alone expressing MIF were not sufficient to induce EAE</td>
<td>71</td>
</tr>
<tr>
<td>5.1</td>
<td>MIF deficient mice have elevated levels of testosterone</td>
<td>83</td>
</tr>
<tr>
<td>5.2</td>
<td>Administration of a corticosterone inhibitor reduced severity of EAE in MIF knockout animals</td>
<td>84</td>
</tr>
<tr>
<td>5.3</td>
<td>Gonadectomy of MIF-deficient mice did not increase severity of disease</td>
<td>85</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>5.4</td>
<td>An inhibitor of MIF did not increase serum corticosterone or testosterone</td>
<td>87</td>
</tr>
<tr>
<td>6.1</td>
<td>The absence of MIF prevents infiltration into the CNS</td>
<td>95</td>
</tr>
<tr>
<td>6.2</td>
<td>An inhibitor of MIF reduced ongoing migration</td>
<td>96</td>
</tr>
<tr>
<td>6.3</td>
<td>A small molecule inhibitor does not prevent the onset of EAE</td>
<td>97</td>
</tr>
<tr>
<td>6.4</td>
<td>A small molecule inhibitor of MIF is available in the brain</td>
<td>98</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

ALCAM activated leukocyte cell adhesion molecule
AP activator protein
APC allophycocyanin
BBB blood brain barrier
BSA bovine serum albumin
CD cluster of differentiation
CDI cumulative disease index
CNS central nervous system
CPM counts per minute
CSF cerebrospinal fluid
DMSO dimethyl sulfoxide
EAE experimental autoimmune encephalomyelitis
EBV Epstein-Barr virus
EIA enzyme immunoassay
ELISA enzyme-linked immunosorbent assay
ERK extracellular signal-related kinase
FBS fetal bovine serum
FITC fluorescein isothiocyanate
Foxp3 forkhead box P3
FSH follicle stimulating hormone
GM-CSF granulocyte-macrophage colony-stimulating factor
H&E hematoxylin and eosin
HLA human leukocyte antigen
HPLC high performance liquid chromatography
ICAM intercellular adhesion molecule
IFN interferon
Ig immunoglobulin
IL interleukin
IM infection mononucleosis
i.p. intraperitoneal
i.v. intravenous
KO knockout
LH leutinizing hormong
LFA leukocyte function-association antigen
LPS lipopolysaccharide
MAPK mitogen-activated protein kinases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>ND</td>
<td>not detected</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OCT</td>
<td>optimum cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PML</td>
<td>progressive multifocal leukoencephalopathy</td>
</tr>
<tr>
<td>p.o.</td>
<td>per os (orally)</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneously</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>SRY</td>
<td>sex-determining region Y</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
</tbody>
</table>
Jean-Martin Charcot distinguished multiple sclerosis (MS) from other neurological diseases in 1868 as a pattern of tremors and paralysis in young adults, differing from James Parkinson’s description of paralysis agitans in elderly patients (Murray 2005). Though at the time little was known about the pathogenesis of multiple sclerosis, the current consensus is that MS is an autoimmune disease of the brain and spinal cord. Currently, there are 30 autoimmune diseases, including diabetes mellitus, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE), to name a few (Rosen and Mackay 2001). Together they affect nearly five percent of the population (Rosen and Mackay 2001). Identifying the cause of each, or devising a reliable therapy, has been exceedingly difficult. The factors that determine whether the immune system tilts towards autoimmunity or immunosuppression are still relatively unknown. Understanding the complicated nature of the immune system and determining mechanisms that keep it in balance are active areas of biomedical research.
1.1 Multiple Sclerosis

Multiple sclerosis is a chronic, demyelinating disease of the central nervous system (CNS) affecting more than 400,000 individuals in the United States and 2.1 million worldwide, the majority of which are female (Anderson, Ellenberg et al. 1992; Peterson and Trapp 2005). Typically diagnosed during the second and third decades of life, it is the primary cause of non-traumatic disability in the United States (Peterson and Trapp 2005).

MS can be difficult to diagnose since patients present with a variety of symptoms, including ocular and sensory disturbances, limb weakness, and clumsiness (Noseworthy, Lucchinetti et al. 2000). Presentation in the clinic is patently heterogeneous, but most patients fall under one of four primary subtypes; the characteristics of each depend on the course of disability. For more than 85 percent of patients, symptoms appear over several days and improve spontaneously, often with no lasting neurological deficits. This type of MS, termed relapsing-remitting, is by far the most common (Noseworthy, Lucchinetti et al. 2000). Within 25 years of diagnosis, nearly 90 percent will progress to a more chronic form of disease known as secondary-progressive MS (Weinshenker, Bass et al. 1989). Disability for these patients is permanent and progresses continuously. At the time of diagnosis, a few patients will experience progressive decline without improvement. Called primary progressive, this type of MS is observed in 10-15 percent of patients and has an equal incidence in both men and women (Miller and Leary 2007). A fourth subtype of MS, progressive relapsing, affects five percent of patients and is characterized by steady
progression of disability punctuated by acute periods of worsening disease (Peterson and Trapp 2005). Across all subtypes, the severity of MS at diagnosis largely predicts the rate of disease progression (Confavreux, Vukusic et al. 2003). However, nearly half of all patients will require a wheelchair within 15 years of initial diagnosis (Weinshenker, Bass et al. 1989).

Early observations of the worldwide prevalence of MS suggested a strong environmental influence (Noseworthy, Lucchinetti et al. 2000). Prevalence is highest in northern Europe, southern Australia, and mid-North America, which initially suggested that susceptibility was inversely associated with sunlight exposure (Ebers 2008). Additional evidence showed that vitamin D deficiency increased the risk of developing several autoimmune diseases, including MS (Cantorna 2000; Munger, Zhang et al. 2004; Munger, Levin et al. 2006). Vitamin D is produced in the skin following contact with ultraviolet B radiation, the amount of which decreases as one moves away from the equator. Closer analyses revealed that the prevalence of MS in northern Norway, which receives very little sunlight in the winter, is much lower than other countries farther south, including Scotland and England. This, and other examples of low prevalence in northern regions, points to a significant genetic contribution, which can partially explain the higher incidence of MS along Scandinavian migration patterns (Ebers 2008).

One of the earliest studies looking at familial MS patterns showed that monozygotic twins had a concordance rate exceeding 25 percent versus 2.3 percent in dizygotic twins or 1.9 percent in other siblings (Ebers, Bulman et al.)
Subsequent familial aggregation studies linked susceptibility to the human leukocyte antigen (HLA) DR2 locus (Ebers, Bulman et al. 1986; Wingerchuk, Lucchinetti et al. 2001). More recent studies have identified other genetic regions, including single-nucleotide polymorphisms in the interleukin-7 receptor α, interleukin-2 receptor α, and matrix metalloproteinases-9 and 2 (Ebers, Sadovnick et al. 1995; Gregory, Schmidt et al. 2007; Hafler, Compston et al. 2007; Lundmark, Duvefelt et al. 2007; Benesova, Vasku et al. 2008). However, no single genetic locus has been as consistently and strongly associated as HLA DR2, suggesting that major histocompatibility complex II (MHC II) has a key role in the susceptibility to MS. These genetic factors may relate to the environmental observations. The vitamin D receptor can act as a transcription factor and bind to vitamin D response elements within the HLA-DRB1*1501 promoter (Ramagopalan, Maugeri et al. 2009). This supports the general hypothesis that MS occurs in genetically susceptible individuals following exposure to certain environmental factors. For example, an increased risk for MS has been reported in persons following infectious mononucleosis (IM) (Nielsen, Rostgaard et al. 2007). Infection with Epstein-Barr virus (EBV), the causative agent for IM, has been linked to an increased prevalence of MS (Ascherio, Munger et al. 2001; Goldacre, Wotton et al. 2004; Sundstrom, Juto et al. 2004; Thacker, Mirzaei et al. 2006; Farrell 2007). Together, these studies show the interplay of genetics and environmental factors in MS but demonstrate that additional work needs to be done in order to better understand the interactions between them.
Although the etiology is unclear, the pathology of MS has been well described. MS is generally believed to be caused by the activation of autoreactive T lymphocytes, which subsequently gain access to the central nervous system (CNS) where they orchestrate an attack on the myelin sheath surrounding neurons and oligodendrocytes, the cells that myelinate axons (McFarland and Martin 2007). Within the CNS, these lymphocytes proliferate, produce inflammatory cytokines and chemokines, cause edema, and recruit additional leukocytes (Noseworthy, Lucchinetti et al. 2000). The accumulation of inflammatory products and edema cause multiple foci visible on magnetic resonant imaging (MRI) in the cortex, optic nerves, brain stem, and spinal cord of those affected (Xiao and Link 1999; Wingerchuk, Lucchinetti et al. 2001). MS lesions most often occur around blood vessels of the white matter, where mononuclear infiltrates can be identified within the perivascular cuffs (Peterson and Trapp 2005). However, MRI-identified white matter lesions alone have not always been adequate to explain clinical symptoms. Post mortem analysis and more sophisticated MRI studies have shown significant demyelination in the gray matter as well (Bo, Vedeler et al. 2003; Kutzelnigg, Lucchinetti et al. 2005). The significance of this inflammation for the progression of MS has yet to be elucidated.

CD4⁺ lymphocytes were the first cell type specifically linked to MS pathogenesis, but other cell types have since been implicated, including CD8⁺ lymphocytes, CD4⁺CD25⁺ regulatory T lymphocytes, monocytes/macrophages, and B lymphocytes (Jurewicz, Biddison et al. 1998; Archelos, Storch et al. 2000;
Medana, Martinic et al. 2001; Giuliani, Goodyer et al. 2003; Greter, Heppner et al. 2005; McDole, Johnson et al. 2006; Lambracht-Washington, O'Connor et al. 2007; Dalakas 2008; Venken, Hellings et al. 2008). Auto-antibodies directed against a number of CNS antigens are present in the cerebrospinal fluid (CSF) of MS patients, but only recently, after improvement in MS was noted following treatment with B cell-depleting antibodies, has more attention been focused on the B lymphocyte (Kabat, Glusman et al. 1948; Hauser, Waubant et al. 2008).

Infiltrating inflammatory cells were once thought to mediate most of the CNS damage in MS, but we now know that a significant amount of neurological deficit can be attributed to cortical edema following compromise of the blood brain barrier (BBB) (Noseworthy, Lucchinetti et al. 2000). The blood brain barrier normally protects the CNS but can be damaged during MS. The ensuing entry of leukocytes and inflammatory products causes both direct and indirect damage to the myelin sheath and underlying axons. Though demyelination is one of the early hallmarks of MS, sustained loss of the myelin sheath leads to axonal transection, causing permanent injury (Trapp, Peterson et al. 1998). The irreversible loss of axons in turn causes the progressive motor and sensory loss characteristic of late secondary-progressive MS.

The autoimmune nature of MS was first suggested by early animal studies in which self-myelin antigens were used to immunize rodents causing an MS-like disease, later termed experimental autoimmune encephalomyelitis (EAE). Although the classification of MS as an autoimmune disease is still debated, no other experimental model has proven more valuable for the study of MS.
1.2 Experimental Autoimmune Encephalomyelitis

In the 1920s, Thomas M. Rivers designed a series of experiments based on observations of patients who had received early formulations of the rabies vaccine. At the time, rabies vaccines were derived from virus grown in nervous tissue cultures from rabies-infected rabbits. Occasionally, patients would experience a mild form of paralysis, usually causing facial palsy or limb paresis. Hypothesizing that paralysis was due to the components of the inoculum and not a rabies infection, Rivers injected rhesus monkeys with all the components of the vaccine except the rabies virus. As he suspected, the paralysis was not due to rabies virus but rather an autoimmune reaction to the CNS proteins found in the inoculum.

Rivers’ original experiments required more than 50 injections of CNS material into monkeys. Later experiments required as few as 26 injections in rabbits, but it was not until the addition of adjuvant with *Mycobacterium tuberculosis* that EAE could be induced with a single injection. Jules T. Freund and others found that the addition of adjuvant to the CNS preparations increased the titer of antibodies, which they hypothesized were the cause of demyelination. Subsequent histological analysis showed that EAE resembled human demyelinating diseases, including MS. EAE is viewed as the major animal model for MS, since the two diseases have in common loss of the myelin sheath, concentration of perivascular lesions within the brain stem and spinal cord, detection of immunoglobulin in the CNS and CSF, accumulation of autoreactive...
T lymphocytes, and production of inflammatory cytokines and chemokines (Gold, Hartung et al. 2000; Gold, Linington et al. 2006).

Most contemporary EAE studies are performed in rodents, primarily mice, and are induced by the injection of purified myelin proteins or peptides together with adjuvant. There are two commonly used models of EAE: female SJL mice, immunized with proteolipid protein (PLP) peptide, are used to model relapsing-remitting EAE. C57Bl/6 mice, immunized with myelin oligodendrocyte glycoprotein (MOG) peptide, and B10.PL mice, immunized with myelin basic protein (MBP) peptide, are used to model chronic-progressive EAE. SJL mice exhibit an acute phase of paralysis, followed by recovery and further relapses, eventually progressing without recovery. C57Bl/6 and B10.PL mice typically do not have remissions during EAE, however in certain models groups have reported relapsing-remitting disease in B10.PL mice (Ando, Clayton et al. 1989; Meyer, Benson et al. 1996). Instead, disease is marked by an initial acute phase followed by uninterrupted paralysis. Both models are frequently used to explore the pathogenesis of MS.

Between and within strains of mice, pronounced sex differences in terms of type and severity of EAE are observed, which reflects the sex dimorphism in MS. Papenfuss et al (Papenfuss, Rogers et al. 2004) found female SJL mice showed greater disease severity of the relapsing-remitting type. Male SJL mice, on the other hand, exhibit a more chronic form of EAE that is less severe. This parallels observations in MS patients, in which women are more frequently diagnosed with relapsing-remitting MS, whereas 50 percent of new primary-
progressive cases of MS are in men (Miller and Leary 2007). Thus, many sex hormone studies utilize SJL mice. C57Bl/6 mice do not show sex differences in EAE, while in the B10.PL strain, male mice show more severe EAE than their female counterparts (Papenfuss, Rogers et al. 2004). These sex differences between and within strains suggest a dual role for both hormones and genetics. The cause of these differences has been the source of ongoing studies seeking to understand the sex dimorphism in MS patients.

1.3 Hormone Regulation of the Immune System

Much of the initial interest in sex hormones followed epidemiological evidence showing that women were three times more likely than men to be diagnosed with MS (Anderson, Ellenberg et al. 1992). Testosterone was a logical candidate for therapy since men were less susceptible to MS, and 24 percent of male MS patients had significantly lower levels of testosterone (Wei and Lightman 1997). These observations were corroborated in studies of EAE. Male SJL mice had lower levels of testosterone during relapses of EAE, and following gonadectomy, male mice had more severe disease that matched their female counterparts (Foster, Daniels et al. 2003; Palaszynski, Loo et al. 2004). These data have led to recent clinical trials in which men with relapsing-remitting MS were given topical testosterone for 12 months. Initial results suggest that testosterone therapy may be neuroprotective, but ongoing work is directed at determining whether the sex hormone is able to slow the progression of MS (Sicotte, Giesser et al. 2007).
Even though women are typically more susceptible to MS, several studies have shown that pregnancy can be protective (Birk, Ford et al. 1990; Bernardi, Grasso et al. 1991; Davis and Maslow 1992; Runmarker and Andersen 1995; Damek and Shuster 1997). A comprehensive analysis showed that relapse rate improved over the course of pregnancy, especially during the third trimester, but that disease flared during the immediate post-partum period (Confavreux, Hutchinson et al. 1998). Detailed studies of the role of sex hormones during pregnancy, primarily estrogen and progesterone, have increased our understanding of these hormones and their effect on the inflammatory response. Experiments in EAE showed that pregnancy induced a shift in the immune response, from a proinflammatory T\textsubscript{H}1 environment characterized by high levels of interferon-\(\gamma\) (IFN-\(\gamma\)) and interleukin-17 (IL-17), to a regulatory environment high in IL-10 (McClain, Gatson et al. 2007). More complex mechanisms may be present, as pregnancy studies in mice have shown an increase in oligodendrocyte precursor cells and enhanced remyelination of axons (Gregg, Shikar et al. 2007). Pregnancy may not only induce an anti-inflammatory environment but may also be neuroprotective or favor neuroregeneration.

Further studies with estrogen and progesterone in mice have attempted to recreate the pregnancy environment. In EAE, addition of exogenous 17\(\beta\)-estradiol lessened the severity of disease and was accompanied by reduced activation of autoreactive lymphocytes and inflammatory cytokines (Offner, Adlard et al. 2000). Estradiol was shown to act via the estrogen receptor \(\alpha\), identifying a potential pharmaceutical target (Polanczyk, Zamora et al. 2003). As
a result of these studies, current clinical trials are evaluating hormone replacement as a potential therapy for women diagnosed with MS. Interestingly, other studies suggest that sex hormones may not have a primary role in the susceptibility to autoimmune diseases. Using XY mice lacking the sex-determining region Y gene (sry) coding for testes, researchers have shown that the XX complement conferred greater susceptibility to EAE compared to XY mice (Smith-Bouvier, Divekar et al. 2008). This study suggests a genetic rather than hormonal basis for observed sex differences. Although there has been much studied on the role of sex hormones in autoimmune disease, there is likely an additional and significant genetic role.

1.4 Current Multiple Sclerosis Therapies

Sex hormones may represent potential future therapies, but one of the earliest treatments for MS patients was another family of hormones, the glucocorticoids. These potent anti-inflammatory hormones shorten the duration of relapses and lengthen remissions (Noseworthy, Lucchinetti et al. 2000). Studies in patients receiving methylprednisolone showed an expanded CD4+CD25+Foxp3+ population of regulatory T lymphocytes within 48 hours of treatment (Braitc, Harikrishnan et al. 2008). However, side effects of long-term glucocorticoid treatment prevent their extended use. Other non-glucocorticoid therapies have been developed that can be given for much longer periods.

