THE ROLE OF MIF PRODUCTION BY B LYMPHOCYTES IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

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ABSTRACT

The Role of MIF Production by B Lymphocytes in Experimental Autoimmune Encephalomyelitis

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Chairperson of the Supervisory Committee: Dr. Caroline Whitacre
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Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) which is characterized by demyelination and axonal loss when autoreactive T lymphocytes gain access to the CNS. This leads to clinical symptoms of decreased motor and sensory functions. Macrophage migration inhibitory factor (MIF) has been shown to be critical for the progression of experimental autoimmune encephalomyelitis (EAE), an animal model for MS. When immunized with the encephalitogenic peptide of myelin oligodendrocyte glycoprotein (MOG) 35-55, mice deficient in the pro-inflammatory cytokine MIF are protected from EAE. . We show the clinical signs of EAE can be reversed by the use of small molecule inhibitors of MIF. Yet, MIF is constitutively expressed in all cell types and important in innate immune function, thus global inhibition of MIF could potentially cause adverse side effects. Thus, we investigate the cellular source of the biologically relevant MIF for EAE. Through a series of bone marrow chimera and adoptive transfer experiments, we

concluded the necessary MIF is bone marrow derived. These experiments eliminated T lymphocytes as the source of the biologically relevant MIF.

Recently, MIF has been linked to B lymphocyte maturation. Although the role of B lymphocytes in MS pathology remains unclear, the recent success of Rituximab, a monoclonal antibody against human B lymphocytes, argues for a strong role of B lymphocytes in MS. The purpose of the latter part of this study is to investigate the autocrine role of MIF production by B lymphocytes in EAE as a future specific therapeutic target. We observed significantly fewer mature B lymphocytes in the immune compartments of MIF deficient mice when compared to wild type counterparts. Yet, significantly more progenitor B lymphocytes were observed in an MIF deficient mice, leading to the hypothesis that MIF may be crucial for B lymphocyte survival. We also observed impaired proliferation of B lymphocytes in the bone marrow of a MIF knockout mouse. Next, we performed a series of transfer experiments where the only source of MIF is B lymphocytes to determine if this source alone could induce EAE; therefore providing a specific cell type to target MIF production in EAE. We observed protection of MIF deficient recipients regardless of MIF production by the B lymphocytes. Our data suggests that although MIF is important for B cell survival, it does not act in an autocrine manner.

DEDICATION

"No can be one of the most positive words in the world. No, I will not give up. No, I will not be defeated."

This work is dedicated to my determined mother who passed away due to complications of MS. She never let the disease take her positive attitude, no matter the challenges and falls. She was a dedicated mother and wife whose lessons have shaped my approach to life.

To my mother and the many other loved ones who are battling this unpredictable disease. May a cure come quickly.

ACKNOWLEDGMENTS

I want to express sincere appreciation to Dr. Whitacre, my guiding mentor throughout this project. Her support and encouragement was integral to the progression of this project. She allowed me to see the passion inside myself and to never lose sight of that ambitious and passionate eighteen-year-old who first set foot in a research lab four years ago. She taught me how research can and should be conducted. And for that, I am most thankful and inspired

Dr. Aaron Kithcart was my fearless leader through this project. As a MD/PhD student in the lab, Aaron allowed me to work with him at the start of this project. His patience and calm approach was ever so helpful as I learned the ways of the lab and project. His involvement in planning the following project and his constant reminder of the bigger picture were crucial.

I also wish to express sincere gratification to the other members of Dr. Whitacre's lab who have helped and supported me throughout my project; Dr. Na Tosha Gatson for believing in my spirit and helping me obtain a position in the lab, Kristen Smith for her positive attitude, Jessica Williams for her patient teaching moments, Gina Mavrikis-Cox for her literature knowledge of MIF, Todd Shawler for his help with the flow cytometry experiments and experimental designs, and Ingrid Gienapp for her teaching spirit.

I also owe sincere appreciation to Lori Martensen and Dr. Bruce Biagi for their vision of the Biomedical Science Major. Their hard work to help students gain not only research experience, but to become comfortable with independent thinking and research presenting has been integral to my success with this research project.

Thank you to the members of my undergraduate research committee who helped me during this adventure. Their support, flexibility, and suggestions have made this document a success.

Lastly, thank you to the most important guys in my life: my dad, brother, and boyfriend. They were the ones who encouraged me after failed experiments and reminded me of the biggest picture of all: happiness in life. They were guinea pigs for the many presentations and papers I had to present and write during this project. They kept my attitude positive and my thinking straight.

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ABBREVIATIONS

APC: antigen presenting cell
BBB: blood brain barrier
CD: cluster differentiation
CDI: cumulative disease index
CNS: central nervous system
CSF: cerebral spinal fluid
EAE: experimental autoimmune encephalomyelitis
IL: Interleukin
IFN: interferon
iNOS: inducible isoform of nitric oxide synthase
i.p.: intraperitoneally
i.v.: intravenously
MIF: macrophage migration inhibitory factor
MOG: myelin oligodendrocyte glycoprotein
MS: multiple sclerosis
PML: progressive multifocal luekencephalopathy
RAG: recombination activating gene
TLR: toll-like receptor
VCAM-1: vascular cell adhesion molecule-1

Chapter 1

INTRODUCTION AND BACKGROUND

Meaning "many scars," multiple sclerosis (MS) was named for the many lesions (scars) found in the brain and spinal cord of affected individuals. The disease was first described by Dr. Jean Martin Charcot in the mid-1800s when he differentiated it from other neurological disorders by its central nervous system (CNS) damage. Despite the scientific advancements made in understanding both the nervous and immune systems, the pathology of multiple sclerosis remains unknown and is an area of active research. Yet, the disease is widely accepted as an autoimmune disease of the CNS. Both the initial event causing autoimmunity and the subsequent events mediating its tissue damage remain poorly understood today. These events must be better understood for the development of targeted therapies to treat the debilitating symptoms of MS.