The most widely used therapy for MS has been the interferon family of drugs, Betaseron, Avonex, and Rebif. Despite interferons being shown to reduce
relapses and delay disease progression, the mechanism of action is still relatively unknown (Kappos, Polman et al. 2006). Most data suggest that interferon-β reduces permeability of the blood brain barrier, thus reducing inflammation in the brain and spinal cord. Regardless of the mechanism, interferons have been shown to be safe over many years and are a staple of MS treatment (Rio, Tintore et al. 2005; Kinkel, Kollman et al. 2006).

Glatiramer acetate, also known as Copaxone, is another frequently used treatment for MS. Similar to the interferons, the exact mechanism of action of glatiramer acetate is unknown but is believed to be anti-inflammatory. Composed of random polymers of L-glutamic acid, L-alanine, L-tyrosine, and L-lysine, it contains the same amino acids as MBP, which may explain some of its mechanism. Glatiramer acetate may act as an immunological decoy, shifting the immune response away from myelin. Also like the interferons, it has been shown to be safe over long treatment periods (Khan, Tselis et al. 2001; Haas and Firzlaff 2005).

Recently, more aggressive therapies have been introduced to target the autoreactive immune system. Autologous haemopoietic stem cell transplantation aims to replace the dysfunctional immune system of MS patients and reconstitute it with self stem cells. Until now, the therapy was thought to be too risky for most patients; however, recent advances and the use of nonmyeloid ablation therapy have improved results. A clinical trial at Northwestern University showed that of 21 patients receiving the therapy, none progressed clinically and only five relapsed (Burt, Loh et al. 2009). Larger studies need to be
done, but initial results suggest that this could be a promising new therapy for early MS patients. Additional means of targeting the immune system have also been employed. The use of a chemotherapeutic drug, mitoxantrone, has recently been approved for patients with secondary progressive MS (Hartung, Gonsette et al. 2002; Krapf, Morrissey et al. 2005). Mitoxantrone disrupts DNA synthesis in actively replicating cells, including those of the immune system. Though the drug slows the progression of MS, it has significant side effects including adverse cardiac events (Avasarala, Cross et al. 2003). Its efficacy in other types of MS is being explored.

One of the most promising new therapies for MS is natalizumab. Sold under by the trade name Tysabri, it is a monoclonal antibody directed against \( \alpha_4 \)-integrin, a critical mediator of leukocyte migration into tissues, including the brain and spinal cord. Administration of natalizumab was shown to be effective in reducing MRI lesion load and delaying disease progression (Polman, O'Connor et al. 2006; Rudick, Stuart et al. 2006). Unfortunately, it has also been associated with a higher risk for acquiring progressive multifocal leukoencephalopathy (PML), with an estimated incidence of 1 in 1000 treated patients (Yousry, Major et al. 2006; Hartung 2009). PML occurred as a result of inhibition of normal immune surveillance in the CNS, which allowed replication of latent viruses that are typically kept in check by the immune system. Identifying mechanisms to reduce inflammation in the brain without increasing the risk of opportunistic infections is an important area of ongoing MS research.
1.5 The Blood Brain Barrier

The BBB plays a key role in regulating leukocyte infiltration into the CNS. The patency of the BBB is one of the primary differences between MS patients and healthy individuals. Migration of leukocytes across the vascular endothelium is largely determined by the expression of adhesion molecules and their ligands. Entry into the CNS is a two-step process. First, leukocytes are tethered and roll along the vasculature, mediated by E-selectin and P-selectin expressed on endothelial cells and P-selectin glycoprotein ligand 1 (PSGL-1) expressed on leukocytes. Second, transendothelial migration occurs through firm adhesion between integrins on leukocytes and vascular ligands on endothelial cells, including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). The expression of these molecules along the vasculature is increased during periods of inflammation.

Initial studies with lymphocytes from MS patients showed that α4-integrin and PSGL-1 were required for CD4+ and CD8+ T cell rolling along endothelial cells, respectively (Battistini, Piccio et al. 2003). Multiple animal studies have corroborated these results, showing that the interaction between α4-integrin and VCAM-1 is required for the recruitment of inflammatory cells into the CNS (Baron, Madri et al. 1993; Steffen, Butcher et al. 1994; Kent, Karlik et al. 1995). The role of selectins is still being explored. Despite upregulation during EAE and inflammation, E- and P-selectin deficient mice are susceptible to EAE that is indistinguishable from wild type animals (Engelhardt, Vestweber et al. 1997; Kerfoot, Norman et al. 2006; Doring, Wild et al. 2007). One of the ligands
for P-selectin, PSGL-1, is not required for EAE induction (Engelhardt, Kempe et al. 2005; Osmers, Bullard et al. 2005). Thus, while the importance of the selectin family of adhesion molecules is still relatively unknown, more focus remains on interactions with ICAM-1 and VCAM-1.

During MS and EAE, ICAM-1 is expressed on endothelial cells, microglia, and astrocytes within the inflammatory lesion (Dopp, Breneman et al. 1994; Bo, Peterson et al. 1996; Lee and Benveniste 1999; Carrithers, Visintin et al. 2000). ICAM-1 engages leukocyte function-associated antigen 1 (LFA-1) on leukocytes and may play a role in activation of effector functions, leading to neuroinflammation (Greenwood, Etienne-Manneville et al. 2002). Based on knockout studies, ICAM-1 is necessary for susceptibility to EAE (Bullard, Hu et al. 2007). Additional adhesion molecules, including activated leukocyte cell adhesion molecule (ALCAM) have been linked to transmigration across the BBB. ALCAM binds with CD6 on leukocytes, which originally was thought to stabilize interactions with antigen presenting cells (Zimmerman, Joosten et al. 2006). However, new data suggest ALCAM may also be expressed by vascular endothelial cells and facilitates trafficking of leukocytes into the CNS (Cayrol, Wosik et al. 2008).

In most cases, the expression of adhesion molecules on the endothelium of the BBB is strongly influenced by the presence of inflammatory cytokines. Tumor necrosis factor α (TNF-α) is present in MS lesions and is associated with the expression of ICAM-1 (Sharief and Thompson 1992). Inflammatory cytokines, including TNF-α, can upregulate adhesion molecules on a number of
different endothelial cell types, such as those along the BBB (Aird 2007). The source of TNF-α during inflammation can be from cells infiltrating the CNS, including T lymphocytes and macrophages. Resident cells of the CNS can also produce TNF-α upon activation. Some of these resident cells include microglia, which are the principal resident antigen presenting cells in the brain and spinal cord. These cells have also been implicated in the pathogenesis of MS (Gay 2007). Following stimulation, microglia can produce a number of inflammatory cytokines. Multiple studies, in MS and spinal cord injury, suggest that reducing inflammation early in disease can prevent ongoing and irreversible neuronal damage (Bartholdi and Schwab 1995; Xu, Fan et al. 1998; Fox and Ransohoff 2004). However, there are also conflicting reports showing that microglia and macrophages may be necessary for axonal repair and regrowth (Rapalino, Lazarov-Spiegler et al. 1998; Li, Setzu et al. 2005; Yin, Henzl et al. 2006). Modulating the CNS environment through regulation of inflammatory cytokines and chemokines is a critical component of recovery from neurodegenerative diseases.

1.6 Chemokines and Cytokines

There are a multitude of studies in both MS and EAE showing that inflammatory chemokines and cytokines are critical in the pathogenesis of neuroinflammation. Cytokines can both directly damage the myelin sheath and activate other cells of the immune system. Chemokines attract leukocytes to the inflammatory lesion and are critical for migration of immune cells into the CNS.
Multiple inflammatory chemokines and cytokines have been the target of developing pharmaceutical therapies.

IFN-γ was among the first inflammatory cytokines implicated in MS. Initial studies in mice suggested that IFN-γ might be beneficial (Willenborg, Fordham et al. 1996; Willenborg, Fordham et al. 1999), but MS patients exhibited exacerbations of disease following administration of exogenous IFN-γ (Panitch, Hirsch et al. 1987; Voorthuis, Uitdehaag et al. 1990). Intrathecal injection of IFN-γ in mice increased migration of lymphocytes into the CNS through the production of CXCL10 and CCL5, two potent chemokines (Voorthuis, Uitdehaag et al. 1990; Millward, Caruso et al. 2007). CCL5 drives migration of mononuclear cells into the perivascular space of the CNS during MS (Ubogu, Callahan et al. 2006). IFN-γ is produced by T<sub>H</sub>1-type CD4<sup>+</sup> T lymphocytes following exposure to IL-12 from antigen presenting cells. IL-12 is a heterodimer of the p40 and p35 subunits (Podlaski, Nanduri et al. 1992). MS patients have elevated IL-12, and treatment with anti-IL-12p40 antibodies reduces the incidence of EAE in mice (Leonard, Waldburger et al. 1995; Nicoletti, Patti et al. 1996; Ichikawa, Koh et al. 2000).

While IL-12 can stimulate IFN-γ production, IFN-γ itself is not the only cytokine required for the progression of EAE. Studies in mice showed that blocking IFN-γ or deleting it genetically could not completely protect against EAE induction (Billiau, Heremans et al. 1988; Duong, St Louis et al. 1992; Ferber, Brocke et al. 1996). Additional cytokines have since been shown to be involved in the pathogenesis of EAE and MS.
Within the last five years, much attention has been focused on a second inflammatory cytokine, IL-17. Like IFN-γ, IL-17 can be detected in inflammatory lesions of MS patients (Tzartos, Friese et al. 2008). IL-17 is produced by T_{h17} CD4^+ lymphocytes, which are expanded by IL-6 and transforming growth factor-β (TGF-β) (Harrington, Hatton et al. 2005; Park, Li et al. 2005). A third cytokine, IL-23, has been hypothesized to stabilize the T_{h17} phenotype (Aggarwal, Ghilardi et al. 2003; Harrington, Hatton et al. 2005; Langrish, Chen et al. 2005; Park, Li et al. 2005). Similar to IL-12, IL-23 is composed of two subunits, p19 and p40, of which the p40 subunit is in common with IL-12. Early studies that inhibited IL-12 targeted the p40 subunit, thereby blocking IL-12 as well as IL-23. Experiments in mice lacking the p19 or p40 subunits show that IL-23, but not IL-12, is required for the development of another inflammatory disease, colitis (Yen, Cheung et al. 2006). IL-23 is also critical for susceptibility to EAE (Segal, Dwyer et al. 1998; Cua, Sherlock et al. 2003). Surprisingly, recent clinical trials in MS with an anti-p40 antibody failed to produce clinical or radiological improvement, however the drug was given late in disease and needs further testing (Segal, Constantinescu et al. 2008; Longbrake and Racke 2009).

The BBB itself, through the secretion of TGF-β and granulocyte-macrophage colony-stimulating factor (GM-CSF), may preferentially expand T_{h17}-polarizing dendritic cells. These dendritic cells have been found capable of promoting the proliferation of both IFN-γ- and IL-17-producing T lymphocytes and have been identified within both MS and EAE lesions (Bailey, Schreiner et al. 2007; Ifergan, Kebir et al. 2008). Evidence shows that T_{h17} cells may more
readily cross the blood brain barrier (Kebir, Kreymborg et al. 2007). However, one study suggests T\(_{H1}\) lymphocytes are among the first to migrate into the CNS and their arrival may facilitate the entry of T\(_{H17}\) lymphocytes (O'Connor, Prendergast et al. 2008). Subsequent increases in trafficking could be due to coexpression of IL-6 with IL-17. IL-6 was shown to upregulate VCAM-1 in a rat model of EAE (Linker, Luhder et al. 2008). Thus, although many of the initial studies focused on IFN-\(\gamma\), more recent data suggests that IL-17 may be a crucial mediator of MS.

While both IFN-\(\gamma\) and IL-17 have been documented to worsen EAE and MS, a number of cytokines have been shown to improve symptoms. IL-4 and IL-10 are correlated with remission during EAE (Kennedy, Torrance et al. 1992). Both can induce a T\(_{H2}\)-type inflammatory environment, but IL-10 may have a stronger role in protection. When IL-4 deficient mice were immunized for EAE, they showed moderate disease, but mice lacking IL-10 had severe non-remitting EAE (Bettelli, Das et al. 1998). IL-10 can be produced by a number of cell types, but most attention has been focused on B lymphocytes and a regulatory population of T lymphocytes that express CD4, CD25, and a transcription factor Foxp3. Fillatreau et al (Fillatreau, Sweeney et al. 2002) implicated B lymphocytes as a source of IL-10 during recovery from EAE by utilizing bone marrow chimeras in which B cells lacked the ability to produce IL-10. These mice failed to recover following induction of EAE and had higher proliferative responses to MOG peptide. Other studies showed that exogenous IL-10 was therapeutic when administered directly into the CNS during EAE (Croxford, Feldmann et al.
Another IL-10-producing cell type correlated with EAE and associated with recovery is the CD4^+CD25^+Foxp3^+ population of regulatory T cells. These cells produce high levels of IL-10 (Segal, Dwyer et al. 1998). Depletion of this regulatory population of T cells increased the severity of EAE (Kohm, Carpentier et al. 2002). These cells are expanded by exposure to TGF-β in the absence of IL-6. Depending on the local environment, TGF-β can expand encephalitogenic T_{eff}17 cells or protective regulatory T cells.

Recently, a third population of cells that play a role during EAE was identified. These cells are expanded by TGF-β with IL-4 and produce IL-9 and IL-10. They are not suppressive suggesting they may have a role in the pathogenesis of EAE and MS (Dardalhon, Awasthi et al. 2008; Veldhoen, Uyttenhove et al. 2008). It is still unclear whether IL-4- and IL-9-producing cells mediate the progression of either EAE or MS. Identifying factors that regulate the coexpression of cytokines with TGF-β could lead to new therapies that modulate autoimmunity.

Modulation of chemokines and cytokines can dictate the development of or recovery from MS. A study of MS patients showed that an additional cytokine, macrophage migration inhibitory factor (MIF), might also play a role in pathogenesis. This pro-inflammatory cytokine was elevated in the CSF of patients undergoing a relapse of MS, as well as in the CNS of mice following the induction of EAE (Niino, Ogata et al. 2000; Gao, Wang et al. 2008). These data together with other studies demonstrating that MIF acts both as a cytokine and as a chemokine, suggest a critical role for MIF in the progression of MS.
1.7 Macrophage Migration Inhibitory Factor

The term “cytokine” covers a large and diverse family of proteins. The first cytokine discovered was MIF. Identified as a product of activated T lymphocytes that inhibited the migration of macrophages from capillary tubes, it was reported simultaneously by two groups in 1966 (Bloom and Bennett 1966; David 1966). Since then, much has been learned about the protein. Cloned in 1989 by John David, mature MIF protein is a highly conserved 114-amino acid polypeptide arranged as a homotrimer (Weiser, Temple et al. 1989; Bernhagen, Mitchell et al. 1994). Unlike other pro-inflammatory cytokines, MIF is upregulated by glucocorticoids, which are known immunosuppressive hormones (Fingerle-Rowson, Koch et al. 2003). MIF, in turn, limits the physiological action of glucocorticoids.

Initial studies suggested MIF has a critical enzymatic function. In fact, three intrinsic enzymatic activities have been ascribed to MIF, specifically D-dopachrome tautomerase, phenylpyruvate tautomerase, and thiol-protein oxidoreductase, but no physiologically relevant substrates have yet to be identified and MIF’s enzymatic activities have not been linked to any specific biological function (Rosengren, Bucala et al. 1996; Kleemann, Kapurniotu et al. 1998). Inhibitor and tautomerase-null knock-in studies in cancer validate that the enzymatic activity of MIF does not play a significant role in the growth of prostate and lung cancers (Meyer-Siegler, Vera et al. 2007; Winner, Meier et al. 2008; Fingerle-Rowson, Kaleswarapu et al. 2009). This suggests an alternative mechanism of action for MIF.
More recent studies have demonstrated that most of MIF’s function may be mediated through extracellular binding with CD74 and CD44 (Leng, Metz et al. 2003; Shi, Leng et al. 2006). Although an exact intracellular signaling pathway is still unclear, MIF may have a role in cell recruitment and migration. MIF can form trimeric complexes with CD74 and CXCR2 or CXCR4 (Bernhagen, Krohn et al. 2007; Weber, Kraemer et al. 2008). By signaling through these surface receptors, MIF likely induces activation of Src-family kinase and MAPK/extracellular signal-related kinase (ERK) to activate the PI3K/Akt pathway or inhibit apoptosis via p53 (Shi, Leng et al. 2006; Lue, Thiele et al. 2007). These signaling pathways can trigger a number of cellular changes, including activation of inflammatory cells such as macrophages and T lymphocytes. Additionally, MIF can be endocytosed and inhibits MAPK through JAB-1 (Kleemann, Hausser et al. 2000; Lue, Thiele et al. 2007). This function is likely a feedback mechanism since subsequent studies have shown that MIF is not only produced in T lymphocytes, but virtually every cell type (Baugh and Bucala 2002). Autocrine regulation could be a mechanism to prevent further release of MIF.

Perhaps most telling of MIF’s diverse function is the large number of diseases that have been associated with the ubiquitously expressed cytokine. One of the early studies that evaluated the role of MIF in inflammatory disease utilized a mouse model in which MIF was deleted. Mice lacking MIF were protected from an experimental model of sepsis (Bozza, Satoskar et al. 1999). Later studies using MIF-deficient mice showed that these mice were also
protected from colitis (de Jong, Abadia-Molina et al. 2001). On the other hand, MIF is required for protection against *Leishmania major*, *Taenia crassiceps*, and *Toxoplasma gondii* infections (Satoskar, Bozza et al. 2001; Rodriguez-Sosa, Rosas et al. 2003; Flores, Saavedra et al. 2008). All the knockout studies showed a decreased ability of macrophages to produce TNF-α, IL-1β, and IL-6 (Riedemann, Guo et al. 2003). Taken together, the data confirm MIF’s function as a proinflammatory cytokine: in the absence of MIF mice are protected from diseases in which the immune system is a key mediator, whereas in diseases where the immune system is required for protection, deficiency of MIF increases susceptibility.