1.1 Problem Statement

Though many specific aspects of MS remain unknown, inflammation is involved in mediating the damage seen within the CNS, which is primarily mediated by auto-reactive T lymphocytes (Glass CK, 2010). Unaffected individuals also have auto-reactive T lymphocytes, yet in unaffected individuals, these cells do not gain access to the CNS and are held in check. Access to the CNS is prevented by the blood brain barrier (BBB), an extremely tight barrier of endothelial cells at the

interface between the periphery (blood) and the CNS (brain). However in MS, the BBB is compromised, allowing the auto-reactive T lymphocytes to cross into the CNS, where they are activated and mediate damage. Although the mechanism behind BBB damage is unknown, cytokines and adhesion molecules play a large role in recruiting damaging cells into the CNS. Macrophage migration inhibitory factor (MIF) was first described in 1976 as a pro-inflammatory cytokine secreted by activated T lymphocytes. It was named for its ability to prevent the movement of macrophages out of a capillary tube (Bloom, 1966 and David, 1967). Recently, MIF has been re-evaluated as an important link between the innate and adaptive immune responses. Data from our lab has shown that MIF is important for the progression of experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Gold, 2000). Thus, my problem statement is: to determine the specific cell type through which MIF mediates its pro-inflammatory effects in MS. The ensuing chapters will specifically explore B lymphocytes as a candidate cell type.

1.2 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a disease of the central nervous system that is characterized by demyelination and axonal loss, leading to clinical symptoms of decreased motor and sensory functions (Noseworthy, 2000). The clinical symptoms include muscle weakness and spasms; difficulties with coordination,

balance, and movement; fatigue; acute or chronic pain syndromes; and bladder and bowel difficulties.

Although the exact mechanism of damage in MS is not well understood, it is generally accepted that myelin specific auto-reactive T lymphocytes gain access to the CNS. Once in the CNS, the T lymphocytes are activated and together with other immune cells mediate functions such as antigen presentation, cytotoxicity effects, cytokine release, and activation of additional cells. Not only do the immune cells mediate damage directly, but they also cause a generalized site of inflammation which expands tissue damage. The influx of auto-reactive and activated immune cells is believed to result in damage to the myelin sheath surrounding neurons. The inflammation and demyelination are thought to cause the majority of the symptoms associated with MS. More recent evidence suggests oligodendrocytes (myelin producing cells) and axons may be targeted in MS as well (Trapp, 1998).

1.3 Experimental Autoimmune Encephalomyelitis (EAE)

Many of the current therapies for, understanding of, and research on MS is made possible through utilization of the animal model experimental autoimmune encephalomyelitis (EAE), which mimics many of the clinical, pathological, and immunologic events seen in MS. In susceptible mouse strains, EAE presents with ascending paralysis as activated T lymphocytes cross the BBB and infiltrate

the white matter of the CNS (Papenfuss, 2004). EAE is induced by immunization with myelin antigens together with immune stimulators known as adjuvants. In the C57Bl/6 mouse strain (which is the genetic background of all mice used in the following study), EAE is induced by immunization with the encephalitogenic myelin oligodendrocyte glycoprotein (MOG) peptide.

1.4 B Lymphocytes

B lymphocytes are a subtype of white blood cells which are responsible for antibody production in the adaptive immune response. Despite their well defined role in antibody production, the role of B lymphocytes in MS remains unclear. Support for their involvement has been shown in studies of antigen presentation, cytokine production, and auto-antibody production (McLaughlin, 2008). A recent study by Magliozzi et al. showed that ectopic follicles containing B lymphocytes are found in mice with progressive relapsing EAE as well as in the meninges of patients with secondary-progressive MS (Magliozzi, 2007). The location of these follicles suggests that B lymphocytes migrate to the CNS. It is not clear whether the B lymphocytes are activated locally where they present antigens, and differentiate into memory B cells, rather that they are being activated in the periphery and migrating to the CNS in a fully mature state (McLaughlin, 2008). Other investigators have shown that antigen specific B lymphocytes are required to prime the pathogenic CD4+ T lymphocytes with low doses of antigen, similar to the levels in MS (Riveria, 2001).

Perhaps the most striking evidence arguing for the importance of B lymphocytes in MS arose from a study by Hauser et al. in which Rituximab, a monoclonal antibody directed against the B lymphocyte specific surface protein CD20, was used to treat MS patients. The treated patients experienced significantly less lesion development, resulting in clinical improvement (Hauser S., 2008). Taken together with the above suggested roles of B lymphocytes, this evidence revived the investigation of B lymphocytes in multiple sclerosis.

1.5 Macrophage Migration Inhibitory Factor (MIF)

The pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF), has recently been re-discovered as an important link between innate and adaptive immune function (Denkinger, 2003). MIF was found to be present in a number of immune cells, including monocytes, T and B lymphocytes, neutrophils, macrophages, dendritic cells, mast cells, and basophils. Additionally, MIF is found in epithelial cells lining the lung and gastrointestinal tract, as well as in endothelial cells (Denkinger, 2003). MIF is ubiquitously produced during inflammatory immune responses and plays a significant role in the up-regulation of inflammatory cytokines involved in the pathogenesis of EAE. Importantly, MIF is elevated in the cerebrospinal fluid (CSF) of MS patients during relapses (Niino, 2000). Our laboratory reported that MIF deficient mice show decreased signs of EAE compared with control mice. Specifically, MIF knockout mice

show a mild acute phase of disease, but do not show progression of the disease (Powell N.D., 2005 and Figure 1.1). Interestingly, MIF has been shown to be critical for mature B cell survival (Gore Y., 2008). Also, recent evidence shows that MIF signals in an autocrine fashion (Lue H., 2007). These results led MIF to be studied with an emphasis on B lymphocytes as a possible therapeutic target for the treatment of MS.

1.6 MS Therapies

Despite the many uncertainties with respect to MS pathogenesis, treatments are available and can be marginally successful for some patients. The first wave of therapies focused on hormonal administration, primarily glucocorticoids. Glucocorticoids are a class of steroids which suppress the immune system, thereby lessening the autoimmune attack of MS. Glucocorticoids can shorten relapses and lengthen remissions (Noseworthy J. L., 2000).

The true staple of current MS therapy is beta interferon. The interferon family of drugs includes Betaseron, Avonex, and Rebif. Though these drugs have been shown to reduce relapses and delay the progression of MS, the mechanism of action is poorly understood. Yet, they remain integral to most MS therapy plans (Kinkel, 2006 and Rio, 2005).

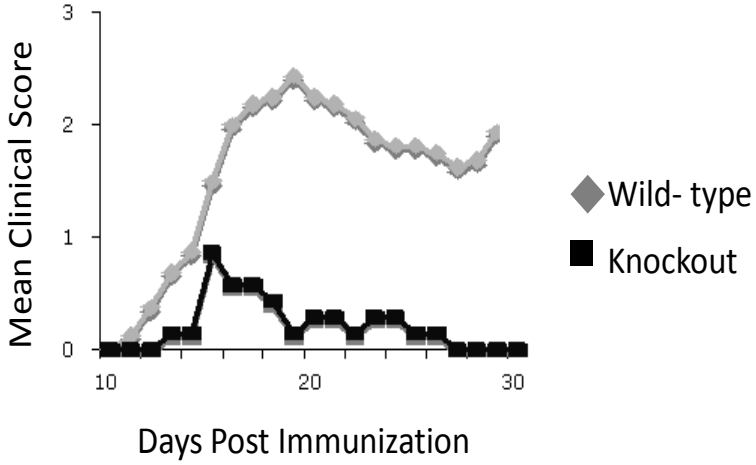
Newer therapies include monoclonal antibodies, such as Natalizamab and Rituximab. Natalizamab is a monoclonal antibody directed against α4-integrin which is a critical mediator of leukocyte entry into tissue (Polman, 2006 and Rudick, 2006). Unfortunately, its administration has been linked to an increased risk of progressive multifocal luekencephalopathy with an estimated incidence of 1 in 1000 treated patients (Yousry, 2006 and Hartung, 2009). Rituximab, a monoclonal antibody directed against CD20, has shown promising results in clinical trials. This evidence provides a solid foundation for the re-examination of B lymphocytes in MS pathology.

Despite the number of current therapies, a cure or holistic treatment remains absent from MS therapy options. Research continues to search for a targeted therapy which leaves the remaining immune system intact and effective to fight opportunistic infections, while quieting the autoimmune attack of MS.

1.7 Significance and Objectives

MS is the most common demyelinating disease of the CNS. Presently, the etiology is unknown but the presumed mechanism is autoimmune as demonstrated by the presence of autoreactive myelin specific T lymphocytes in the CNS. Currently, no treatment has proven curative; however, B lymphocyte depletion therapies such as Rituximab have proven beneficial. Understanding the role of B lymphocytes and MIF production in EAE pathogenesis will be

significant for the treatment of MS as both appear to play a vital role in disease progression. Several small molecule inhibitors for MIF are available, however if the target could be further narrowed to MIF producing B lymphocytes through the use of a small interfering RNA (siRNA) for example, fewer side effects would accompany the treatment.



<u>Figure 1.1:</u> The genetic deletion of MIF is protective against EAE. Wild type (◆, n=18) and MIF ko (■, n=13) mice were immunized for EAE with 200 μg MOG(35-55) peptide in adjuvant. MIF ko mice are protected from the progression of EAE and exhibit less severe EAE relative to wild-type controls. Data are representative of four separate experiments.

*This work was completed by Aaron P. Kithcart, PhD.

Chapter 2

METHODS

2.1 Mice

Male C57Bl/6 wild-type mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and housed five per unit in standard ventilated cages. Age- matched male C57Bl/6 recombination activating gene (RAG) 2 knockout mice were purchased from Taconic and bred in house (The Ohio State University). MIF deficient mice were developed as previously described (Bozza, 1999).

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 the vivarium in 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 *ad libitum*. Mice were randomly assigned to experimental groups and ear tagged for identification purposes.

2.2 Antigens

Myelin oligodendrocyte glycoprotein (MOG) 35-55 (MEVGWYRSPFSRVV

HLYRNGK) peptide used for the induction of EAE in C57Bl/6 mice and 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.

2.3 Cell Purification

CD4+ T lymphocytes were purified by the negative selection using a 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 conjugated to biotin. Anti-biotin microbeads were used to magnetically separate the antibody-labeled cells. Purity, confirmed by flow cytometry, was greater than 95%.

B lymphocytes were also purified by the negative selection using a magnetic bead kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Single cell suspensions were depleted of non-B220+ B cells using anti- CD8α, -CD11b, -CD4, -CD43, and -Ter-119 monoclonal antibodies directly conjugated to biotin. Anti-biotin microbeads were used to magnetically separate the antibody-labeled cells. Purity, confirmed by flow cytometry, was greater than 90%.

Purified splenocytes were cultured at $4x10^5$ cells per well in a 96 well culture plate. Media was supplemented with 2 μg/ml anti-CD3.

2.4 Induction of Experimental Autoimmune Encephalomyelitis

For the induction of EAE in wild type mice, the mice were immunized with 200 μg MOG (35-55) peptide (Princeton Biomolecules) emulsified in complete Freund's adjuvant (containing 200 μg *Mycobacterium tuberculosis* Jamaica strain). The emulsion was 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).

For the induction of EAE in RAG knockout mice, the mice were given purified CD4+ T lymphocytes from male wild type or $MIF^{-/-}$ donor mice. Purity following the cell isolation protocol as determined by flow cytometry, was greater than 95 percent, and the cells were transferred into male recipient $RAG^{-/-}$ mice by intravenous (i.v.) injection in phosphate buffered saline. Recipient mice were immunized for EAE as described above with MOG (35-55) peptide on the day of or two days following T lymphocyte transfer.

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 or treatment.

CDI was reported as an average within each group. Peak score was reported as the average maximum clinical score within each group over the observed period. EAE incidence was calculated based on individual animals and reported as a mean within each group.

2.5 Transfer of Experimental Autoimmune Encephalomyelitis

EAE was adoptively transferred following *in vitro* culture of splenocytes for some experiments. Donor male wild-type or MIF knockout mice were 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 were isolated, 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 ug/ml streptomycin, and $5x10^{-5}$ M 2-ME) with 20 μg/ml MOG (35-55) peptide and 0.5 ng/ml IL-12p70 (BD Biosciences, San Jose, California, USA). The MOG (35-55) peptide and IL-12p70 were used to reactivate the T lymphocytes *in vitro*. Following culture, cells were washed twice in phosphate buffered saline (PBS), and $10x10^{6}$ cells were injected i.v. into male recipient mice.

2.6 Drug Administration

The small molecule inhibitors of MIF (CPSI-00155S, -00155F, -00156S, gifts from Cytokine PharmaSciences, King of Prussia, Pennsylvania, USA) were administered to mice following induction of EAE. Inhibitors were given either i.p. or orally by gavage. For i.p. administration, 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 and the total volume administered was 50 µl. Orally administered inhibitor was dissolved in 15 percent DMSO in 0.1 percent methylcellulose in water. Mice were fed a total of 50 µl at a given concentration of inhibitor by gavage. 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.