It stands to reason that MIF has a critical function in the pathogenesis of autoimmune diseases. A unique cytokine that is upstream of many other inflammatory cytokines, MIF may play a key regulatory role in inflammation and additionally may also function as a chemokine. Increased levels of MIF have been associated with several autoimmune diseases, including diabetes, rheumatoid arthritis, celiac disease, and MS (Niino, Ogata et al. 2000; Morand, Leech et al. 2002; Radstake, Sweep et al. 2005; Herder, Kolb et al. 2006; Rueda, Nunez et al. 2006; Herder, Klopp et al. 2008). This has been confirmed in mouse models in which the absence or blockade of MIF leads to decreased severity of experimental models of diabetes, arthritis, and EAE (Santos, Hall et al. 2001; Denkinger, Denkinger et al. 2003; Powell, Papenfuss et al. 2005; Stosic-Grujicic, Stojanovic et al. 2008). New insights into the mechanisms of MIF action during neuroinflammation could lead to new therapeutic targets for MS patients.
1.8 Objectives

MS represents one of the most complex autoimmune diseases. Though much has been learned about its pathology and factors that increase risk for developing MS, new therapies have been slow to arrive. The most recently approved drug is natalizumab, but cases of PML in several patients have put the future of this drug in question. One of the largest obstacles facing new drug discovery is identifying a therapy that slows the progression of MS without creating a globally immunosuppressive environment. Part of the difficulty has been that no single cytokine has been identified which alone can either explain or prevent ongoing MS. Thus, the focus of this project has been to better understand the underlying mechanisms that lead to neuroinflammation.

MIF is one of the earliest known markers of immune cell activation. Its conservation across a large number of species and its role in a number of inflammatory processes suggest that MIF is an important early regulator of inflammation. A handful of studies have correlated MIF with disease progression in EAE and MS. The objectives of this thesis are to determine the role of MIF in EAE using genetically deficient mice, to identify the cellular source of biologically relevant MIF, and to identify potential mechanisms of protection. This project culminates in the identification of a new therapeutic inhibitor of MIF that could represent a novel pharmaceutical therapy for MS.
CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

Age-matched male and female C57Bl/6 and SJL mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and housed five per unit in standard ventilated cages. Male C57Bl/6x129 mice were purchased from Taconic (Germantown, New York, USA). Male and female C57Bl/6 recombination activating gene (RAG) 2 knockout mice (B6.129S6-Rag2<sup>tm1Fwa</sup>) were purchased from Taconic and utilized in bone marrow chimera studies. Mouse strains lacking the MIF gene (B6;129S4-Mif<sup>tm1Dvd</sup>) were developed as previously described (Bozza, Satoskar et al. 1999) and extensively backcrossed onto C57Bl/6, and C57Bl/6 RAG<sup>−/−</sup> backgrounds.

All mice were allowed a seven-day acclimation period prior to any experimental procedures and were between six and eight weeks of age at the time of use. Mice were cared for according to policies established by The Ohio State University and housed in vivariums at Graves Hall and the Biomedical Research Tower. Mice were maintained on a 12-hour light/dark cycle with lights on at 0600 h and off at 1800 h. Food and water were given <i>ad libitum</i>. Mice were
randomly assigned to experimental groups and ear tagged for identification purposes.

2.2 Antigens

Myelin oligodendrocyte glycoprotein (MOG) 35-55 (MEVGWYRSPF SRVHVHLRYNGK) peptide used for the induction of EAE in C57Bl/6 mice was purchased from Princeton Biomolecules Corporation (Langhorne, Pennsylvania, USA). This peptide was purified by high performance liquid chromatography (HPLC) and had a purity of greater than 90 percent. For the induction of EAE in SJL mice, proteolipid protein (PLP) 139-151 (HCLGKWLGHPDKF) peptide was purchased from Sigma-Genosys (The Woodlands, Texas, USA). Purity was greater than 90 percent.

2.3 Induction and Transfer of Experimental Autoimmune Encephalomyelitis

For the induction of EAE in C57Bl/6 mice, male and female animals were immunized with 200 µg MOG$_{35-55}$ peptide (Princeton Biomolecules) emulsified in complete Freund’s adjuvant (containing 200 µg Mycobacterium tuberculosis Jamaica strain), injected intradermally in each of four flanks. Pertussis toxin (List Biological Labs, Campbell, CA, USA) was injected as an additional adjuvant intraperitoneally (i.p.) on the day of immunization and 48 hours later (200 ng in 0.2 ml phosphate buffered saline). Female SJL mice were immunized with
150 µg PLP$_{139-151}$ peptide (Sigma-Genosys) emulsified in complete Freund’s adjuvant. Pertussis toxin was not used for the induction of EAE in SJL mice.

Prior to the induction of EAE in RAG$^{-/-}$ mice, CD4$^+$ T lymphocytes, purified from splenocytes of naïve male wild type or MIF$^{-/-}$ C57Bl/6 mice, were transferred into naïve RAG$^{-/-}$ recipients. T lymphocytes were purified using a negative selection magnetic bead kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Single cell suspensions were depleted of non-CD4 cells using anti-CD8α, -CD11b, -CD45R, -CD49b, and -Ter-119 monoclonal antibodies directly conjugated to biotin. Anti-biotin microbeads were used to magnetically separate the antibody-labeled cells. Purity following depletion, determined by flow cytometry, was greater than 95 percent, and 2x10$^6$ cells were transferred into male recipient RAG$^{-/-}$ mice by intravenous (i.v.) injection. Recipient mice were immunized for EAE as described above with MOG$_{35-55}$ peptide on the day of T lymphocyte transfer.

For some experiments, EAE was adoptively transferred following in vitro culture of splenocytes. Donor male wild type or MIF$^{-/-}$ C57Bl/6 mice were actively immunized as described above with MOG$_{35-55}$ peptide and adjuvants. Ten days following immunization, spleens and draining lymph node cells from the sites of injection (inguinal, axillary, brachial, cervical, popliteal, and periaortic) were harvested, dissociated into a single cell suspension, and placed into culture for 72 hours with RPMI 1640 medium (containing 10 percent fetal bovine serum, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 5 x $10^{-5}$ M 2-ME) with 20 µg/ml MOG$_{35-55}$ peptide and 0.5
ng/ml IL-12p70 (BD Biosciences, San Jose, California, USA). Following culture, cells were washed twice in phosphate buffered saline (PBS), and $10 \times 10^6$ cells were injected i.v. or i.p. into male recipient mice. The route of cell transfer did not affect the severity or incidence of EAE.

All animals were observed daily for EAE clinical signs and scored according to degree of paralysis; 0 = no paralysis, 1 = limp tail or ataxia, 2 = limp tail with ataxia, 3 = partial hind limb paralysis, 4 = complete hind limb paralysis, and 5 = death. Cumulative disease index (CDI) was calculated as the sum of daily clinical scores from each animal during the course of observation and reported as an average within each group. Peak score was reported as the average maximum clinical score within each group over the observed period. Additional outcome measures included EAE incidence and mortality and were calculated based on individual animals reported as a mean within each group.

2.4 Gonadectomy

Male wild type and MIF$^{-/-}$ C57Bl/6 mice were anesthetized and the testes removed as follows: A small (5 mm) median incision was made through the scrotum, followed by another small (2.5 mm) incision through the scrotal sacs. The spermatic blood vessels were ligated and testes excised. Mice were allowed one week to recover before immunization for EAE. For sham surgeries, scrotal incisions were made but the spermatic blood vessels were not ligated and the testes were not excised.
2.5 Bone Marrow Chimeras

Male donor wild type or MIF<sup>-/-</sup> C57Bl/6 mice were euthanized, and the femur, tibia, and humerus from each mouse were collected aseptically. Bone marrow was flushed from the medullary cavity with supplemented RPMI 1640 using an 18-gauge needle and syringe. The immune system of recipient C57Bl/6 RAG<sup>-/-</sup> or MIF<sup>-/-</sup>RAG<sup>-/-</sup> mice was ablated by irradiation using a Gammarcell GC40 irradiator (MDS Norion, Ottawa, ON, Canada) at 350 rads, followed by a second dose of 350 rads two hours later. A single cell suspension of 10x10<sup>6</sup> bone marrow cells in PBS was transferred i.v. into recipient mice immediately following irradiation. Water was supplemented with 0.2 percent enrofloxacin (Baytril, Shawnee Mission, Kansas, USA) to prevent infection for two weeks following irradiation.

To verify immune reconstitution, at six weeks post-irradiation, blood was drawn from the submandibular vessel bed using a sterile lancet. Blood was analyzed by flow cytometry for the presence of CD4<sup>+</sup> T and CD19<sup>+</sup> B lymphocytes.

2.6 Drug Administration

For the administration of flutamide and mifepristone, pellets were implanted in wild type and MIF<sup>-/-</sup> C57Bl/6 mice. Animals were anesthetized and 60-day release flutamide (37.5 mg), mifepristone (75 mg), or placebo pellets
(Innovative Research of America, Sarasota, Florida, USA) were implanted subcutaneously over the shoulder blades. Mice were allowed to recover seven days before immunization for EAE. Alternatively, mifepristone was injected daily subcutaneously (s.c.). Mifepristone (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in 10 percent sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich) in polyethylene glycol (PEG) 400 (Sigma-Aldrich). A total of 50 µl was injected s.c. at a dose of 50 mg/kg per mouse per day.

Several small molecule inhibitors of MIF (gift from Cytokine PharmaSciences, King of Prussia, Pennsylvania, USA) were administered to mice prior to or following induction of EAE. Inhibitors were given either i.p. or orally (p.o.) for 10 to 21 days. CPSI-2705 and CPSI-1306 inhibited tautomerase assay as previously described (Aroca, Solano et al. 1991) at concentrations ranging from 1 to 10 µM. For i.p. administration of inhibitors, drug was dissolved in sterile DMSO, and then diluted in PBS for an overall ratio of 1:3 (DMSO to PBS). The concentration of inhibitor dissolved was based on overall dosing requirements: 10, 25, or 50 mg/kg. The total volume administered was 50 µl of 25 percent DMSO in PBS. Orally administered inhibitor was dissolved in 15 percent DMSO in 0.1 percent methylcellulose in water. Mice were fed by gavage a total of 50 µl at a concentration of inhibitor equal to 0.01, 0.1, 1, 10, or 25 mg/kg. Vehicle controls received 15 percent DMSO in 0.1 percent methylcellulose and were included in all experiments. The time of day of inhibitor administration was kept constant, between 1000 h and 1200 h.
Dexamethasone was administered as a positive therapeutic control. Preparations were given i.p. daily. Dexamethasone was dissolved in DMSO and diluted with PBS to a ratio of 1:3 (DMSO to PBS). The overall volume given to each mouse was 50 µl at a dose of 10 mg/kg. Vehicle controls were given the same preparation without dexamethasone at a ratio of 1:3 (DMSO to PBS).

2.7 Inhibitor Assays

Brains and serum were collected from mice receiving MIF inhibitors orally for five days. To collect serum, mice were anesthetized and blood drawn from the retro-orbital sinus using heparinized Natelson blood collecting tubes. Samples were centrifuged at 10,000 g for 15 minutes and collected serum was stored at -20°C. Analysis was performed by Cytokine PharmaSciences.

Proteins from samples of serum or brain homogenates were precipitated from the mixture by the addition of 0.25 mL acetonitrile. Inhibitors of MIF were extracted from the resulting supernatant by an on line extraction. This was followed by conventional liquid chromatography and analysis on a tandem mass spectrometer equipped with a heated nebulizer ion source. Calibration was performed using a standard stock solution of 1 mg/mL CPSI-1306 or CPSI-2705 in methanol. Standard spiking solutions of inhibitor in methanol/water at concentrations of 1000 ng/mL and 100 ng/mL were prepared by dilution of the stock standard solution. The appropriate amount of standard spiking solution was added to 100 mL of 5 percent bovine serum albumin (BSA) in 0.01M PBS (pH 7.4) to prepare calibration standards at the following concentrations: 5
ng/dL, 10 ng/dL, 20 ng/dL, 50 ng/dL, 100 ng/dL, 200 ng/dL, 500 ng/dL, 1000 ng/dL, and 2000 ng/dL. The standards were processed with the sample preparation procedure described above. The standard stock solution and the standard spiking solutions were stored at -20 °C.

HPLC analysis was performed using the Transcend TLX-2 System (Thermo Scientific, San Jose, CA). Samples were injected onto a 4 x 2 mm C18 Guard cartridge that served as an extraction column. Analyte was directly transferred from the extraction column and focused onto the 33 x 4.6 mm analytical column that was packed with particles three microns in diameter. The loading and eluting mobile phases A were water. Loading phase B was methanol, and loading phase C was a solution containing 45 percent acetonitrile, 45 percent isopropanol, and 10 percent acetone which was used to wash the extraction column. The eluting Mobile phase B started at 100 percent water and via a linear gradient over a 45 second period shifted to 100 percent methanol. MS/MS analysis was carried out on a TSQ Quantum Ultra triple stage quadrupole mass spectrometer with an atmospheric pressure chemical ionization (APCI) probe (Thermo Scientific, San Jose, CA).

2.8 Hormone and IgE Assays

Serum was collected as described previously from anesthetized mice. Levels of testosterone and corticosterone were measured by EIA according to the manufacturer’s instructions (Assay Designs, Ann Arbor, Michigan, USA). Serum samples were diluted with assay buffer and plated on 96-well plates pre-coated
with antibodies directed against testosterone or corticosterone. Samples were incubated for two hours at room temperature, washed, and coated with a second antibody directly conjugated to alkaline phosphatase. Following a second incubation for one hour, plates were washed and coated with p-nitrophenyl phosphate. Serum levels of immunoglobulin E (IgE) were also measured by EIA (BD Biosciences). Ninety-six well ELISA plates were coated with anti-mouse IgE antibody and incubated overnight. The next day, plates were washed and then blocked with 10 percent fetal bovine serum (FBS) in phosphate-buffered saline (PBS) for one hour. Samples were added and plates incubated for two hours at room temperature. The amount of IgE in each sample was assayed by adding detection antibody conjugated to biotin and visualized using streptavidin-horseradish peroxidase. Both hormone and IgE levels were measured using a SPECTRAplus ELISA reader and quantified against a standard curve by SOFTmax PRO software (Molecular Devices, Sunnyvale, CA, USA).

2.9 Flow Cytometry

Single cell suspensions derived from draining lymph nodes at the sites of injection and spleens were stained with anti-MHC Class II, -B220, -CD3, -CD4, -CD8, -CD11b, -CD11c, -CD19, -CD25, -CD31, -CD44, -CD45RB, -CD54, -CD62L, -CD69, -CD74, or -CD106 fluorescein isothiocyanate (FITC)-, phycoerythrin(PE)-, allophyco-cyanin (APC)-, or pacific blue-conjugated fluorescent antibodies (BD Biosciences). Isotype control monoclonal antibodies (BD Biosciences) were matched for each fluorochrome. Cells were labeled at $1 \times 10^6$ cells per tube,
incubated for 30 minutes at 4°C, and measured using a FACSCalibur flow cytometer (BD Biosciences). Analysis was performed using CellQuest Pro software (BD Biosciences). Alternatively, some experiments were performed on a FACSCanto II flow cytometer and analyzed using Diva software (both from BD Biosciences).

Intracellular expression of Foxp3 was measured using APC-labeled monoclonal antibodies and processed according to manufacturer’s instructions (eBioscience, San Diego, California, USA). Briefly, $1 \times 10^6$ cells were labeled for surface antigens, including CD4 and CD25, with directly conjugated fluorescent monoclonal antibodies. Following labeling, cells were permeabilized with fix/perm working solution and stained intracellularly for the transcription factor Foxp3 for 30 minutes at 4°C. Cells were washed twice with permeabilization buffer and analyzed using a FACSCalibur flow cytometer.

Measurement of intracellular cytokines, including IFN-γ, IL-10, and IL-17, was also performed by flow cytometry. Cells were harvested from lymph nodes and spleens of mice following EAE and pulsed for four hours with monensin in supplemented RPMI 1640 medium at 37°C and 5 percent CO$_2$. Cells were permeabilized and labeled according to the manufacturer’s instructions (eBioscience, Minneapolis, Minnesota, USA). Cells were first stained with surface monoclonal antibodies, including anti-CD4, -CD8, and -CD19, followed by intracellular cytokine staining. Populations of cells were measured on a FACSCalibur flow cytometer and analyzed using CellQuest Pro software.
2.10 Proliferation and Suppression Assays

Single cell suspensions of leukocytes from lymph nodes and spleens were cultured in supplemented RPMI 1640 with MOG\textsubscript{35-55} peptide (20 µg/ml), anti-CD3 antibody (2 µg/ml), or medium alone for 24, 48, or 72 hours at 37°C and 5 percent CO\textsubscript{2} in 96-well round-bottom plates (4 x 10\textsuperscript{5} cells/well). Cells were pulsed with \textsuperscript{3}H thymidine (1 µCi/ml) 12 hours prior to collection. Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Spatron, Sterling, Virginia, USA). Thymidine incorporation was measured using liquid scintillation on a Wallac Betaplate (LKB, Wallac, Maryland, USA) or a Perkin Elmer TopCount NXT luminescence counter with TopCount NXT software (Perkin Elmer, Waltham, Massachusetts, USA). The means of triplicate wells were determined and reported as counts per minute (CPM) for each group.

Alternatively, in some experiments CD25\textsuperscript{-} and CD25\textsuperscript{+} regulatory T lymphocytes were co-cultured with MOG\textsubscript{35-55} peptide (20 µg/ml), anti-CD3 antibody (µg/ml), or medium alone in supplemented RPMI 1640. For these experiments, lymphocytes from draining lymph nodes and spleens were collected 10 to 12 days following immunization and purified using magnetic bead purification (Miltenyi Biotec). Single cell suspensions were depleted of non-CD4 cells by labeling leukocytes with anti-CD8α, -CD11b, -CD45R, -CD49b, and -Ter-119 monoclonal antibodies directly conjugated to biotin. Anti-biotin
microbeads were used to magnetically separate the antibody-labeled cells. A second monoclonal antibody against CD25 and directly conjugated to PE was used to positively select CD4$^+$CD25$^+$ regulatory T cells. The purity following negative then positive selection was greater than 95 percent as determined by flow cytometry. The number of CD25$^+$ T lymphocytes was kept constant (1x10$^6$ cells/ml) and added to different concentrations of CD25$^+$ regulatory T lymphocytes (0.1, 0.5, 1, and 2x10$^6$ cells/ml) together with antigen presenting cells (3x10$^6$ cells/ml). C57Bl/6 splenocytes served as antigen presenting cells and were irradiated in a Gammacell GC40 irradiator (MDC Nordion) at 3000 rads prior to culture. Cells were cultured together in 96-well round bottom plates and pulsed with $^3$H thymidine 12 hours prior to harvest. After 72 hours, cells were harvested and measured for proliferation as described above.

2.11 Cytokine Assays

Single cell suspensions of leukocytes from lymph nodes and spleens were cultured in supplemented RPMI 1640 with MOG$_{35-55}$ peptide (20 $\mu$g / ml), anti-CD3 antibody (2 $\mu$g / ml) or medium alone for 48 or 72 hours at 37°C and 5 percent CO$_2$ in 96-well round-bottom plates (4 x 10$^5$ cells/well). Following culture, supernatants were harvested and evaluated for the presence of IL-10 and IFN-γ by EIA according to manufacturer’s instructions (BD Biosciences). Additionally, levels of IL-17 were measured by EIA (R&D Systems, Minneapolis, Minnesota, USA). For all kits, primary antibodies specific for the cytokine of interest were coated on 96-well ELISA plates overnight. Following incubation,
plates were washed and blocked with 0.1 percent BSA. Supernatants were added to the coated plates for two hours at room temperature, followed by the addition of a secondary antibody conjugated to horseradish peroxidase. Levels of cytokines were measured using a SPECTRAplus ELISA reader and quantified against a standard curve by SOFTmax PRO software (Molecular Devices). Results are represented as means of duplicate wells.

Additionally, levels of TNF-α, IFN-γ, IL-5, IL-4, and IL-2 were measured in culture supernatants by cytometric bead array (BD Biosciences). Inflammation and T\textsubscript{H1}/T\textsubscript{H2} kits from BD Biosciences were used according to the manufacturer’s instructions. Briefly, 50 µl of supernatant was cultured for two hours with antibody-coated beads specific for the cytokines of interest. Samples were measured with a BD FACSCalibur flow cytometer and analyzed by BD Cytometric Bead Array software.

2.12 Histopathologic Assessment

Immunohistochemical, hematoxylin and eosin (H&E), luxol fast blue with silver contrast, and Bielschowsky silver staining were carried out by The Ohio State University Veterinary Sciences core facility. Brains and spinal cords were removed at various time points following immunization and either frozen at -80°C in OCT media or fixed in 4 percent paraformaldehyde and paraffin embedded. In some experiments, animals were perfused first with cold 0.1M PBS, followed by fixation with 4 percent paraformaldehyde. H&E sections were graded by a blinded pathologist for mononuclear cell infiltration on a scale of
zero (no inflammation) to three (diffuse parenchymal infiltration). Immunohistochemical stains were labeled with anti-CD4, -CD8, or -F4/80 antibodies and evaluated by a blinded pathologist.

2.13 Endothelial Cell Expansion

Brains of adult wild type and MIF\textasciitilde/\textasciitilde C57Bl/6 mice were collected aseptically and processed for purification of microvascular endothelial cells as previously reported (Wu, Hofman et al. 2003). Briefly, brain tissues were homogenized in supplemented MCDB 131 medium (2 percent fetal bovine serum with 100 U/ml penicillin and 100 \( \mu \)g/ml streptomycin), followed by centrifugation in 15 percent dextran. The pellet was digested for six hours in 0.1 percent collagenase and dispase at 37\(^\circ\)C and 5 percent CO\(_2\). A final centrifugation in 45 percent isotonic Percoll separated purified endothelial cells, which were cultured on collagen-coated flasks in MCDB 131 (with 10 percent fetal bovine serum, 30 \( \mu \)g/ml endothelial cell growth supplement, 15 U/ml heparin, 325 \( \mu \)g/ml glutathione, 1 \( \mu \)l/ml 2-mercaptoethanol, 100 U/ml penicillin, and 100 \( \mu \)g/ml streptomycin). Cells were washed in fresh medium every two to three days.

\textit{In vitro} manipulations of the culture conditions were done 10 to 14 days following initial incubation. Cells were cultured for two to three hours in varying concentrations of TNF-\(\alpha\) (R&D Systems), recombinant mouse MIF (R&D Systems), or small molecule inhibitors of MIF (Cytokine PharmaSciences) before analysis by flow cytometry.
2.14 Statistical Analysis

All statistical references were made based on appropriate methods as outlined in the literature (Fleming, Bovaird et al. 2005). Statistical significance between groups for cumulative disease index, mean clinical score, and day of onset was calculated using the Students’ t test. Measures of lymphocyte populations, cytokine production, and proliferation also used the t test. Significance for incidence was calculated using a Chi-square. Significance was recorded as p<0.05.
CHAPTER 3

MIF IS A KEY MEDIATOR OF EAE

A number of studies have utilized animal models to determine the role of cytokines and chemokines in MS. From this early work, it was determined that IFN-γ, once thought to be a potential therapeutic target for MS, worsened disease in patients receiving exogenous protein (Panitch, Hirsch et al. 1987). Other cytokine and chemokine targets, including IL-17, have been identified in MS lesions but have yet to give rise to new therapies (Tzartos, Friese et al. 2008). In this chapter, we will explore the role of the inflammatory cytokine MIF in two mouse models of EAE and identify potential therapeutic strategies using small molecule inhibitors of MIF.

3.1 MIF Does Not Preferentially Expand Populations of Leukocytes

MIF has a number of reported roles in the regulation of inflammation. It was initially described as inhibitor of macrophage migration. Subsequent studies have identified MIF during lymphocyte and macrophage activation and proliferation. We evaluated whether MIF preferentially expanded specific populations of cells in naïve mice. We obtained mice in which part of the second
and the complete third exons of MIF had been deleted (Bozza, Satoskar et al. 1999). These mice were backcrossed multiple times onto the C57Bl/6 background.

Several previous reports suggested that MIF played a role in the establishment of both innate and adaptive immune responses. To evaluate whether deleting MIF creates an inherent defect in the immune system, we harvested lymph nodes and spleens from naïve, age-matched male MIF knockout and wild type mice and enumerated populations of lymphocytes and antigen presenting cells, both vital components of the immune response. We found comparable numbers of CD4+ and CD8+ T lymphocytes between groups in both lymph nodes and spleen (Figure 3.1). Populations of the key antigen presenting cells, macrophages (CD11b+) and dendritic cells (CD11c+), were similar in lymph nodes. The only statistically significant difference between groups was a decrease in B lymphocytes in the spleen of MIF knockout animals, but this difference was small. Populations of other antigen presenting cells in the spleen were similar. This led us to conclude that although MIF is an important cytokine in the activation of many leukocyte populations, its absence does not skew the overall distribution of immune cells in naïve mice. Activation, measured by the expression of surface molecules, was also unchanged between groups (data not shown). We could not identify differences in the populations of CD4+CD25+ regulatory lymphocytes in either naïve wild type or MIF knockout mice (data not shown).
An additional observation was made in MIF knockout mice on the C57Bl/6 background. Naïve, MIF knockout mice had significantly higher levels of serum IgE (Figure 3.2). IgE is an immunoglobulin associated with allergic responses and is linked to a $T_{H2}$ environment. We measured IgE after noting that some MIF knockout mice had what appeared to be allergic dermatitis. However, IgE was highly variable among MIF knockout mice suggesting it was not directly due to the MIF knockout background. The significance of this finding is unclear, but when IgE levels were compared with incidence or severity of EAE there was no correlation. No differences were observed between groups in other subtypes of immunoglobulin (data not shown).

3.2 MIF is Required for Susceptibility to EAE

Although there were no observed differences in the naïve immune system following deletion of MIF, we predicted that knockout animals would have reduced EAE. Previous reports had shown that antibody depletion of MIF reduced the severity of disease using both active induction and adoptive transfer of EAE (Denkinger, Denkinger et al. 2003). Furthermore, studies in our lab using another genetic knockout mouse strain, the C57Bl/6x129, showed that MIF was required for disease progression (Powell, Papenfuss et al. 2005). We sought to evaluate EAE in the MIF knockout on the C57Bl/6 background, a better model to assess EAE than the C57Bl6x129. We immunized age-matched, male MIF knockout and wild type control mice with MOG$_{35-55}$ peptide and adjuvants. Knockout mice showed less severe EAE relative to wild type controls (Figure
Furthermore, MIF knockout mice also had a lower incidence of EAE, reduced cumulative disease index, and a lower peak clinical score relative to controls (Table 3.1). Interestingly, the absence of MIF did not affect the day of onset, which was similar between groups. The severity of EAE in MIF knockout mice on the C57Bl/6 background was comparable to that of C57Bl/6x129 mice (data not shown).

We examined whether the presence of MIF promoted an inflammatory environment. Ten days following immunization, we collected lymph nodes draining the sites of injection and spleens from MIF knockout and wild type mice. The number of cells within the lymph nodes and spleens were similar between groups (data not shown). We assessed the ability of leukocytes to proliferate in response to the peptide used for immunization. There was a comparable proliferation to MOG$_{35-55}$ peptide after 72 hours as measured by $^3$H thymidine incorporation (Table 3.2). Proliferation to anti-CD3 antibody was similar between wild type and MIF knockout mice. Our data show that MIF is not required to generate antigen-specific responses to MOG peptide, and lymphocytes from MIF knockout mice do not have any significant defects in proliferation to antigen.

We also measured cytokine production by leukocytes from draining lymph nodes in both wild type and knockout mice. There was less IL-10 production from leukocytes of wild type mice versus knockout mice following 72 hours of stimulation in vitro with MOG$_{35-55}$ peptide. There was also a trend toward more IFN-γ, but IL-17 production was low in both groups. Thus, we
concluded that MIF did not enhance or hinder the ability of lymphocytes to produce cytokines in response to antigen, a key part of the pathogenesis of EAE. However, increases in IL-10 could suggest an anti-inflammatory environment.

Finally, we compared lymphocyte and antigen presenting cell populations following immunization. We found no differences in CD4+ or CD8+ T lymphocytes and CD19+ B lymphocytes. Populations of other antigen presenting cells were also comparable between groups. A striking difference was the elevation of CD4+CD25+ lymphocytes in MIF knockout mice. This population of cells can exert regulatory activity through the transcription of Foxp3 and secretion of IL-10. The greater numbers of these cells, plus the enhanced production of IL-10 in response to antigen in MIF-deficient lymphocytes, led us to hypothesize that this population of regulatory cells is negatively affected by MIF.

3.3 Functional Activity of Regulatory T Cells in MIF Deficient Mice

We further characterized the CD4+CD25+ population of T lymphocytes from immunized mice. These two markers are frequently found together on regulatory T lymphocytes, along with the transcription factor Foxp3. In EAE, regulatory cells can be protective because they inhibit proliferation of activated T lymphocytes in response to antigen stimulation. We found that wild type mice had a smaller population of CD4+CD25+ regulatory lymphocytes, as well as a smaller percentage of cells expressing Foxp3 (Figure 3.4A). We noted that within the CD4+CD25+ population of cells, more than 80 percent expressed Foxp3 in...
both groups (Figure 3.4B). We were concerned that the absence of MIF could decrease the function of regulatory cells. To determine the function of this population, we co-cultured varying ratios of wild type and MIF deficient CD4$^+$CD25$^+$ regulatory lymphocytes with autoreactive CD25$^-$ effector T cells. Regulatory T cells from MIF knockout mice were as effective at inhibiting proliferation as those from wild type animals on a per cell basis (Figure 3.4C). Thus, in the presence of MIF, populations of regulatory T cells were reduced, and in knockout mice these cells were as functional as wild type regulatory cells. This population of CD4$^+$CD25$^+$Foxp3$^+$ regulatory lymphocytes could be a critical component of protection against EAE, due to their increased numbers in the absence of MIF. Determining how MIF inhibits the differentiation of regulatory cells could be an important step in understanding MIF’s role in EAE.

3.4 A Small Molecule Inhibitor of MIF Reduces Ongoing EAE

All studies thus far explored EAE in mice genetically lacking MIF with wild type controls. The progression of EAE requires both the induction of the peripheral immune response and subsequent trafficking of autoreactive cells into the CNS. We explored whether administration of an inhibitor of MIF after onset of acute disease could reduce ongoing EAE. Through collaboration with Cytokine PharmaSciences, we used two small molecule inhibitors of MIF, CPSI-2705 and CPSI-1306, that disrupt the tertiary structure of MIF and reduce its biological activity. Beginning 17 days following immunization with MOG$^{35-55}$ peptide, we orally administered 1.0 mg/kg of CPSI-1306 daily for 21 days. Mice
receiving inhibitor had less severe EAE within three days after beginning treatment (Figure 3.5A). The cumulative disease index during the treatment period was lower in those mice receiving inhibitor, and the mean score between groups during this period was significantly lower (Table 3.3). We concluded that an inhibitor of MIF could be therapeutic during ongoing EAE. We also gave CPSI-1306 at multiple lower doses and found that the inhibitor was still therapeutic at 0.01 mg/kg (data not shown).

We also assessed the ability of an MIF inhibitor to reduce relapses in a second, relapsing-remitting model of EAE. Using SJL mice, we started CPSI-2705, a second MIF inhibitor, 23 days after immunization for EAE. This corresponded to the first remission of disease. We found that administration of an inhibitor prevented the onset of a second relapse of disease (Figure 3.5B). Mice receiving inhibitor had less severe clinical disease relative to vehicle treated mice and a lower mean clinical score during the treatment period (Table 3.3). These results show that MIF inhibitors can extend periods of remission and prevent the onset of new relapses.

We examined lymphocyte proliferation and cytokine production in response to antigen from splenocytes isolated after 21 days of inhibitor administration in C57Bl/6 mice. As in knockout mice following immunization, there was no inherent deficit in proliferation to MOG_{35-55} peptide or anti-CD3 antibody from leukocytes of inhibitor treated mice (Table 3.4). Administration of an MIF inhibitor reduced IFN-γ and IL-17, but these differences were small. IL-10 was not detected from either group of mice.
We observed that inhibitor-treated mice had a significantly greater proportion of both CD4$^+$ and CD8$^+$ T lymphocytes, while B lymphocytes were significantly reduced in inhibitor-treated mice. Populations of other antigen presenting cells, including dendritic cells and macrophages, were comparable. Notably, MIF inhibitor increased the population of CD4$^+$CD25$^+$ cells, supporting our observations in knockout mice (Figure 3.4).

Following inhibitor treatment, mice not only had a larger population of CD4$^+$CD25$^+$ lymphocytes but also a larger population of cells expressing Foxp3 (Figure 3.6A). Among the population of CD4$^+$CD25$^+$ double positive cells, in both groups the transcription marker Foxp3 was expressed in more than 75 percent of cells (Figure 3.6B). We believe that an inhibitor of MIF may allow the expansion of regulatory T cells, which could reduce clinical severity. Further investigations on the function of these regulatory cells and where they are located is needed to tell us more about the role of regulatory T lymphocytes during recovery from EAE.

3.5 MIF Functions Through Its Ligand-Binding Activity

The absence of MIF activity, whether through genetic deletion or pharmacologic inhibition, had a profound effect on the progression of EAE. However, the mechanism by which MIF functions, including its biological activity in vivo, remains unknown. MIF has two distinct activities: first characterized was an enzymatic activity, although a biological substrate has not been identified (Rosengren, Bucala et al. 1996; Kleemann, Kapurniotu et al. 1998).
MIF’s other activity is through noncognate binding with CD74, CXCR2, and CXCR4 (Bernhagen, Krohn et al. 2007; Weber, Kraemer et al. 2008). Although it is unclear how MIF signals intracellularly, several chemokine functions have been attributed to MIF released from sites of inflammation.

We utilized two additional inhibitors from Cytokine PharmaSciences to determine which activity was relevant in EAE. CPSI-0112 selectively interrupts the tertiary structure of MIF but is a much poorer inhibitor of enzymatic activity. CPSI-0202 specifically inhibits the tautomerase activity of MIF. We immunized wild type C57Bl/6 mice with MOG\textsubscript{35-55} peptide and administered both compounds, in addition to a vehicle control, 17 days after immunization. We found that only the ligand-binding inhibitor was able to reduce the severity of EAE (Figure 3.7). Mice receiving CPSI-0112 had a lower CDI than vehicle-treated mice (5.9 versus 10.6). Administration of the enzymatic inhibitor had no effect on disease course compared with vehicle control (CDI of 10.3). We believe that during EAE, MIF functions exclusively through binding with ligands, including CD74, and other potential biological ligands that may have not been identified yet. The enzymatic activity, regardless of its substrate, does not appear to be important in the progression of EAE.