2.7 Generation of Bone Marrow Chimeras

Male donor wild-type or $MIF^{-/-}$ 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 $RAG^{-/-}$ or $MIF^{-/-}$x $RAG^{-/-}$ mice was ablated by irradiation using a Gammacell 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 $10x10^6$ 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 and analyzed by flow cytometry for the presence of CD4+ T and CD19+ B lymphocytes.

2.8 Flow Cytometry

Single cell suspensions from draining lymph nodes, spleens, and bone marrow were stained with anti-B220, -CD4, -CD25, -CD19, -CD80, -CD86, -IgM, or -IgD fluorescein isothiocyanate (FITC)-, phycoerythrin(PE)-, allophycocyanin (APC)-, or pacific blue-conjugated fluorescent antibodies (BD Biosciences). Isotype control monoclonal antibodies (BD Biosciences) were matched for each fluorochrome. Cells were labeled at $1x10^6$ cells per tube, incubated for 20 minutes at 4°C, and measured using a FACSCalibur flow cytometer (BD Biosciences). Analysis was performed on a FACSCanto II flow cytometer and analyzed using Diva software (both from BD Biosciences).

2.9 CFSE Proliferation Assay

Single cell suspensions from the organs of interest from donor mice were purified for CD4+ T lymphocytes using magnetic separation described previously. The purified cells were then washed with 5 percent FBS in PBS. CFSE was added to a final concentration of 5 μM. The staining cells were cultured at room temperature in the dark for seven minutes. The CFSE cell solution was then diluted to 50x its volume with 5 percent FBS in PBS and washed three times. CFSE staining was confirmed by flow cytometry.

The cells were either transferred i.v. into recipient mice as described above or were placed into culture. At the specified time point, the CFSE stained cells were recovered and analyzed using flow cytometry. If they were transferred *in vivo*, the recipient mouse was sacrificed and single cell suspensions were derived from the organs of interest. Proliferating cells were denoted by discrete peaks shifted to the left in the flow cytometric histogram.

2.10 Immunohistochemical Staining

Samples were prepared by removing brains and spinal cords post immunization. The organs were flash frozen at -80°C in OCT media. Antibody staining for CD4+ T lymphocytes was performed by The Ohio State University Veterinary Sciences core facility.

Chapter 3

A SMALL MOLECULE INHIBITOR OF MIF CAN REVERSE EAE CLINICAL SIGNS

MIF is a key mediator in EAE pathogenesis as evidenced by the reduced disease severity observed in MIF deficient mice (Figure 1.1). Other cytokines, including IFNγ and IL-17, have also been investigated as possible therapeutic targets (Panitch, 1987 and Tzartos, 2008). Our laboratory has shown a definite role for MIF in EAE progression using different mouse strains and immunization regimens which result in both chronic and relapsing-remitting EAE. In this chapter, we will explore MIF as a therapeutic target by exploring the efficacy of two small molecule inhibitors. The route of administration of the inhibitors, either orally or intraperitoneally, will also be explored, since no established oral therapy is currently available for the treatment of MS.

3.1 A Small Molecule Inhibitor of MIF Reduces Ongoing EAE

To this point, we had explored mice genetically lacking MIF to wild-type controls. MIF deficient mice experience less severe EAE with very little progression of disease compared to wild-type mice (Figure 1.1). We now sought to explore MIF as a possible therapeutic target by the use of a small molecule inhibitor. Ongoing EAE was targeted because the differences in clinical severity between MIF deficient and wild-type mice were greatest during the progression

of EAE. Additionally, ongoing EAE represents the most clinically relevant time point, as it is after diagnosis and when therapy would begin for a patient.

Two small molecule inhibitors of MIF, CPSI-00155F and CPSI-00155S were obtained through collaboration with Cytokine PharmaSciences. The two different inhibitors have the same chemical composition but differing stereochemistry. Both disrupt the tertiary structure of MIF, preventing both its ligand binding and enzymatic functions. We explored the effectiveness of the inhibitors to reduce ongoing EAE. Additionally, the efficacy differences between the two inhibitors were explored (Figure 3.1). Wild-type C57Bl/6 mice were immunized for EAE with 200 μg MOG (35-55) peptide. Seventeen days later during the peak of the induction phase, mice in the treated group were given 10.0 mg/kg i.p. of the inhibitor daily for fourteen consecutive days. Vehicle controls were given 25 percent DMSO in 0.5 percent methylcellulose daily for the same fourteen days as the inhibitor. Mice receiving the CPSI-00155F inhibitor exhibited reduced severity of EAE as soon as four days after inhibitor treatment began. Mice in the CPSI-00155F inhibitor treated group had a reduced cumulative disease index (CDI) relative to both CPSI-00155S treated mice and vehicle controls (7.50 compared to 10.36 and 11.82, respectively; $p<0.05$, Table 3.1). Additionally, the mice in the CPSI-00155F inhibitor treated group followed a trend of a lower mean score relative to both (0.47 compared to 0.78 for the CPSI-00155S treated mice and 0.65 for the vehicle treated mice, Table 3.1). We

concluded that a small molecule inhibitor of MIF can be therapeutic when administered during ongoing EAE.

3.2 CPSI-00155S is Not as Effective Given Orally

No oral therapies are currently approved for multiple sclerosis treatment; however, research is advancing in this area with the recent positive results from two large-scale phase III clinical trials for oral Fingolimod. Administration of Fingolimod was shown to significantly reduce the frequency of MS relapses and may slow progression. The desirability of an oral treatment led us to explore the oral effectiveness of two different small molecule inhibitors of MIF from Cytokine Pharmasciences.