3.6 Conclusions

MIF levels were previously found to be elevated in the CSF of MS patients during relapse and the CNS of mice with EAE (Niino, Ogata et al. 2000; Gao, Wang et al. 2008). We have shown here that lack of MIF does not substantially
bias any specific population of leukocytes in naïve knockout mice. Following induction of EAE, MIF deficient mice have profoundly reduced EAE and demonstrate a larger population of the CD4⁺CD25⁺Foxp3⁺ regulatory T cells. This population of cells was shown to inhibit the proliferation of effector T lymphocytes equal to that of wild type regulatory cells. When cultured in vitro following immunization, both wild type and MIF knockout lymphocytes proliferated to antigen and produced inflammatory cytokines. IL-10 was slightly higher in knockout mice, supporting a larger regulatory population of cells.

These data have been corroborated using a small molecule inhibitor of MIF. We have shown that an inhibitor reduces the severity of ongoing EAE and expands regulatory T lymphocytes. There was no change in proliferation to antigen or cytokine production in inhibitor-treated mice. Furthermore, we have determined that the ligand-binding function of MIF is required for progression for EAE. Although certain biological functions may be mediated by the enzymatic activity of MIF, they do not appear important during EAE.

We have shown MIF is a critical mediator of EAE. MIF appears to inhibit the differentiation of the regulatory population of lymphocytes, which may prevent wild type C57Bl/6 from recovering from EAE. Interestingly, despite a larger population of regulatory cells in MIF knockout and inhibitor-treated mice, there were no differences in the peripheral response to antigen. This strongly suggests regulatory lymphocytes are most active in other anatomical areas, particularly the CNS. Whether these cells are expanded in the lymph nodes and traffic to the CNS or are produced locally still needs to be determined.
Given MIF’s ubiquitous expression, the relevant source of MIF during EAE is critical to understanding its function. Pinpointing the expression of MIF to a certain cell type could help elucidate mechanisms in which MIF mediates inflammation. We will next discuss approaches in which we limited the production of MIF to specific cellular lineages in order to learn more about MIF’s function.
Figure 3.1: The genetic deletion of MIF does not cause a shift in the populations of T lymphocytes. Lymph nodes and spleens were collected from naïve age-matched male wild type and MIF knockout mice. Despite the absence of MIF, there were no significant differences in the majority of populations of T lymphocytes or antigen presenting cells. There was a slight reduction in CD19⁺ B lymphocytes in the spleens of MIF knockout animals (n=3 per group).

*p<0.05
Figure 3.2: Mice lacking MIF have higher serum levels of IgE. Blood from naïve wild type and MIF-deficient mice was collected and analyzed for IgE by ELISA. MIF knockout mice had greater serum levels of IgE (n=3 per group).

*p<0.05
Figure 3.3: The genetic deletion of MIF is protective against EAE. Wild type (◆) and MIF knockout (□) mice were immunized for EAE with 200 µg MOG 35-55 peptide in adjuvant. MIF knockout mice had less severe EAE relative to wild-type controls. Data are representative of four separate experiments (n=18 in C57Bl/6 group, n=13 in MIF-/- group).
**Table 3.1**: MIF knockout mice have reduced incidence and severity of EAE. Wild type and MIF knockout mice were immunized with MOG 35-55 peptide in adjuvant.

<table>
<thead>
<tr>
<th></th>
<th>INCIDENCE</th>
<th>ONSET&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CDI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PEAK SCORE&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>15/18 (83%)</td>
<td>15.9±4.6</td>
<td>24.8±18.1</td>
<td>2.2±1.3</td>
</tr>
<tr>
<td>MIF-/-</td>
<td>7/13 (54%)**</td>
<td>14.6±2.3</td>
<td>6.4±8.1**</td>
<td>0.9±1.0**</td>
</tr>
</tbody>
</table>

<sup>a</sup> Day of onset was calculated as the mean of the first day of clinical scores among mice that developed EAE, ±SD.

<sup>b</sup>Cumulative disease index (CDI) was calculated as the sum of clinical scores over the duration of disease per animal and averaged within each group, ±SD.

<sup>c</sup>Peak score was measured over the duration of disease per animal and averaged, ±SD.

**p<0.01**
Table 3.2: Lymphocytes from MIF knockout mice respond to antigen stimulation. Draining lymph nodes from wild type and MIF knockout animals were collected 10 days post-immunization and cultured in vitro for 72 hours with MOG 35-55 peptide or anti-CD3 antibody. Proliferation to antigen was similar between both groups. Cytokine production was also similar and measured after culture with MOG 35-55 peptide. Alternatively, single cell suspensions of draining lymph nodes were analyzed by flow cytometry. Knockout mice had an elevation in the regulatory CD4^+CD25^+ population of T lymphocytes (n=3 per group).
Figure 3.4: Mice lacking MIF have a larger population of regulatory cells that are functional. Draining lymph nodes from wild type and MIF knockout animals were collected 10 days post-immunization. (A) Knockout mice had an elevation in regulatory CD4+CD25+ population of T lymphocytes and Foxp3 positive cells. (B) Of those lymphocytes that were CD4+CD25+ greater than 80 percent were Foxp3 positive (n=3 per group). (C) CD25 positive and CD25 negative cells were co-cultured to assess inhibition of proliferation by regulatory T lymphocytes. All CD25 negative cells were wild type, but regulatory lymphocytes were either wild type (●) or MIF knockout (□). Regulatory lymphocytes were capable of inhibiting proliferation in the absence of MIF.

*p<0.05
Figure 3.5: A small molecule inhibitor of MIF reduces disease severity in two animal models of EAE. (A) Male C57Bl/6 mice were injected with MOG 35-55 peptide and monitored for clinical signs. At 17 days post-immunization, mice in the inhibitor-treated group (♦, n=18) were fed daily 1.0 mg/kg CPSI-1306. Vehicle controls (♦, n=19) were fed 15 percent DMSO in 0.5 percent methylcellulose. Those mice fed inhibitor had reduced severity of disease relative to vehicle controls. (B) Relapsing-remitting EAE was induced in female SJL mice with PLP 139-151 peptide. Following the first remission, 23 days post-immunization, mice were given CPSI-2705 (○, n=7). Vehicle controls were given 15 percent DMSO in 0.5 percent methylcellulose (♦, n=9). Administration of an MIF inhibitor reduced relapses and severity of EAE.
Table 3.3: MIF Inhibitor-treated mice have reduced severity of EAE. C57Bl/6 mice were immunized with MOG 35-55 peptide and given inhibitor beginning 17 days after immunization. SJL mice were immunized with PLP 139-151 peptide and given inhibitor 23 days after immunization. *Cumulative disease index (CDI) was calculated as the sum of clinical scores over the period of treatment per animal and averaged within each group, ±SD. †Mean score was measured over the duration of treatment per animal and averaged within each group, ±SD. *p<0.05

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>CDIa</th>
<th>MEAN SCOREb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C57Bl/6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEHICLE</td>
<td>18</td>
<td>22.5±18.6</td>
<td>1.1±1.0</td>
</tr>
<tr>
<td>1306</td>
<td>19</td>
<td>12.9±14.9</td>
<td>0.6±0.7*</td>
</tr>
<tr>
<td><strong>SJL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEHICLE</td>
<td>9</td>
<td>28.3±10.9</td>
<td>2.4±0.9</td>
</tr>
<tr>
<td>2705</td>
<td>7</td>
<td>13.9±14.5*</td>
<td>1.2±1.2*</td>
</tr>
</tbody>
</table>
Table 3.4: Lymphocytes from inhibitor-treated mice are able to respond to antigen. Spleens from animals treated with an inhibitor of MIF or vehicle control were collected 36 days post-immunization and cultured in vitro for 72 hours with MOG 35-55 peptide or anti-CD3 antibody. Proliferation to antigen was similar between both groups. Cytokine production was also similar and measured after culture with MOG 35-55 peptide. Alternatively, single cell suspensions of draining lymph nodes were analyzed by flow cytometry. Inhibitor-treated mice had an elevation in the regulatory CD4⁺CD25⁺ population of T lymphocytes (n=3 per group).

ND=not detected
*p<0.05
**p<0.01
Figure 3.6: A small molecule inhibitor of MIF expands a regulatory population of T lymphocytes. C57Bl/6 mice were immunized with MOG 35-55 peptide. Draining lymph nodes were collected following administration of MIF inhibitor or vehicle control for 21 days. (A) Mice receiving an inhibitor of MIF had a larger population of CD4^+ CD25^+ lymphocytes and a higher overall expression of Foxp3. (B) Among the population of CD4^+ CD25^+ cells, the expression of Foxp3 was present in more than 75 percent of cells (n=3 per group).

*p<0.05

**p<0.01
Figure 3.7: The ligand-binding function of MIF is critical for disease progression. Male C57Bl/6 mice were induced for EAE with MOG 35-55 peptide. Groups of mice were given either an enzymatic (□) or ligand-binding (△) inhibitor of MIF at 17 days following immunization. Relative to administration of vehicle (♦) or an enzymatic inhibitor, mice receiving the ligand-binding inhibitor of MIF had less severe EAE (n=10 per group).
CHAPTER 4

MYELOID CELLS ARE A NECESSARY SOURCE OF MIF

When MIF was initially described in 1966, it was characterized as an inflammatory protein produced by activated T lymphocytes (David 1966). Since then, MIF has been linked to most cell types, including macrophages, endothelial cells, and cells of the pituitary gland (Calandra, Bernhagen et al. 1994; Bacher, Meinhardt et al. 1998; Nishihira, Koyama et al. 1998). MIF exhibits two distinct and dichotomous functions: it acts as a hormone, present at detectable levels in the serum of even healthy adults (Bruchfeld, Carrero et al. 2009). It also has autocrine and paracrine functions, i.e. it can activate cells in close proximity with or even the cells that produced it (Lue, Thiele et al. 2007).

We have shown that MIF is involved in the progression of EAE. MIF may play a role in the differentiation of regulatory T lymphocytes, but we noted that lymph node and splenic lymphocyte responses to antigen are intact in MIF knockout mice. This suggests these regulatory cells are more active in other areas. It is a possibility that regulatory cells expanded in the absence of MIF may traffic to sites of inflammation, including the CNS. It is also plausible that MIF
production by specific cell types within the CNS directs the inhibition of regulatory cells. An absence of MIF in these areas would allow the expansion of CD4⁺CD25⁺Foxp3⁺ T lymphocytes. Local activity of regulatory T cells in the CNS would reduce clinical scores, as we observed in MIF knockout and inhibitor-treated mice.

In this chapter, we discuss the relevant cellular source of MIF during EAE. Identifying the source of MIF would not only help to elucidate the function of MIF during EAE, but also shed new light on the mechanisms by which MIF mediates inflammation.

4.1 Expression of MIF from Bone Marrow-Derived Cells is Required for EAE

The bone marrow is the source of nearly all immune cell populations relevant to EAE, including lymphocytes and macrophages. Like other cells in the body, cells derived from the bone marrow are capable of producing MIF upon activation. In order to determine whether MIF necessary for EAE was originating from cells of the bone marrow, we created bone marrow chimeric mice to restrict the expression of MIF to certain cell types. We used this technique to create mice in which bone marrow-derived cells did not express MIF, while all other cells in the mouse were capable of MIF expression. We utilized RAG knockout and MIF /RAG double knockout mice for bone marrow recipients. RAG knockout mice lack the recombination activating gene (rag), which is responsible for rearrangement of the immunoglobulin and T cell
receptor genes. Mice lacking the RAG gene do not possess B or T lymphocytes. Utilization of RAG knockout mice as recipients ensured that lymphocytes were derived solely from donor bone marrow following reconstitution.

RAG knockout mice which had been reconstituted with MIF knockout bone marrow were immunized for EAE with MOG\textsubscript{35-55} peptide. We found that the absence of MIF in the bone marrow significantly reduced susceptibility to EAE (Figure 4.1A). Mice reconstituted with MIF knockout bone marrow had a lower incidence than mice reconstituted with wild type bone marrow (28.6% versus 71.4% in WT->WT mice, p<0.05). The severity of EAE was also reduced (CDI of 4.7 versus 18.9 in WT->WT mice). We concluded from this experiment that the expression of MIF by bone marrow-derived cells was critical for the induction and progression of EAE. These cells include erythrocytes, macrophages, B lymphocytes, and T lymphocytes, which were one of the first cell types implicated in the pathogenesis of EAE and MS.

We also created bone marrow chimeric mice in which MIF knockout mice were reconstituted with wild type bone marrow. We found that these mice were much more susceptible to EAE than knockout controls (Figure 4.1B). The incidence of EAE was significantly higher than in knockout controls reconstituted with knockout bone marrow (75.0% versus 33.3% in KO->KO mice, p<0.01). In these mice, MIF was completely absent from the CNS and all other non-immune cells of the periphery. However, reconstitution of the bone marrow with cells producing MIF restored susceptibility to EAE in the C57Bl/6 mice.
The results of these experiments suggest that bone marrow-derived cells are a critical source of MIF during EAE (Table 4.1). MIF from other sources including the pituitary gland and additional endocrine organs is not required. Interestingly, MIF expression from other cell types was not sufficient for EAE, even if MIF was only absent from bone marrow-derived cells. This strongly suggests that MIF is a local factor in inflammation produced by a specific cell type at the site of damage. We next focused on determining the cell type within the bone marrow that was the likely source of MIF during EAE.

4.2 Lymphocytes Alone Lacking MIF are not Sufficient to Transfer EAE

T lymphocytes were one of the early cell types linked to the production of MIF (Bloom and Bennett 1966; David 1966). In order to determine whether MIF from lymphocytes alone was sufficient for the induction of EAE, we utilized an adoptive transfer system. Wild type and MIF knockout C57Bl/6 mice were immunized for EAE with MOG\textsubscript{35-55} peptide. Lymph nodes draining the sites of injection and spleens were harvested 10 days following immunization. Single cell suspensions of total lymphocytes were cultured in vitro for 72 hours with antigen. Cultured lymphocytes were transferred into naïve wild type or MIF knockout C57Bl/6 recipients. We found that donor lymphocytes from wild type mice were unable to transfer EAE to MIF deficient recipients (Figure 4.2A). The incidence of disease in these recipients was substantially reduced (25.0% versus 73.7% in wild type lymphocytes into wild type recipients, p<0.01). We concluded that although wild type lymphocytes are encephalitogenic in wild
type recipients, MIF knockout recipient mice lacked critical factors determining EAE susceptibility. T lymphocytes produce MIF upon activation, yet this MIF was insufficient to induce EAE when MIF was absent from the environment.

Reciprocal experiments with knockout lymphocytes into wild type and knockout recipients yielded expected results. Lymphocytes lacking MIF could induce EAE if recipient mice expressed MIF (Figure 4.2B). The incidence of EAE in wild type mice receiving knockout lymphocytes was greater than the control adoptive transfer (47.6% versus 31.3% in knockout mice receiving knockout lymphocytes, p<0.05). Overall, the incidence and severity of EAE were reduced in this series of experiments. This was likely due to reduced EAE in donor knockout mice in which the lymphocytes for adoptive transfer were expanded. However, the differences between wild type and knockout recipients were clear following adoptive transfer of MIF knockout lymphocytes.

In all the above experiments, the MIF knockout recipient mouse was universally protected. The cells of these mice that lack MIF include all of the cell types of the CNS, both those bone marrow- and resident-derived, as well as cells of the periphery, including all other cells of the recipient mouse (Table 4.2). This strengthens earlier experiments in which we showed that very specific cell types produce MIF during EAE, even though MIF is ubiquitously expressed. We have shown here that MIF knockout mice lack critical mechanisms for the induction of EAE. Furthermore, MIF from lymphocytes alone is not sufficient to restore these mechanisms.
4.3 Conclusions

Determining the cellular source of MIF enables better understanding of the mechanisms and function of MIF during EAE. With the use of chimeric mice, we determined that bone marrow derived cells, as opposed to other somatic cells, are a critical source of MIF for the development of EAE. Subsequent experiments ruled out lymphocytes as potential sources of MIF. Adoptively transferred lymphocytes from immunized knockout mice failed to induce EAE in wild type recipients.

These experiments taken together lead us to exclude bone marrow-derived lymphoid cells as the relevant source of MIF during EAE. While these cells may in fact produce MIF, MIF is not sufficient to induce adoptively transferred EAE. Myeloid cells are the other major lineage of immune cells derived from the bone marrow. Our data suggests that myeloid cells are the critical source of MIF during EAE. Myeloid cells are found in the brain and spinal cord. This lineage of cells includes macrophages and dendritic cells, two populations of cells that have known roles in the pathogenesis of MS and EAE. In the CNS, bone marrow-derived monocytes can differentiate into microglia. This has important relevance for our studies, as microglia are the major antigen presenting cells in the CNS. Monocytes can also differentiate into macrophages, which reside in the perivascular space within the CNS and direct migration and antigen presentation. They also have a critical role in both EAE and MS.
Any of the above mentioned cells, dendritic cells, macrophages, or microglia, could be the critical source of MIF during EAE. Determining which one(s) is a critical step in understanding the mechanism of MIF. At this point, we have not identified this cell type. Current work is focused on isolating distinct populations of myeloid-derived cells and studying their importance during EAE. Other work has given us new insights on the function of MIF. The remainder of this thesis will center on additional aspects of MIF, including supplementary mechanisms by which MIF directs the progression of EAE.
Figure 4.1: The expression of MIF by bone marrow-derived cells is required for progression of EAE. Wild type and MIF-deficient RAG knockout mice were irradiated and reconstituted with wild type or MIF-deficient bone marrow. Six weeks later, mice were immunized with MOG 35-55 peptide. (A) MIF produced from bone marrow-derived cells was sufficient for the induction and progression of EAE (O), but knockout bone marrow in wild type recipients greatly reduced susceptibility to EAE (●). (B) Wild type bone marrow increase susceptibility to EAE in knockout recipient mice (△). Data are representative of two separate experiments (n=5 per group per experiment).
Table 4.1: The expression of MIF from myeloid and lymphoid cells was sufficient for the induction of EAE. Bone marrow chimeric mice expressing MIF from bone marrow cells were susceptible to EAE.
Figure 4.2: Lymphocytes alone expressing MIF are not sufficient to induce EAE. Following 72 hours of *in vitro* reactivation, 10x10⁶ splenocytes from wild type or MIF-deficient C57Bl/6 donor mice were adoptively transferred into naïve recipient wild type or MIF knockout mice. (A) Wild type lymphocytes were unable to induce EAE if MIF was absent in recipient mice (△). (B) Lymphocytes from MIF knockout mice could induce EAE in wild type recipient mice (●). Data are representative of four separate experiments (n=5 per group per experiment).
<table>
<thead>
<tr>
<th>BONE MARROW</th>
<th>CNS</th>
<th>MYELOID</th>
<th>LYMPHOID</th>
<th>EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT→WT</td>
<td>MIF</td>
<td>MIF</td>
<td>MIF</td>
<td>YES</td>
</tr>
<tr>
<td>KO→WT</td>
<td>MIF</td>
<td>MIF</td>
<td>NO MIF</td>
<td>YES</td>
</tr>
<tr>
<td>WT→KO</td>
<td>NO MIF</td>
<td>NO MIF</td>
<td>MIF</td>
<td>NO</td>
</tr>
<tr>
<td>KO→KO</td>
<td>NO MIF</td>
<td>NO MIF</td>
<td>NO MIF</td>
<td>NO</td>
</tr>
</tbody>
</table>

Table 4.2: MIF is required in recipient mice for the induction of EAE by adoptive transfer. Expression of MIF from CNS and myeloid cells was required for adoptive transfer of EAE by activated leukocytes.
CHAPTER 5

MIF REGULATES HORMONE HOMEOSTASIS

We have utilized MIF knockout mice to show that MIF is not required for the induction of EAE. Using an adoptive transfer model, we showed that lymphocytes from MIF deficient mice could induce EAE in wild type recipients. We also proposed a mechanism by which MIF knockout mice are protected after the induction of EAE. We showed that MIF knockout and inhibitor-treated mice had larger numbers of CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes, a population of cells that is protective during EAE. However, these cells were only expanded after induction of EAE. They were not present at the time of immunization. Thus, other mechanisms of protection must be present in naïve animals. We explored other factors that could reduce susceptibility in MIF knockout mice.

One known function of MIF is as an antagonist to the glucocorticoid family of hormones (Calandra, Bernhagen et al. 1995; Santos, Hall et al. 2001). Glucocorticoids are potent anti-inflammatorys, and MIF is unique in that glucocorticoids stimulate its synthesis. MIF blocks some intracellular signaling pathways and stimulates others, hindering glucocorticoid inhibition of AP-1 and
NF-κB (Aeberli, Leech et al. 2006). Activation of transcription events downstream of AP-1 and NF-κB stimulates inflammation in leukocytes. Glucocorticoids are commonly used in MS patients to manage exacerbations. High levels of MIF could impede their therapeutic benefit.

MIF may also play a role in the homeostasis of other immunomodulatory hormones, including testosterone. MIF has been localized to Leydig cells within the testis, and MIF expression is high in other endocrine organs (Meinhardt, Bacher et al. 1996). Testosterone is currently being explored as a topical treatment for MS. This chapter will evaluate MIF’s role in the function of corticosterone, the predominant glucocorticoid hormone in mice, and testosterone during the progression of EAE.

5.1 MIF Reduces Serum Levels of Corticosterone and Testosterone

Earlier experiments with naïve C57Bl/6 mice showed that MIF knockout mice had similar populations of T and B lymphocytes, as well as other leukocytes including dendritic cells and macrophages. However, it remained that MIF knockout mice were protected after induction of EAE. This was despite similar proliferation and cytokine responses to immunizing antigen. We have shown that wild type mice have fewer regulatory T lymphocytes, and in MIF knockout mice these cells are expanded following immunization. We explored whether other factors could explain why MIF knockout mice had reduced susceptibility to EAE.
We measured serum levels of corticosterone and testosterone in naïve wild type and MIF knockout C57Bl/6 mice. Both are known immunosuppressive hormones. We found higher serum levels of both corticosterone and testosterone in MIF knockout mice. Corticosterone was almost 50 percent higher in MIF-deficient mice compared to wild type mice (Figure 5.1A). This is likely due to MIF’s antagonistic action against glucocorticoids. Genetic deletion of MIF removed an important inhibitory feedback mechanism that facilitated the increase in corticosterone. High levels of an anti-inflammatory hormone like corticosterone could be protective during the induction of EAE.

Testosterone was significantly increased in MIF knockout mice. We observed a four-fold increase in basal testosterone levels in naïve knockout mice compared to wild type controls (Figure 5.1B). Testosterone is an important immunoregulatory hormone, but MIF is not a known suppressor of testosterone production. However, MIF is produced in the pituitary gland and could play a role in the regulation of leutinizing and follicle stimulating hormones (LH and FSH, respectively). Both hormones are important for testosterone synthesis in the Leydig cells of the testis. The extent of MIF’s regulation of these hormones has not yet been examined and is not the focus of these studies. Instead, the rest of this chapter will discuss the role of corticosterone and testosterone in MIF knockout mice in determining susceptibility to EAE.

Naïve MIF knockout mice had elevations in two immunosuppressive hormones: corticosterone and testosterone. MIF knockout mice were also
resistant to the induction of EAE. We hypothesized that the higher basal levels of these two hormones could be a mechanism of protection in MIF knockout mice.

5.2 Inhibition of Corticosterone Does Not Increase the Severity of EAE

Previous data in both MS and EAE show that glucocorticoids are immunosuppressive. We predicted that the higher levels of corticosterone in MIF knockout mice at the time of immunization could prevent the onset of EAE. We used mifepristone (RU486), an antagonist of the glucocorticoid receptor, to inhibit the anti-inflammatory effects of corticosterone prior to the induction of EAE. Slow release pellets of mifepristone were implanted in wild type and MIF knockout mice. Mice were allowed to recover seven days and then immunized for EAE with MOG35−55 peptide and adjuvants. We found that mifepristone reduced the severity and delayed the onset of EAE in wild type mice (Figure 5.2). Wild type mice receiving mifepristone had a reduced cumulative disease index (14.0 versus 24.9 in vehicle-treated mice, p<0.05). The incidence of EAE between groups was similar (80% versus 66.7% in mifepristone-treated mice).

Knockout mice had generally less severe disease regardless of treatment (Figure 5.2). MIF knockouts receiving mifepristone had a lower incidence of disease (50.0% versus 100% in vehicle control mice). However, the administration of mifepristone did not affect the progression of EAE in knockout animals. Both groups in which MIF was absent failed to develop significant
clinical scores (CDI of 6.3 versus 4.3 in mifepristone-treated knockout mice). Thus, mifepristone had a minimum effect in these animals.

Interestingly, the administration of a glucocorticoid antagonist reduced severity of EAE in wild type mice. Since glucocorticoids are immunosuppressive hormones, we predicted mifepristone would enhance disease. In knockout mice, mifepristone reduced the incidence of EAE but had little effect on progression. Mifepristone inhibits the glucocorticoid receptor but also competitively blocks progesterone at the progesterone receptor and has a weak affinity for the androgen receptor. Because mifepristone delayed the onset of EAE in wild type mice and reduced incidence in MIF knockouts, we believe the drug may be functioning through other pathways. Determining the mechanism in which mifepristone reduced severity of EAE in wild type and MIF knockout animals needs additional exploration.

Although our results with corticosterone contradicted what we had observed previously in C57Bl/6x129 mice, we also noted an elevation in a second hormone, testosterone, in MIF knockout mice. We explored whether testosterone had a role in susceptibility to EAE.

5.3 MIF Antagonizes Testosterone and Reduces Incidence of EAE

Testosterone has long been of interest in MS as women are three times as likely as men to be diagnosed with MS (Anderson, Ellenberg et al. 1992). Furthermore, 24 percent of men with MS have significantly lower serum levels of
testosterone (Wei and Lightman 1997). These studies have led to new clinical trials with testosterone, the results of which are still being explored.

We observed that MIF knockout mice had four-fold higher levels of testosterone. We predicted that high serum testosterone at the time of immunization could block important mechanisms of susceptibility. We used surgical gonadectomy to reduce serum testosterone in animals prior to the induction of EAE. The testes of wild type C57Bl/6 and MIF knockout mice were removed, and seven days later animals were immunized for EAE. A subset of mice received sham surgery. Gonadectomy significantly increased the severity of EAE in wild type C57Bl/6 mice (Figure 5.3), supporting other studies that have shown testosterone is protective. Gonadectomized wild type mice had a larger CDI and higher peak score (103.2 versus 59.9, and 3.5 versus 2.6, respectively, p<0.05 for both).

Gonadectomy did not increase the severity of disease in knockout mice (CDI of 26.7 versus 23.8 in sham surgery mice, Figure 5.3). However, gonadectomy significantly increased the incidence of EAE (100% versus 33.3% in sham surgery mice, p<0.01). The incidence in gonadectomized knockout mice was equal to that of sham surgery or gonadectomized wild type mice (100% in both sham and gonadectomized wild type mice, p<0.01). Testosterone reduced incidence of EAE in knockout mice but did not reduce the cumulative disease index or affect the progression of EAE (Table 5.1).

These gonadectomy studies confirm that testosterone has a significant immunosuppressive effect. In MIF knockout mice, testosterone is highly
The increase in testosterone protects MIF-deficient mice from induction of EAE. However, without testosterone, MIF knockout mice have an incidence of EAE approaching wild type animals. There was no effect on total cumulative disease index. This leads us to conclude that high levels of testosterone in the absence of MIF are highly protective during antigen sensitization. Gonadectomized mice had a higher incidence of EAE but no more severe disease. We also conclude that testosterone alone cannot further reduce the severity of disease in MIF knockout mice. These data strongly suggest additional mechanisms of protection in MIF knockout mice.

5.4 An MIF Inhibitor Did Not Increase Corticosterone or Testosterone

To further characterize the effects of MIF on hormone regulation, we looked at corticosterone and testosterone in inhibitor-treated mice following EAE induction. Studies in knockout mice showed that corticosterone was not required for protection from EAE. An inhibitor of corticosterone, mifepristone, had a negligible effect on the severity of disease. However, mifepristone blocks a number of hormone receptors and could be a confounding factor in these studies. Testosterone reduced severity of disease in wild type mice but had no significant effect on severity in MIF knockout mice. Testosterone did, however, reduce the incidence of EAE in MIF knockout mice. From these studies, testosterone appeared to play a critical role in disease induction. It was still unclear whether either hormone had a role in prolonged protection, and data suggested neither corticosterone nor testosterone were the primary mediators of protection.
We used an inhibitor of MIF, CPSI-2705, to treat wild type mice following the acute phase of EAE. We tested whether inhibitor treatment caused an elevation in either corticosterone or testosterone. Treatment with an inhibitor profoundly reduced clinical scores, but only small increases in both corticosterone and testosterone were observed after 21 days of inhibitor administration (Figure 5.4). We expected that if either hormone had a role in reducing clinical scores, we would observe significant elevations in serum levels. We can conclude from these studies that neither hormone has a significant role in recovery from EAE following inhibitor administration. However, inhibition of MIF over longer periods of time may have a more significant effect.

5.5 Conclusions

MIF has known regulatory functions with regard to glucocorticoid activity. Following deletion of MIF, we noted elevations in corticosterone, the major glucocorticoid hormone in mice. Blockade of the glucocorticoid receptor reduced severity of EAE in wild type mice but had no effect in knockout animals. Mifepristone was surprisingly therapeutic, despite being antagonistic for the glucocorticoid receptor. However, mifepristone has significant cross reactivity with other nuclear hormone receptors, suggesting we may have unintentionally inhibited other pathways. Glucocorticoids deserve future study, as MS patients frequently will be prescribed a member of the glucocorticoid family to reduce exacerbations. Since glucocorticoids themselves stimulate MIF production, an
inhibitor of MIF could be synergistic with glucocorticoid therapy. The combination warrants further research.

We also observed elevations in testosterone, a second immunosuppressive hormone. Testosterone protected mice and gonadectomy worsened clinical scores. However, gonadectomy in knockout mice produced little change in clinical scores yet significantly increased the incidence of EAE. These studies suggest that testosterone plays a lesser role in progression of EAE but has a larger role in the susceptibility for developing EAE. These are important data to consider for future pharmacological therapies in multiple sclerosis patients. Most men with MS have lower basal levels of testosterone relative to healthy males. Although we saw no significant increase in testosterone after 21 days of inhibitor administration, long-term treatment could elevate testosterone in these patients and create secondary benefits. More studies are needed to evaluate the long-term influence of MIF inhibitors on testosterone levels.

Though the effects of mifepristone are still unclear, it appears from these studies that testosterone is critical during the induction of disease. MIF, which is upregulated during inflammation but also constitutively expressed at low levels, may decrease serum testosterone, increasing susceptibility to disease. Other disease models show that MIF reduces testosterone during inflammation (Morales-Montor, Baig et al. 2002). In our model, we did not note significant decreases in testosterone following EAE, but male MS patients are more likely to be low in testosterone. While other factors mediated by MIF may be more important for disease susceptibility, like inhibition of regulatory lymphocytes,
testosterone could be a secondary factor that is therapeutically relevant. The remainder of this thesis will explore additional mechanisms in which MIF mediates susceptibility to EAE.
Figure 5.1: MIF deficient mice have elevated levels of testosterone. Blood was drawn from naïve wild type and MIF knockout C57Bl/6 mice. (A) Analysis by ELISA showed MIF deficient mice had elevated levels of the immunosuppressive hormone corticosterone. (B) MIF knockout mice had significantly elevated levels of testosterone (n=3 per group). $^{**}p<0.01$
Figure 5.2: Administration of a corticosterone inhibitor reduced severity of EAE in MIF knockout animals. Wild type and MIF-deficient mice were immunized for EAE with MOG 35-55 peptide following implantation of slow release pellets of mifepristone (RU486) or vehicle control. Mifepristone reduced the severity of EAE in both wild type and MIF knockout mice. Data are representative of three separate experiments (n=5 per group per experiment).
Figure 5.3: Gonadectomy of MIF-deficient mice did not increase severity of disease. Wild type and MIF-deficient mice were immunized for EAE with MOG 35-55 peptide following gonadectomy. Wild type mice had significantly worse EAE following gonadectomy, but EAE in MIF knockout mice did not change significantly following gonadectomy. However, gonadectomy increased the incidence of EAE in MIF knockout mice (n=5 per group).
<table>
<thead>
<tr>
<th>INCIDENCE</th>
<th>CDI⁹</th>
<th>PEAK SCORE⁹⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI/6 SHAM</td>
<td>5/5  (100%)</td>
<td>59.9±38.8</td>
</tr>
<tr>
<td>BI/6 GONAD</td>
<td>5/5  (100%)</td>
<td>103.2±18.2*</td>
</tr>
<tr>
<td>MIF/- SHAM</td>
<td>1/3  (33%)</td>
<td>23.8±41.3</td>
</tr>
<tr>
<td>MIF/- GONAD</td>
<td>5/5  (100%)**</td>
<td>26.7±20.4</td>
</tr>
</tbody>
</table>

Table 5.1: Gonadectomy increases the incidence of EAE in MIF knockout mice. Wild type and MIF knockout mice were gonadectomized or received sham surgery. Seven days later, mice were immunized for EAE with MOG 35-55 peptide.

⁹ Cumulative disease index (CDI) was calculated as the sum of clinical scores over the duration of disease per animal and averaged within each group, ±SD.

⁹⁺ Peak score was also measured over the duration of disease per animal and averaged, ±SD.

* p<0.05, vs. SHAM control

** p<0.01, vs. SHAM control
Figure 5.4: An inhibitor of MIF did not increase serum corticosterone or testosterone. Wild type C57Bl/6 mice were immunized for EAE with MOG 35-55 peptide. At 17 days post-immunization, mice were treated for 21 days with MIF inhibitor CPSI-2705. There was no significant elevation in either corticosterone or testosterone following treatment (n=3).
A number of cytokines and chemokines mediate the progression of EAE in MS. We have shown that MIF is required for induction of EAE and progression of chronic disease. Despite profoundly reduced clinical signs, there were no differences peripherally in either proliferation or cytokine production in MIF knockout or inhibitor-treated mice. We determined that myeloid cells are the source of macrophage migration inhibitory factor and a small molecule inhibitor of MIF is therapeutic during chronic EAE. However, while MIF deficient mice have elevated serum levels of corticosterone and testosterone, they do not contribute to the progression of disease. Testosterone may protect mice from EAE induction, but testosterone levels do not increase following 21 days of MIF inhibitor treatment. Another mechanism of protection must be present.

Thus far we have shown that inhibition of MIF protects against EAE. We have proposed two separate mechanisms by which MIF may mediate susceptibility: inhibition of regulatory T lymphocytes and negative regulation of testosterone. Both factors are important in regulating the function of autoreactive leukocytes. However, experimental autoimmune encephalomyelitis
has two distinct parts: first an induction phase occurs during which a peripheral immune response is generated by injection of myelin antigens. The second component of EAE is responsible for clinical signs. Autoreactive cells generated during the induction phase migrate into the CNS and damage the myelin sheath. Gaining access to the brain and spinal cord is critical for this step of EAE pathogenesis.

We have shown that MIF is not required for the peripheral immune response during EAE. Lymphocytes from MIF knockout animals produce inflammatory cytokines in response to antigen and are capable of inducing EAE following adoptive transfer into wild type recipients. This chapter will focus on the next step in disease pathogenesis: migration into the central nervous system.