First, CPSI-00155F and S were tested for oral administration after observing the effectiveness of CSPI-00155F when administered i.p. (Figure 3.2). Wild-type C57Bl/6 mice were immunized for EAE with 200 μg MOG (35-55) peptide. Seventeen days later during the peak of the induction phase, mice in the treated group were gavage- fed 1.0 mg/kg of the inhibitor daily for fourteen consecutive days. Vehicle controls were given 15 percent DMSO in 0.5 percent methylcellulose daily for the same fourteen days as the inhibitor was given. The mice were fasted prior to inhibitor administration. Mice fed CPSI-00155F exhibited less severe EAE during inhibitor treatment , but the protection was less dramatic than with the i.p. administration route (Figure 3.1). Still, mice in the

CPSI-00155F oral inhibitor treated group followed the trend of a lower cumulative disease index relative to both the CPSI-00155S orally treated mice and vehicle controls (22.38 compared to 30.25 and 25.31, respectively; Table 3.2). Additionally, the mice in the CPSI-00155F inhibitor treated group had a reduced mean score relative to the CPSI-00155S treated mice (1.60 compared to 2.16; $p<0.05$, Table 3.2).

A second small molecule inhibitor of MIF from Cytokine Pharmasciences was tested for its effectiveness when administered orally. CPSI-00156F was found to be as effective as CPSI-00155F when administered via i.p. injection. We sought to determine if CPSI-00156S is effective at treating ongoing EAE when given orally (Figure 3.3). Wild-type C57Bl/6 mice were immunized for EAE with 200 μg MOG (35-55) peptide. Seventeen days later during the peak of the induction phase, mice in the treatment groups were fed either 0.1, 1.0, or 10.0 mg/kg of CPSI-00156S daily for fourteen consecutive days. Vehicle controls were given 15 percent DMSO in 0.5 percent methylcellulose daily for the same fourteen days as inhibitor administration. We observed the inhibitor to be effective at the lowest administered dose of 0.1 mg/kg, with its most effective dose at 1.0 mg/kg dose. Mice in the 1.0 mg/kg CPSI-00156S inhibitor treated group had a reduced CDI relative to the vehicle controls (10.00 compared to 15.60, $p<0.05$, Table 3.3). Additionally, the mice in the 1.0 mg/kg CPSI-00156S inhibitor treated group had

a lower mean score relative to vehicle controls (0.71 compared to 1.14, $p<0.05$, Table 3.3).

We concluded that a small molecular inhibitor of MIF was effective at reducing ongoing EAE when administered orally. This finding was corroborated with two additional inhibitors from Cytokine Pharmasciences, CPSI-1306 and CPSI-2705 (data not shown). These inhibitors proved effective at an oral dose of 1.0 mg/kg.

3.3 Discussion

Previous studies in mouse models with the genetic deletion of MIF showed the importance of MIF in the progression of EAE. Taken together with the observation of increased MIF levels in the cerebrospinal fluid of MS patients during relapses, this data argues for the importance of MIF in MS pathology as well (Gao, 2008). We sought to explore the role of MIF in EAE and MS by utilizing several small molecule inhibitors of MIF through collaboration with Cytokine Pharmasciences. We found that small molecule inhibitors of MIF are effective at reducing the progression of EAE.

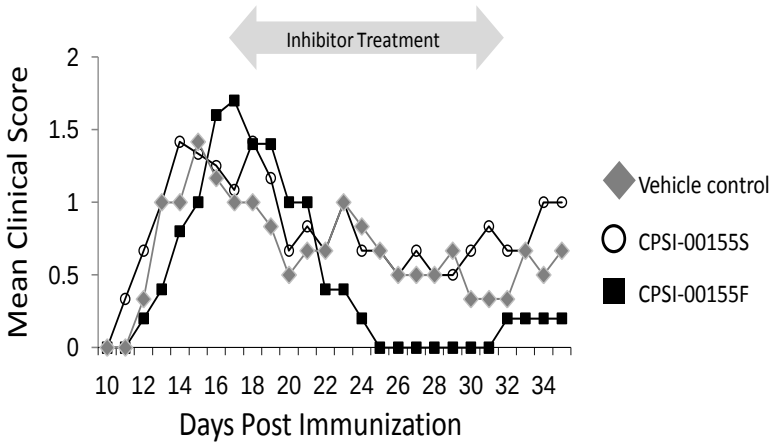
We investigated the oral effectiveness of the inhibitors. With the exception of Fingolimod, the promising oral therapy in phase III clinical trials, oral therapies for MS are nonexistent. The success of the inhibitors at reducing EAE clinical signs in ongoing EAE when administered orally is very promising and novel. A limitation to the oral studies involved the necessary fasting of the mice. Mice

from both the treated and vehicle groups were fasted before the treatment was administered. During the inhibitor studies, we observed a slight recovery in the vehicle treated groups in place of the predicted worsening of disease. Recent data shows reduced caloric intake through fasting may be protective in MS patients (Piccio L., 2008). Fasting presents a stress on the body, calling for increased production of stress steroids which may suppress the immune system thereby serving as protective in the autoimmune attack of MS.

The oral effectiveness of the inhibitors was investigated at three different doses: 0.1 mg/kg, 1.0 mg/kg, and 10 mg/kg. The treatment trended to lower clinical signs at all doses, but the effective dose was found to be the 1.0 mg/kg oral dose. The low-dose efficacy presents a large advantage for therapy, reducing toxicity and side effects. Additionally, testing performed by Cytokine Pharmasciences showed the MIF inhibitors to be safe for patient administration.

Given MIF's ubiquitous expression, determining the biologically relevant source of MIF would provide a more specific and safer therapeutic target. MIF has been shown to be important in the innate immune response, thus total depletion of MIF may have adverse side effects on patients (Terrazas C.A., 2010). Identification of a specific cell type which produces the biologically relevant MIF in EAE would allow for a specific therapeutic target, perhaps through the use of a small-interfering RNA. Additionally, because MIF is a pro-inflammatory

cytokine, identifying the relevant source of MIF would help elucidate the mechanism of how MIF mediates the inflammatory response seen in MS.

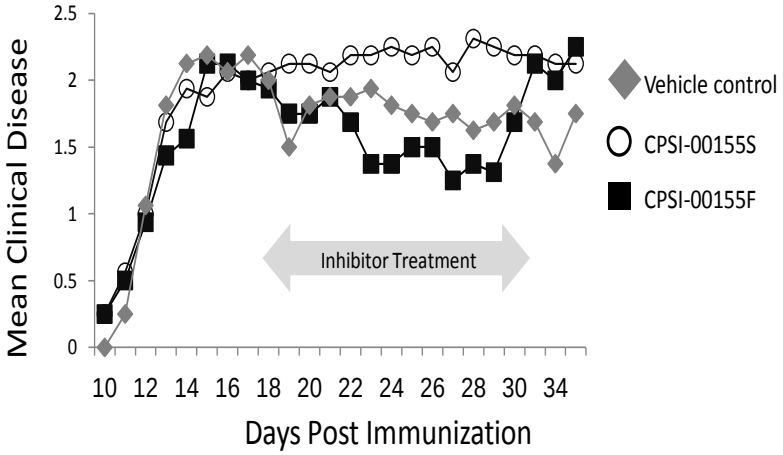


<u>Figure 3.1:</u> A small molecule inhibitor of MIF reduces disease severity in EAE.