6.1 MIF Knockout Mice Have Reduced Mononuclear Infiltration

To evaluate whether MIF was required for leukocyte migration, we immunized wild type and MIF-deficient C57Bl/6 mice with MOG\textsubscript{35-55} peptide. After 17 days, we evaluated the CNS for the presence of inflammatory infiltrates. We found reduced perivascular infiltration in MIF knockout mice (Figure 6.1A). In wild type mice, there was a considerable presence of inflammatory cells in the perivascular cuffs of blood vessels in the cerebellum. However, knockout mice had significantly less inflammation (Figure 6.1C). Given the identical peripheral response in both groups, this strongly suggested that MIF is required for migration into the CNS.
We also examined damage in the brain following immunization. Again, wild type and MIF-deficient C57Bl/6 mice were immunized for EAE. After 35 days, damage was assessed by luxol fast blue staining with a silver counterstain. Luxol fast blue measures the presence of myelin, which was reduced in wild type mice (Figure 6.1B). Wild type mice also developed enhanced axonal severing, appearing as numerous retraction bulbs along axons. Knockout mice, on the other hand, had much less demyelination and axonal severing. This correlated with reduced clinical severity observed in knockout mice. Together with data from peripheral lymphocytes, these data suggest MIF not only prevents migration into the CNS but also subsequent neuronal damage.

These data suggest a third mechanism of MIF mediating susceptibility to EAE. We have previously described MIF’s ability to prevent expansion of regulatory T lymphocytes. We also propose MIF inhibits production of testosterone. However, these factors alone are not known to inhibit migration or extravasation from the vasculature. Here we show that MIF knockout mice have fewer inflammatory infiltrates within the brain and spinal cord. MIF likely plays a role in the trafficking and transendothelial migration of leukocytes, including T lymphocytes and macrophages. To test whether MIF is required for migration, we utilized the small molecule inhibitors of MIF.

6.2 An Inhibitor of MIF Prevents Ongoing Migration

We have previously shown that an MIF inhibitor could reduce ongoing EAE, but it was unknown whether an inhibitor could reverse or even slow
ongoing migration into the CNS. Following antigen presentation in the CNS, leukocytes release inflammatory cytokines that upregulate adhesion molecules on the blood brain barrier. This allows trafficking by additional leukocytes into the brain and spinal cord. Although MIF inhibitors reduce clinical severity, it is unclear whether they reduce migration.

We immunized wild type C57Bl/6 mice with MOG\textsubscript{35-55} peptide and started administering CPSI-2705 at 17 days post-immunization. Following 21 days of drug administration, we harvested brains and spinal cords to evaluate the degree of mononuclear infiltration. We found few differences in the spinal cords of vehicle and inhibitor-treated mice (Figure 6.2A and B). This was unexpected as MIF knockout mice have very few perivascular infiltrates in both the spinal cord and brain. However, inhibitor was not given until after the acute phase of EAE, when inflammation had already begun in the spinal cord. From these studies, it appears inhibition of MIF does not reduce ongoing inflammation in the spinal cord.

Analysis of brain tissues revealed comparatively few infiltrates in inhibitor treated mice relative to vehicle controls (Figure 6.2C and D). This suggests an MIF inhibitor is able to inhibit future migration into subsequent tissues, including the brain. Further study showed that F4/80\textsuperscript{+} macrophages were specifically prevented from entering the brain (Figure 6.2E and F). This strongly supports earlier studies about the sequence of events in EAE. T lymphocytes are early arrivers in the brain and spinal cord. Following reactivation, they recruit other leukocytes, including macrophages, to the site of
inflammation. Treatment with CPSI-2705 stopped the migration of additional leukocytes into the CNS.

Our data here support that MIF is a critical mediator of migration. Other mechanisms we have identified, including regulatory T cells and testosterone, are present following inhibitor treatment but less pronounced. Here, an inhibitor of MIF significantly prevented new inflammation in the brain. This suggests the MIF inhibitor acts quickly following administration and halts migration into new inflammatory sites. We next explored whether an inhibitor could prevent the onset of EAE if given before the appearance of clinical signs.

### 6.3 MIF is Not Required for the Induction of EAE

Initial experiments with the C57Bl/6x129 strain of mice in which MIF had been deleted showed a similar acute phase of disease with wild type C57Bl/6x129 mice (Powell, Papenfuss et al. 2005). However, chronic EAE was severely reduced relative to wild type mice. Our own data showed mice lacking MIF had both reduced incidence and clinical severity of EAE. While we could show an inhibitor of MIF reduced ongoing EAE and leukocyte migration, we were interested in whether pretreatment with the MIF inhibitor could prevent or reduce the severity of subsequent EAE. We treated C57Bl/6 mice with an inhibitor of MIF for 10 days following the induction of EAE. We ended the treatment period before the expected onset of clinical signs.

Following treatment, we observed no change in disease course between groups (Figure 6.3). The day of disease onset was similar (13.2 versus 14.2 in
vehicle-treated mice). Interestingly, the severity of disease was slightly worse in mice pretreated with MIF inhibitor, although the difference was not statistically significant (CDI of 44.0 versus 36.0 in vehicle-treated mice). These data confirm previous experiments that showed MIF is not required for the induction of EAE. However, administration of inhibitor following the onset of EAE supports that MIF expression is critical for disease progression, likely through mediation of leukocyte trafficking into the brain and spinal cord.

The efficacy of any pharmaceutical intervention depends not only on the mechanism of action of the drug but also its availability at the site of disease. We evaluated the presence of CPSI-2705 in the serum and brain after five days of oral administration. We found significant amounts of inhibitor in both tissues (Figure 6.4). However, there was more inhibitor present in the brain of mice with EAE than those mice that were healthy controls. This strongly suggests patency of the BBB improves availability of MIF inhibitor in the CNS tissues. This could imply CPSI-2705 was not available in the CNS when administered as a pretreatment. Further work evaluating MIF inhibitors in the brain and spinal cord before the onset of EAE is needed.

6.4 Conclusions

MIF knockout mice are protected from EAE. Following immunization, these mice have significantly fewer perivascular infiltrates and less neuronal damage measured by myelin staining and the degree of axon severing. Administration of an inhibitor of MIF has comparable results. However,
inhibition of MIF did not reverse prior inflammation nor could it prevent the onset of EAE when administered during the induction phase. An inhibitor of MIF did prevent new infiltration in the brain and reduced clinical signs. This strongly suggests MIF could be a regulator of leukocyte migration.

We have described here several mechanisms of protection in MIF knockout and MIF inhibitor-treated mice. The importance of each appears to depend largely on the timing of disease. Testosterone is elevated early in naïve MIF knockout mice and may play a significant role during the induction of disease. Following immunization, MIF knockout and inhibitor-treated mice have a larger population of regulatory T cells. These CD4+CD25+Foxp3+ regulatory lymphocytes have important suppressive functions that are critical for recovery following inhibition of MIF. We now propose an inhibitor of MIF also reduces ongoing migration, suggesting a third mechanism in which MIF controls the susceptibility and progression of EAE and MS. MIF has been documented to be a potent inflammatory cytokine. We propose that MIF has important migratory functions, in addition to other studies that show MIF acts as a short-range chemokine. Thus, MIF likely plays a significant role in the extravasation of leukocytes during acute and chronic EAE through a number of different mechanisms. The therapeutic efficacy of the MIF inhibitors during EAE suggests they could be novel regulators of disease progression in MS.
Figure 6.1: The absence of MIF prevents infiltration into the CNS. At 17 days following immunization with MOG 35-55 peptide, brains were taken from wild type and MIF-deficient animals. (A) Hematoxylin and eosin (H&E) staining of brain sections showed less perivascular infiltration in MIF knockout mice versus wild type controls. (B) At 35 days post-immunization, luxol fast blue with silver counterstain revealed more axonal degeneration in wild type mice versus MIF knockouts. (C) Inflammation was significantly reduced in MIF-deficient mice, as graded by a blinded pathologist (n=5 per group). Results are representative of three separate experiments.

*p<0.05
Figure 6.2: An inhibitor of MIF reduced ongoing migration. Male C57Bl/6 mice were induced for EAE with MOG 35-55 peptide and monitored for clinical signs. At 17 days post-induction, mice in the inhibitor-treated group were fed daily 1.0 mg/kg CPSI-1306. Vehicle controls were fed a dose of 15 percent DMSO in 0.5 percent methylcellulose. (A,B) There was no difference in infiltration by H&E stain at the end of treatment period in the spinal cord. (C,D) Inhibitor treated mice had significantly less infiltration in the brain. (E,F) F4/80-stained macrophages were not identified in inhibitor treated mice.
Figure 6.3: A small molecule inhibitor does not prevent the onset of EAE. A small molecule inhibitor of MIF was administrated 10 days following the induction of EAE with MOG 35-55 peptide. Inhibitor did not prevent the onset of EAE or lessen the severity of disease (n=8 per group).
Figure 6.4: A small molecule inhibitor of MIF is present in the brain. Following five days of administration of CPSI-2705, blood and brain homogenates were collected from mice in which some were immunized for EAE with MOG 35-55 peptide. Inhibitor was present in both the plasma and brain of mice (n=4 per group).
Autoimmune diseases result from complex processes in which the immune system attacks the body, confusing the distinction between self and non-self. MS is a debilitating autoimmune disease that affects nearly 2.1 million individuals worldwide. Many of the key mediators of MS pathogenesis have been actively investigated as therapeutic targets but, at present, no single factor such as an individual cytokine or chemokine has been identified which alone can slow progression of disease. Most treatments have focused on managing the inflammatory response, and nearly all are immunosuppressive. More effective therapies must target the complete pathogenesis of MS, while keeping the fundamental role of the immune system intact. Targeting a specific cytokine that mediates an inflammatory response represents a more focused therapy than global immunosuppression.

One such specific cytokine is MIF, a cytokine widely conserved across many species that plays a role in balancing inflammation with suppression and regulation of an immune response. The expression of MIF has been implicated in a number of diseases, many autoimmune. In MS, concentration of MIF is
elevated in the CSF of patients during an exacerbation of disease, suggesting a role in progression (Niino, Ogata et al. 2000). Studies in animal models of MS strongly support that MIF has a critical inflammatory role. One report showed higher levels of MIF in the CNS of mice following EAE, and another described the use of anti-MIF antibodies to inhibit disease (Denkinger, Denkinger et al. 2003; Gao, Wang et al. 2008). Many of our own observations described here corroborated these studies and suggest the underlying importance of MIF in regulating the inflammatory response. The summation of this work is that MIF is a required cytokine for the progression of EAE and may play a significant role in the pathogenesis of MS.

The mechanisms by which MS develops are still unclear but a number of factors have been correlated with the development of disease. MS occurs in genetically susceptible individuals following unknown environmental exposures. Autoreactive T cells, identified even in normal individuals, become activated during MS. The BBB, whose function is to limit migration into the brain, is overwhelmed as activated lymphocytes enter the brain and spinal cord at accelerated rates. These cells, and others recruited to the lesions, begin the inflammatory cascade that produces the symptoms of MS. Regulatory T lymphocytes, which normally inhibit antigen-specific proliferation, are reduced in MS, and pathogenic T cells are allowed to mediate the autoimmune attack unchecked. Numerous cytokines and chemokines accumulate in the CNS, damaging myelin and exposing axons underneath. Long-term demyelination causes irreversible axonal severing with subsequent retraction.
Immunosuppressive therapies have sought to delay the progression of inflammation, but no therapies have been able to restore neuronal connections that are lost during inflammation.

In order to better understand the role of MIF in the pathogenesis of MS and EAE, we utilized mice lacking MIF on a C57Bl/6 background, a mouse strain that is susceptible to EAE and commonly used to model MS. Mice lacking MIF are significantly less susceptible to EAE induction. This suggests that MIF plays an important role in influencing susceptibility in these mice to developing EAE. To better understand how MIF knockout mice were protected, we evaluated knockout animals for the presence of important cellular mediators of EAE, including B and T lymphocytes, dendritic cells, and macrophages. We concluded from flow cytometric analyses that there were no significant differences in the number of these cells in knockout mice compared to wild type C57Bl/6 mice. MIF knockout mice had comparable percentages of CD4+ and CD8+ lymphocytes and a reduction in CD19+ B lymphocytes in the spleen. Other antigen presenting populations of cells, including dendritic cells and macrophages, were similar. Activation of lymphocytes, dendritic cells, and macrophages was comparable as measured by the expression of surface molecules. Based on our studies, MIF does not appear to be required for differentiation of lymphocytes or antigen presenting cells and is not a mechanism by which MIF increases susceptibility to EAE. Given that relative numbers of immune cells were not altered in MIF knockout mice, we wanted to
determine whether functional differences may account for the decreased susceptibility in MIF deficient mice.

Phenotypically, the relative numbers of leukocytes from peripheral lymph nodes and the spleen did not differ between wild type and knockout animals either before or following induction of EAE. Proliferation in response to antigen was similar between MIF knockout mice and controls. MIF inhibitor-treated mice also had no differences in proliferation suggesting that lack of T cell activation was not a mechanism protecting these mice. In MIF knockout and inhibitor-treated mice, there was a trend for less IFN-γ and IL-17 compared to control mice. Although these two inflammatory cytokines were increased in wild type mice, the differences were not large enough to explain clinical scores. Other factors must explain clinical observations in knockout mice.

We noted wild type mice had reduced levels of IL-10, a potentially protective cytokine during EAE and MS. IL-10 can be produced by CD19+ B lymphocytes, of which there were similar numbers between wild type and knockout mice following immunization. Additionally, IL-10 can be produced by CD4+CD25+Foxp3+ regulatory T lymphocytes. Regulatory lymphocytes suppress proliferation and effector function through a number of mechanisms, including cell-to-cell contact with activated lymphocytes and antigen presenting cells and release of immunosuppressive cytokines like IL-10, IL-35, and TGF-β (Sakaguchi, Yamaguchi et al. 2008). Both wild type and MIF knockout mice had CD4+CD25+Foxp3+ regulatory cells following immunization, but in knockout and inhibitor-treated mice, these cells were significantly expanded.
The distinction in the number of Foxp3+ cells among wild type mice and MIF knockout or inhibitor-treated mice partially explain differences in susceptibility and clinical score. Prior to immunization, we could not identify significant numbers of regulatory T cells in either wild type or MIF knockout mice, suggesting they were expanded only after EAE induction. This would imply less of a function for these cells during induction of EAE. However, within several days after immunization, MIF knockout mice had more CD4+CD25+Foxp3+ regulatory cells and a higher production of IL-10. We believe the absence of MIF allowed the expansion of regulatory lymphocytes, which inhibited the progression of EAE. On the other hand, wild type mice had less IL-10 and fewer CD4+CD25+Foxp3+ regulatory lymphocytes that correlated with disease progression. We predict increases in the concentration of MIF during EAE inhibited the expansion of regulatory cells in wild type mice. The regulatory T lymphocytes from both wild type and MIF knockout mice were equally capable of in vitro inhibition, suggesting no defect in function in either group of animals. The higher number of regulatory cells in knockout mice, combined with observed suppression in vitro, demonstrates a likely mechanism of protection. An important point of discussion is the anatomical location of these cells during EAE.

The organs in which these regulatory cells reside appear to change over the course of disease. As early as 10 days after immunization, significant numbers of CD4+CD25+Foxp3+ regulatory T lymphocytes were found in the lymph nodes draining the sites of injection. Late in disease, significant numbers
were found in the spleen. As these regulatory cells migrate from the lymph nodes into the blood and are captured by the spleen, we predict a large number of regulatory cells would also be found within the brain and spinal cord. Antigen-specific inhibition of proliferation and effector function in the CNS would significantly decrease proliferation to myelin antigens in knockout mice and be protective. At this point, we can identify very few lymphocytes in the CNS of knockout mice following EAE, however this could be an important factor of protection. Evaluating the CNS for the presence of CD4⁺CD25⁺Foxp3⁺ regulatory T lymphocytes in MIF knockout mice warrants further investigation.

CD4⁺CD25⁺Foxp3⁺ regulatory T cells transferred into EAE recipients reduce disease severity, confirming their ability to mediate the inflammatory environment (McGeachy, Stephens et al. 2005). They also play a role in susceptibility to MS (Viglietta, Baecher-Allan et al. 2004). In our model, CD4⁺CD25⁺Foxp3⁺ cells were relatively absent prior to the induction of EAE, suggesting that the absence of these cells facilitates the induction of disease. CD4⁺CD25⁺Foxp3⁺ regulatory cells were expanded, particularly in knockout mice, following immunization, as well as after administration of an MIF inhibitor, and the increase in the number of these cells corresponded with disease recovery. An increase in the population of these regulatory cells would be protective against EAE. Given our observations, it is important to consider how an MIF inhibitor could cause differentiation of a regulatory T lymphocyte population.
Past studies show MIF increases expression of IL-6 (Bozza, Satoskar et al. 1999). Co-expression of IL-6 with TGF-β expands the T\textsubscript{H}17 population of cells, a subtype of lymphocytes known to mediate several inflammatory diseases. During the induction of EAE and MS, we believe MIF directs production of IL-6 with TGF-β and mediates the proliferation of IL-17-producing lymphocytes. These, and other encephalitogenic cells, can migrate into the CNS and mediate myelin damage. Previous reports have shown MIF can specifically expand T\textsubscript{H}17 lymphocytes (Stojanovic, Cvjetanicin et al. 2009). While driving the expansion of T\textsubscript{H}17 cells, large production of IL-6 inhibits the differentiation of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T cells. The absence of MIF in knockout mice protects this population of regulatory cells.

TGF-β preferentially expands peripherally derived CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T lymphocytes. The induction of EAE, coupled with large increases in cytokine production, drives the expression of TGF-β. Without IL-6, TGF-β causes the differentiation of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{+} regulatory T cells. These cells are produced in the lymph nodes, enter other lymphatic tissues like the spleen, migrate to the CNS, and inhibit proliferation and effector functions of encephalitogenic T cells. The induction of TGF-β production following EAE would help explain why regulatory T cells only appear during the chronic phase of EAE and how they could mediate late suppression. We also anticipate comparable changes in the cytokine milieu following administration of an MIF inhibitor. By inhibiting expression of MIF, IL-6 is reduced, shifting lymphocytes from an encephalitogenic to regulatory population. Thus, in both knockout mice
and following inhibitor administration, MIF’s ability to expand encephalitogenic lymphocytes is severely impaired and protection from EAE is observed.