A: Male wild-type mice were injected with MOG (35-55) peptide and monitored daily for clinical signs. At 17 days post immunization, mice in the inhibitor treated group were injected daily i.p. with 10.0 mg/kg CSPI-00155S (o , n=10) or CPSI-00155F (■, n=10). Vehicle controls (◆, n=8) were given 25 percent DMSO in 0.5 percent methylcellulose. Treatment was given for 14 days. Mice given the inhibitor CPSI-00155F had reduced severity when compared to the inhibitor CPSI-00155S and the vehicle controls. Data are representative of two separate experiments.

TREATMENT	n	CDI[a]	MEAN SCORE[b]
Vehicle	8	11.82	0.65
CPSI-00155S	10	10.36	0.78
CPSI-00155F	10	7.50*	0.47

Table 3.1: A small molecule inhibitor of MIF reduces disease severity in EAE.
Wild-type mice were immunized for EAE with 200 μg MOG (35-55) and given inhibitor seventeen days later for fourteen consecutive days.
[a] Cumulative disease index (CDI) was calculated as the sum of clinical scores during inhibitor administration per animal and averaged within each group.
[b] Mean score was measured over the duration of treatment per animal and averaged within each group.
* $p<0.05$ compared to the vehicle by Student's t- Test

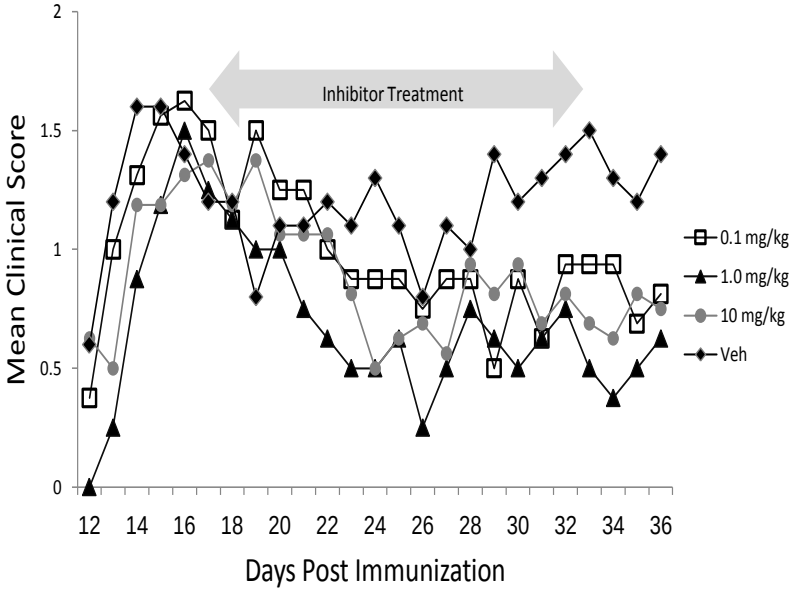


<u>Figure 3.2:</u> CSPI-00155F is not as effective when administered orally.

Male wild-type mice were injected with MOG (35-55) peptide and monitored daily for clinical signs. At 17 days post immunization, mice in the inhibitor treated group were fed daily 1.0 mg/kg CSPI-00155S (O, n=8) or CPSI-00155F (■, n=8). Vehicle controls (◆, n=8) were given 15 percent DMSO in 0.5 percent methylcellulose. Treatment was given for 14 days and mice were fasted approximately eight hours before inhibitor administration. Mice given the inhibitor CPSI-00155F had reduced severity of ongoing EAE but the protection was not as dramatic as seen with the i.p. administration route.

TREATMENT	n	CDI[a]	MEAN SCORE[b]
Vehicle	8	25.31	1.81
CPSI-00155S	8	30.25	2.16
CPSI-00155F	8	22.38*	1.60*

Table 3.2: CSPI-00155F is not as effective when administered orally.
Wild-type mice were immunized for EAE with 200 μg MOG (35-55) and fed inhibitor by gavage seventeen days later for fourteen consecutive days.
[a] Cumulative disease index (CDI) was calculated as the sum of clinical scores during inhibitor administration per animal and averaged within each group.
[c] Mean score was measured over the duration of treatment per animal and averaged within each group.
*$p<0.05$ compared to CPSI-00155S by Student's t Test.



<u>Figure 3.3:</u> Oral inhibitor doses of CPSI-00156S are effective at doses as low as 0.1 mg/kg.

A: Male C57Bl/6 wild-type mice were injected with MOG (35-55) peptide and monitored daily for clinical signs. At 17 days post immunization, mice in the inhibitor treated groups were fed daily 10, 1.0, or 0.1 mg/kg CSPI-00156S (n=8 for each group). Vehicle controls (n=5) were fed 15 percent DMSO in 0.5 percent methylcellulose. Mice were treated for 14 days and mice were fasted approximately eight hours before inhibitor administration. Mice treated with the inhibitor showed less severe EAE. The 1.0 mg/kg (▲) group showed significantly reduced scores compared to the vehicle treated group ($p<0.05$).

TREATMENT	n	CDI[a]	MEAN SCORE[b]
Vehicle	5	15.60	1.14
0.1 mg/kg	8	14.25	1.01
1.0 mg/kg	8	10.00*	0.71*
10.0 mg/kg	8	13.69	0.93

Table 3.3: Oral inhibitor doses of CPSI-00156S are effective at doses as low as 0.1 mg/kg.
Wild-type mice were immunized for EAE with 200 μg MOG (35-55) and gavage fed inhibitor seventeen days later for fourteen consecutive days.
[a] Cumulative disease index (CDI) was calculated as the sum of clinical scores during inhibitor administration per animal and averaged within each group.
[c] Mean score was measured over the duration of treatment per animal and averaged within each group.
*$p<0.05$ compared to the vehicle group by Student's t Test.

Chapter 4

THE RELEVANT MIF IN EAE IS BONE MARROW DERIVED

MIF is constitutively produced and stored throughout the body in a wide variety of immune cells, including monocytes, macrophages, dendritic cells, B lymphocytes, T lymphocytes, neutrophils, mast cells and basophils. Additionally, MIF is found in endothelial cells and epithelial cells lining the lung, gastrointestinal and urogenital tracts (Denkinger, 2003). MIF has been described as a mediator of inflammation and because EAE is mediated by pro-inflammatory cytokines, we hypothesized that the relevant MIF in EAE was likely to be produced by a specific immune cell. In addition to the pro-inflammatory properties, we hypothesized a specific source of MIF because of its local mechanism of action taken with the specific environment of the CNS. Identification of the specific cell type would shed light on the inflammatory pathway in EAE and would allow for a better therapeutic target for multiple sclerosis treatment.

In this chapter, we investigate a possible source of the relevant MIF. We first explore immune cells, since the pro-inflammatory properties of MIF and its involvement in EAE progression suggest an immune linkage. Next we turned our investigation to determining the specific immune cell producing MIF in EAE, starting with the likely source of T lymphocytes.

4.1 The Biologically Relevant MIF is Bone Marrow Derived.

To test our initial hypothesis that the relevant MIF in EAE is from an immune source, we created bone marrow chimera mice. The immune system is derived from the bone marrow. Our bone marrow chimera models allowed us to investigate the immune compartment separate from the nervous system, which is also involved in MS pathology. We started with a $MIF^{-/-}$x $RAG^{-/-}$ double knockout mouse (Figure 4.1A). $RAG^{-/-}$ mice are deficient in the recombination activating gene (RAG) which is necessary for proper B and T lymphocyte development. Thus, $RAG^{-/-}$ mice are deficient in B and T lymphocytes and $MIF^{-/-}$ x $RAG^{-/-}$ mice are deficient in MIF in addition to B and T lymphocytes. We irradiated the $MIF^{-/-}$x $RAG^{-/-}$ mice in order to ablate their immune systems. Immediately following the irradiation, we reconstituted the $MIF^{-/-}$x $RAG^{-/-}$ mice with bone marrow from a wild-type donor (WT→KO). After six weeks, allowing for reconstitution of the immune system, the $MIF^{-/-}$ mouse has the immune system of a wild-type mouse. The mouse remains deficient in MIF in the nervous system but now has MIF present in the immune compartment. The converse situation was also created by reconstituting a $RAG^{-/-}$ mouse with $MIF^{-/-}$ bone marrow (KO→WT). Controls were also included, by reconstituting a $MIF^{-/-}$x $RAG^{-/-}$ mouse with $MIF^{-/-}$bone marrow (KO→KO) and reconstituting a $RAG^{-/-}$ mouse with wild-type bone marrow (WT→WT).

After the chimeric mice were created, we immunized each group with MOG (35-55) to determine susceptibility to EAE (Figure 4.1B and C). The chimeric mice expressing MIF in their immune compartment (WT→KO and WT→WT) exhibited increased susceptibility regardless of MIF production in the nervous system. However, when MIF was absent from the immune system (KO→WT and KO→KO), the mice showed less severe clinical signs.

These results are shown in Table 4.1. The phenotype of the central nervous system is the same as the recipient mouse whereas the source of the antigen presenting cells and T lymphocytes is the same as the donor bone marrow. The shaded boxes highlight the involvement of MIF from bone marrow derived immune cells in EAE, showing disease is more severe when MIF is present in the bone marrow.

4.2 T Lymphocytes Alone Expressing MIF are not Sufficient for EAE Pathogenesis.

Determining that relevant MIF is bone marrow derived narrowed our focus to cells of the immune system. The immune system is divided into two important lineages: myeloid and lymphoid (Figure 4.2). The myeloid lineage gives rise to the antigen presenting cells, most noticeably dendritic cells and monocytes. On the other hand, the lymphoid lineage gives rise to B and T lymphocytes. We

continued our investigation with T lymphocytes because they were one of the early cell types linked to MIF production (David, 1967).

In order to determine whether T lymphocytes are the possible source of MIF, we utilized an adoptive transfer model (Figure 4.3A). We immunized a wild-type mouse with MOG (35-55) peptide and after fourteen days, isolated the T lymphocytes. Next, we re-activated the T lymphocytes *in vitro* with MOG (35-55) peptide. The re-stimulated T lymphocytes from the wild-type donor mouse were next adoptively transferred to a naïve $MIF^{-/-}$ mouse, resulting in a mouse that only produced and expressed MIF in T lymphocytes (WT→KO). The converse situation was created by adoptively transferring re-activated T lymphocytes from a $MIF^{-/-}$ mouse into a naïve wild-type mouse (KO→WT). Appropriate control mice were also included by adoptively transferring re-activated T lymphocytes from a wild-type mouse into a naïve wild-type mouse (WT→WT) and transferring re-activated T lymphocytes from a $MIF^{-/-}$ mouse into a naïve $MIF^{-/-}$ mouse (KO→KO). Mice in each group were monitored daily for clinical scores.

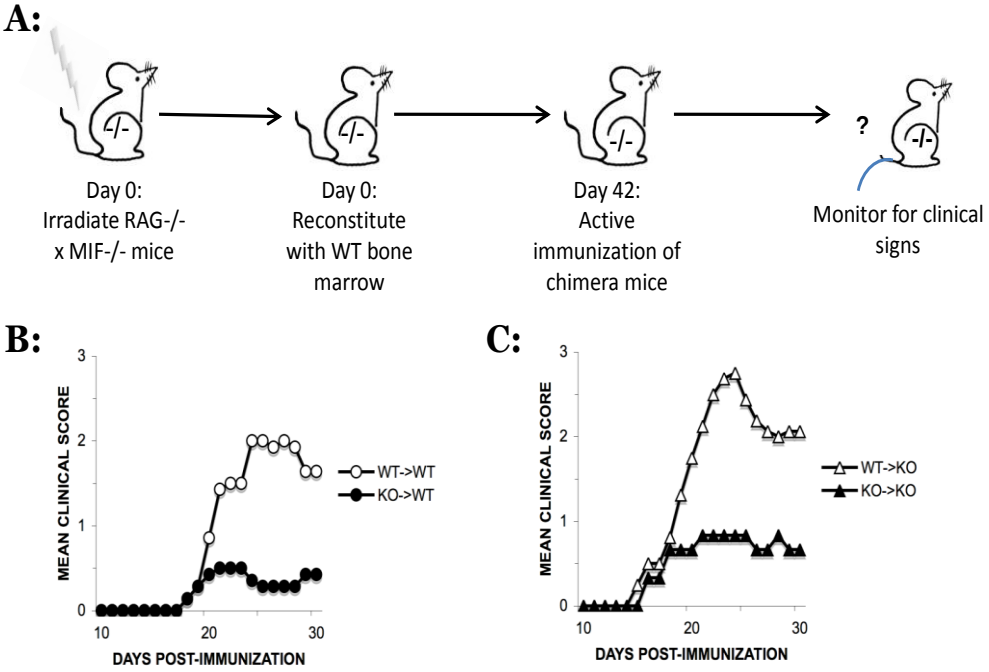
We observed significantly reduced clinical scores in the mice only expressing MIF in the T lymphocytes (WT→KO) when compared to the wild-type control group (WT→WT) (Figure 4.3B). We also observed increased susceptibility of wild-type mice receiving re-activated $MIF^{-/-}$ T lymphocytes (KO→WT) when compared to the knockout control group (KO→KO) (Figure 4.3C). These results are shown