We made our observations in an animal model of MS, but our data suggest MIF could inhibit CD4⁺CD25⁺Foxp3⁺ regulatory T cells in numerous other systems. Regulatory cells are important in a large array of syndromes. A genome-wide survey showed polymorphisms in several genes necessary for regulatory cell development increased susceptibility to a number of autoimmune diseases, including type I diabetes (Wellcome Trust Case Control Consortium 2007). Studies like these identify the critical nature in which regulatory cells normally restrain inflammation. We have found that MIF reduces the population of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. We propose an increase in MIF increases susceptibility to a number of diseases, including autoimmune, through inhibition of the differentiation of regulatory T cells. Polymorphisms in MIF have been associated with several diseases (Martínez, Orozco et al. 2007; Nunez, Rueda et al. 2007; Fei, Lv et al. 2008). An inverse correlation between levels of MIF and the population of CD4⁺CD25⁺Foxp3⁺ has not been explored, but we would predict that polymorphisms that increase the activity of MIF would be associated with decreased numbers of regulatory cells and increased susceptibility.

We showed that regulatory T lymphocytes are important mediators of EAE suppression in knockout mice, but we also identified additional mechanisms that may be present and reduce susceptibility to EAE. Adoptive transfer studies showed naïve MIF knockout recipient mice were protected from
EAE. We could not induce EAE in MIF knockout mice using activated encephalitogenic lymphocytes from wild type animals. The MIF knockout recipient mice had greatly reduced incidence of EAE, as well as reduced severity, suggesting that a critical mechanism necessary for susceptibility to EAE was absent in knockout animals. We did not identify significant populations of CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells in naïve recipient wild type or knockout mice. We predicted an additional factor in knockout mice was responsible for the observed protection in these mice.

Following EAE, migration is significantly reduced in MIF knockout mice. Wild type animals have considerable perivascular cuffing and inflammation within 17 days of induction of EAE. There is no such inflammation in MIF knockout mice despite the few phenotypic differences discussed previously. Regulatory T cells inhibit proliferation and effector function, but they have not been shown to directly preclude trafficking into the CNS. MIF knockout lymphocytes were capable of transferring EAE into naïve wild type recipients. These lymphocytes were taken from immunized MIF knockout mice and expanded in vitro with MOG antigen. Successful transfer of EAE would only be possible if autoreactive lymphocytes were expanded in the MIF knockout donor mice prior to transfer. This again supports our earlier claims that MIF has a reduced role during the induction of EAE. These results suggest that MIF is not required from the transferred activated T cells and more important at the level of the BBB for disease susceptibility. We concluded that there were mechanisms intrinsic to the BBB that prevented leukocyte migration in MIF knockout mice.
We predicted MIF likely plays an essential role in the trafficking of encephalitogenic T cells from the periphery into the CNS. Denkinger et al. (Denkinger, Denkinger et al. 2003) were among the first to propose mechanisms in which MIF might play a role in leukocyte migration. Denkinger proposed that MIF upregulated key adhesion molecules, including VCAM-1. Others have since shown that MIF acts as a chemotactic protein, attracting leukocytes to the site of inflammation (Schober, Bernhagen et al. 2008). Despite its name and initial reported function as an inhibitor of macrophage migration, MIF appears to be an important mediator of leukocyte trafficking. In our studies, deleting MIF prevented migration of leukocytes into the brain and spinal cord. Migration was similarly inhibited in mice receiving a small molecule inhibitor of MIF. We were able to show that inhibition of MIF prevents subsequent migration but cannot reverse previous inflammation. Following inhibitor treatment, leukocyte migration into the brain was significantly reduced, most profoundly by F4/80⁺ macrophages, but migration in the spinal cord that occurred prior to drug administration was not reversed. These observations support conclusions from our adoptive transfer studies, in which MIF knockout recipient mice were universally protected. Without expression of MIF, mice lack an important mechanism that allows the upregulation of adhesion molecules and subsequent migration into the CNS.

It is important to consider how MIF may regulate expression of adhesion molecules and migration. There are a number of means in which MIF mediates inflammation. First, MIF is an inflammatory cytokine that causes the release of
additional cytokines, including IL-6 and TNF-α (Bozza, Satoskar et al. 1999). Both cytokines activate vascular endothelial cells and cause the surface expression of ICAM-1, VCAM-1, and selectins. The upregulation of these markers facilitates tethering and transendothelial migration across the BBB. Without these markers, the incidence and severity of EAE is severely reduced.

Second, MIF itself may activate vascular endothelial cells. MIF stimulates cellular activation through a number of intracellular pathways, including AP-1 and NF-κB (Kleemann, Hausser et al. 2000; Riedemann, Guo et al. 2003). Signaling through these transcription factors can cause an increase in vascular and parenchymal adhesion molecules on astrocytes and microglia. This could be an important mechanism of trafficking outside the vasculature and within the parenchyma of the CNS. Finally, MIF has known chemotactic functions, with mechanisms in atherosclerosis (Schober, Bernhagen et al. 2008). MIF may directly recruit leukocytes to the site of inflammation, causing the trafficking of T and B lymphocyte to the CNS. This mechanism of MIF would be relevant in a number of diseases in which the migration of leukocytes is required for the pathogenesis of disease.

Our studies regarding the cellular source of MIF determined that the myeloid lineage of cells were the source of MIF expression during EAE. Virtually all cells ubiquitously express MIF, yet it was clear that a very specific cell type was producing MIF. We used bone marrow chimera mice to show induction and progression of EAE required MIF expression from bone marrow-derived cells. MIF knockout mice in which the bone marrow was replaced with
wild type marrow were susceptible to EAE. Yet, wild type mice receiving MIF knockout bone marrow were protected. Bone marrow-derived cells include not only erythrocytes and megakaryocytes, but also the cellular repertoire of the innate and adaptive immune system. Our studies indicate at least one of these cell types is the source MIF during EAE. A number of other cell types may produce MIF, but during EAE, MIF produced by bone marrow-derived cells is required.

From these observations, we further explored the potential cellular source of MIF by adoptively transferring activated lymphocytes into naïve recipients. As was mentioned previously, wild type lymphocytes were unable to induce disease in naïve MIF knockout recipients, despite lymphocytes’ ability to produce MIF. We concluded another cell type that is bone marrow-derived must express the relevant MIF during EAE. Through the exclusion of lymphocytes, we have focused on the myeloid lineage of cells. We can presume that MIF acts locally following extracellular release at the sight of inflammation, as MIF production by other cells types does not affect susceptibility to EAE.

Myeloid-derived cells are strong candidates for several reasons. First, they are strongly associated with the induction of both MS and EAE. Dendritic cells and macrophages are important antigen presenting cells that modulate the inflammatory environment. They bridge the innate and adaptive immune system and selectively expand T_{H}1 and T_{H}17 lymphocytes through the release of cytokines, including IL-12, IL-6, and TGF-β. Both types of antigen presenting cells can be identified in MS and EAE lesions. They can be activated by
environmental cues, through engagement of Toll-like receptors (TLRs).

Adjuvants administered during the induction of EAE are potent TLR agonists. Natural agonists following infection could similarly engage TLRs during the onset of MS. We have already shown MIF from myeloid-derived cells strongly mediates susceptibility. We can conclude that this follows TLR activation. MIF is produced by macrophages after they are stimulated by lipopolysaccharide (LPS), a TLR4 agonist. Following its release, MIF can mediate the upregulation of TLR4 (Roger, David et al. 2001). An increase in TLR4 expression would enhance the sensitivity of leukocytes to TLR agonists and increase susceptibility to inflammation. This places MIF at a key junction in the sequence of events leading up to MS.

Second, myeloid lineage cells are important in the constitution of the brain and spinal cord. Myeloid-derived cells comprise the cells of the immune system lining the vascular endothelium in the CNS. These perivascular mononuclear antigen presenting cells detect changes in the peripheral environment. Following the influx of inflammatory cytokines or other mediators, perivascular cells can activate vascular endothelial cells and increase expression of adhesion molecules along the BBB. Perivascular cells, including dendritic cells and macrophages, produce cytokines that upregulate adhesion molecules on vascular endothelial cells, further aiding extravasation. Inhibiting MIF reduces surface expression of VCAM-1 on the BBB (Denkinger, Denkinger et al. 2003). We have already shown a very specific cell, likely in close proximity to target tissues, produces MIF during EAE. We propose that dendritic cells and macrophages
within the perivascular space produce MIF and stimulate endothelial cells to increase expression of adhesion molecules. IL-6 and TNF-α, both downstream cytokines of MIF, increase adhesion molecule expression and are also produced by both dendritic cells and macrophages following activation. Dendritic cells and macrophages can also initiate the production of critical chemokines, which recruit additional leukocytes to the site of inflammation. MIF, through local autocrine and paracrine functions, can mediate the expression of these cytokines and chemokines. These factors place myeloid-derived cells at a second important time point during the development of EAE and MS.

Our work strongly implicates the myeloid lineage of cells as the relevant source of MIF during EAE. We showed that the absence of MIF from bone marrow-derived cells was protective. Furthermore, lymphocytes activated in vivo and adoptively transferred into MIF knockout recipients were insufficient to induce EAE. We concluded from these studies that MIF was a local factor produced by myeloid-derived cells at the site of inflammation. We have described how myeloid cells are associated with EAE and MS, but it is important to consider how an inhibitor of MIF could mediate the function of these cells during disease.

From our studies in knockout mice, we proposed several mechanisms of MIF during EAE. First, we showed that MIF skews T lymphocytes away from encephalitogenic subtypes like T_{h}17 and towards more regulatory populations. CD4^{+}CD25^{+}Foxp3^{+} T lymphocytes are expanded following exposure to TGF-β. Myeloid-derived cells, including dendritic cells and macrophages, can produce a
number of cytokines, including TGF-β, following activation. Without MIF, dendritic cells and macrophages produce less IL-6, a cytokine that is co-expressed with TGF-β to expand T<sub>H</sub>17 lymphocytes. Any reduction in IL-6 without a change in TGF-β would specifically expand regulatory T lymphocytes. Regulatory cells accelerate recovery from EAE and possible MS. We propose that an inhibitor of MIF reduces IL-6 and increases the population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory cells. These cells traffic to the CNS and mediate rapid improvement in clinical scores. We believe an inhibitor of MIF could be protective in MS patients, as well. Inhibition of MIF could skew populations of T lymphocytes towards a more regulatory phenotype, shortening periods of relapse and protecting the CNS environment. Our work suggests an inhibitor of MIF may expand regulatory cells in other inflammatory diseases, as well.

Second, we propose MIF mediates leukocyte trafficking. Myeloid-derived cells are found in locations within that perivascular space that would suggest a role during migration. The expression of adhesion molecules along the BBB is required for progression of EAE and entry into the CNS. Surface expression of adhesion molecules is increased on endothelial cells following activation, which can be caused by the release of inflammatory cytokines in close proximity to vascular cells. MIF, produced by dendritic cells and macrophages, may directly activate endothelial cells, or, through autocrine and paracrine activation, cause the release of other inflammatory cytokines like TNF-α and IL-6. TNF-α and IL-6 in turn upregulate adhesion molecules including VCAM-1. An inhibitor of MIF would decrease subsequent cytokine release in the perivascular space, reducing
the expression of IL-6 and TNF-α. This decrease in inflammatory cytokines would cause a concomitant decrease in the expression of adhesion molecules. During MS, this would significantly reduce new migration into the CNS, slowing progression. This is a second mechanism of MIF that could be targeted for therapeutic intervention in MS patients.

Both mechanisms can be used to explain why an inhibitor of MIF failed to prevent EAE when administered prior to the onset of clinical signs. CPSI-2705 was given to mice for ten days following the induction of EAE with MOG\textsubscript{35-55} peptide. Clinical signs appeared between days 12 and 14 post-immunization. Given the mechanisms proposed here, we can see why an inhibitor failed to prevent disease onset. MIF inhibits differentiation of regulatory T cells and is required for migration from the vasculature. During the induction of EAE and while we were administering inhibitor, autoreactive lymphocytes were expanded in the lymph nodes and may have migrated into the vasculature. However, lymphocytes were unable to migrate into the CNS, likely because of reduced expression of adhesion molecules. Other factors, including the differentiation of regulatory T cells, was probably present, but had less of a role during the initial induction of inflammation. Adjuvants that were administered with MOG peptide, including complete Freund’s adjuvant and pertussis toxin, probably overcame any potential inhibition by regulatory cells, as these adjuvants are strongly mitogenic. In the face of these potent immune stimulators, regulatory cells could not prevent the expansion of autoreactive immune cells. When we stopped administration of an MIF inhibitor after ten
days, rapid increases in MIF activity likely upregulated adhesion molecules and drove differentiation of $T_{H17}$ cells, causing the onset of clinical scores. However, given that MIF knockout mice are protected during both the acute and chronic periods of EAE, a third mechanism must mediate suppression during the initial induction of inflammation.

A final observation in knockout mice deserves discussion. Testosterone is an important regulatory hormone for spermatogenesis and secondary sex characteristics. It is also a potent immunosuppressive hormone as documented by numerous studies in MS and EAE. There is a clear sex dimorphism in MS patients. Men are typically less susceptible to MS. However, half of all new diagnoses of primary progressive MS, which represents approximately 15 percent of total cases of MS, are in men. Women, however, are three times more likely to develop MS and are typically diagnosed with relapsing remitting. Investigations into this discrepancy showed a quarter of all men with MS had lower levels of testosterone than healthy males. Other studies with topically applied testosterone showed therapeutic benefit.

In EAE, gonadectomized male mice have increased incidence and more severe disease. Exogenous testosterone improves EAE in both male and female mice. In our MIF knockout mice, testosterone was more than four fold higher than wild type mice. Gonadectomy in these animals significantly increased the incidence of EAE but did not worsen the severity of clinical scores. We can conclude hormone homeostasis is an additional mechanism in which MIF mediates susceptibility. It is clear that in the absence of MIF testosterone rises
dramatically versus normal serum levels. Higher serum levels of testosterone inhibit inflammation and protect mice from the induction of EAE. The presence of MIF increases susceptibility to EAE through a reciprocal reduction in testosterone.

Gonadectomy in male MIF knockout mice increased incidence but did not increase severity. It appears from these studies testosterone does not prevent subsequent inflammation or inhibit ongoing disease. While still significantly elevated, testosterone at this point in disease is not sufficient to inhibit progression. Other mechanisms in which MIF mediates inflammation likely have stronger roles during chronic disease. We also noted that an inhibitor of MIF, given after acute EAE, reduced clinical scores but did not increase testosterone.

Myeloid-derived cells have not been as directly implicated in the mediation of testosterone synthesis as other cellular sources of MIF. However, numerous studies have linked MIF expression from other cells with the production of testosterone. In particular, MIF expression has been identified in Leydig cells within the testis (Meinhardt, Bacher et al. 1996). MIF has also been found in cells of the pituitary gland, which produce important tropic hormones for the production of testosterone in both the adrenal cortex and testes (Bacher, Meinhardt et al. 1998). MIF has already been shown to inhibit another immunosuppressive hormone, corticosterone. Given MIF’s inflammatory function, it could also inhibit testosterone. Studies in T. crassiceps show wild type male mice infected with the parasite have an 80 percent reduction in testosterone
versus health animals (Morales-Montor, Baig et al. 2002). In MIF knockout mice, there is no subsequent reduction in testosterone following infection. Thus, as MIF increases over the course of disease, it likely inhibits testosterone production, facilitating an increase in inflammation. Though we did not see an increase in testosterone following 21 days of inhibitor administration, we may see higher testosterone following longer treatment. We also would not expect an increase in testosterone when we treated for ten days prior to the onset of clinical scores. This also helps explain why pretreatment with an inhibitor of MIF failed to prevent EAE.

We can conclude that MIF has different mechanisms mediating susceptibility, depending on timing following disease. In naïve mice, MIF inhibited testosterone, increasing susceptibility to EAE. Other factors we have mentioned, regulatory populations of T lymphocytes and expression of adhesion molecules, are most influential during chronic EAE. Following immunization, MIF acted to expand encephalitogenic populations of cells, including T\(_{H1}\) and T\(_{H17}\), as well as upregulate critical adhesion molecules on the endothelium of the BBB. MIF also causes significant release of other inflammatory cytokines, including IL-1\(\beta\), IL-6, and TNF-\(\alpha\). Through all of these mechanisms, at subsequent steps in the disease process, MIF facilitates susceptibility to EAE. It represents one of the most comprehensive cytokine targets for the treatment of MS.

We have shown that an inhibitor of MIF is therapeutic during EAE. Two different inhibitors, CPSI-1306 and CPSI-2705, reduced ongoing disease severity.
This was marked by increases in the number of regulatory T lymphocytes and reduced CNS leukocyte infiltration. Additionally, we expect with long-term treatment an increase in testosterone as well. Any of these three could be primary mechanisms in which inhibition of MIF is therapeutic, and all could be successful for the management of MS. These inhibitors could represent novel therapeutic targets or may be synergistic with current therapies. Testosterone is currently being explored as a neuroprotective adjuvant therapy. Administration of an MIF inhibitor has important subsequent benefits that include an increase in testosterone. Importantly, none of the mechanisms of MIF inhibition appear to suppress the immune system. Rather, MIF is an important mediator of several components of the immune response, including autoregulation through regulatory T cells, trafficking into the peripheral tissues, and hormone homeostasis. Targeting these multiple components could prove most successful for the management of a complicated autoimmune disease like MS.