in Table 4.2. In the above adoptive transfer experiments, we controlled the expression of MIF from T lymphocytes while allowing MIF expression to remain consistent between the central nervous system and the antigen presenting cells (APCs). The observed protection of the MIF knockout environment (CNS and APCs) regardless of MIF production by the T lymphocytes led us to conclude T lymphocytes are not the relevant source of MIF.

4.3 Discussion

We have previously shown MIF plays a role in the progression of EAE. Hypothesizing the relevant MIF has a specific cellular source, we investigated whether this source was derived from the immune system or the nervous system. We utilized a chimera system to investigate MIF from the nervous system and immune system. Our results showed that MIF from the bone marrow was critical for EAE progression. With this result, our focus narrowed to the myeloid and lymphoid compartments within the bone marrow. Given the strong connections between MIF production and T lymphocytes taken with the undisputed connections between T lymphocytes and EAE, we first investigated the impact of MIF production by T lymphocytes. Adoptive transfer experiments showed that MIF production by T lymphocytes was unnecessary for EAE susceptibility. Taken together, these results allow us to conclude that the relevant source of MIF is bone marrow derived, yet not from T lymphocytes.

In the ensuing chapters, we will continue to investigate the lymphoid lineage to determine the relevant source of MIF. The second important lymphocyte in EAE pathology is the B lymphocyte, which will be explored next.



<u>Figure 4.1:</u> The expression of MIF by bone marrow derived cells is required for the progression of EAE.
A: Wild-type and MIF-deficient RAG knockout mice were irradiated and reconstituted with wild type or MIF-deficient bone marrow. Six weeks later (to allow for adequate reconstitution), mice were immunized with MOG(35-55) peptide and monitored daily for clinical signs.
B: MIF produced from bone marrow derived cells was sufficient to induce EAE but MIF knockout bone marrow in wild-type recipients greatly reduced susceptibility to EAE.
C: Wild-type bone marrow increased susceptibility to EAE in knockout recipient mice.
Data are representative of two separate experiments (n=5 per group per experiment)

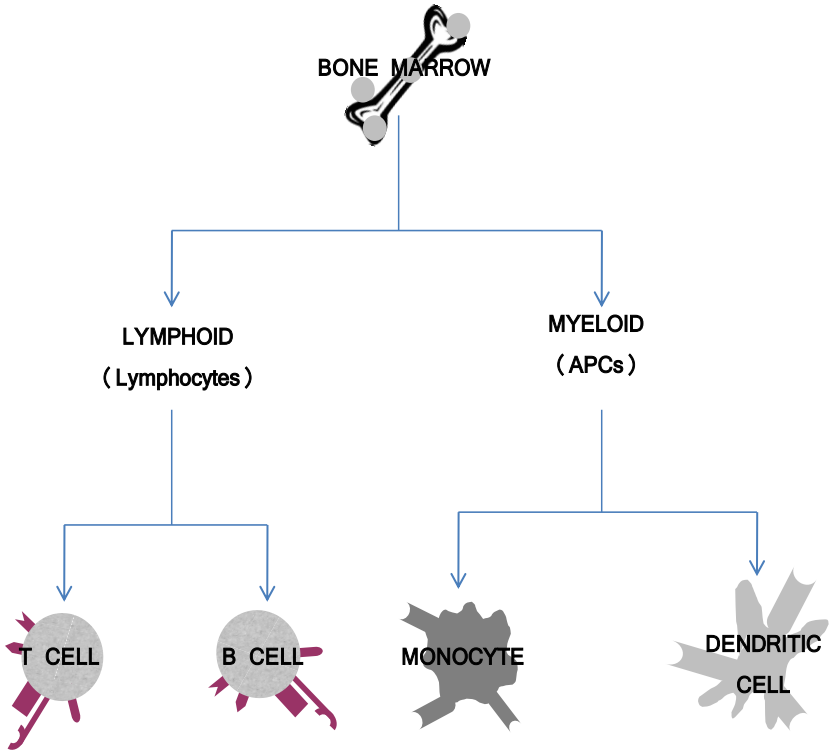
*This work was done in collaboration with Aaron P. Kithcart, PhD.

	CNS	APCs	T cell	EAE
WT→ KO	NO MIF	MIF	MIF	YES
WT→WT	MIF	MIF	MIF	YES
KO→KO	NO MIF	NO MIF	NO MIF	NO
KO→WT	MIF	NO MIF	NO MIF	NO

CNS: central nervous system
APCs: antigen presenting cells

Table 4.1: The expression of MIF from bone-marrow derived cells was sufficient for the induction of EAE.
Bone marrow chimeric mice expressing MIF in bone marrow derived cells are susceptible to EAE.

*This work was done in collaboration with Aaron P. Kithcart, PhD.



APCs: antigen presenting cells

Figure 4.2: Bone Marrow Derived Lineages and Cell Types
Bone marrow derived cells serve as the source for the lymphoid and myeloid compartments. The lymphoid lineage gives rise to lymphocytes, namely B and T lymphocytes. The myeloid line gives rise to antigen presenting cells, such as monocytes and dendritic cells.

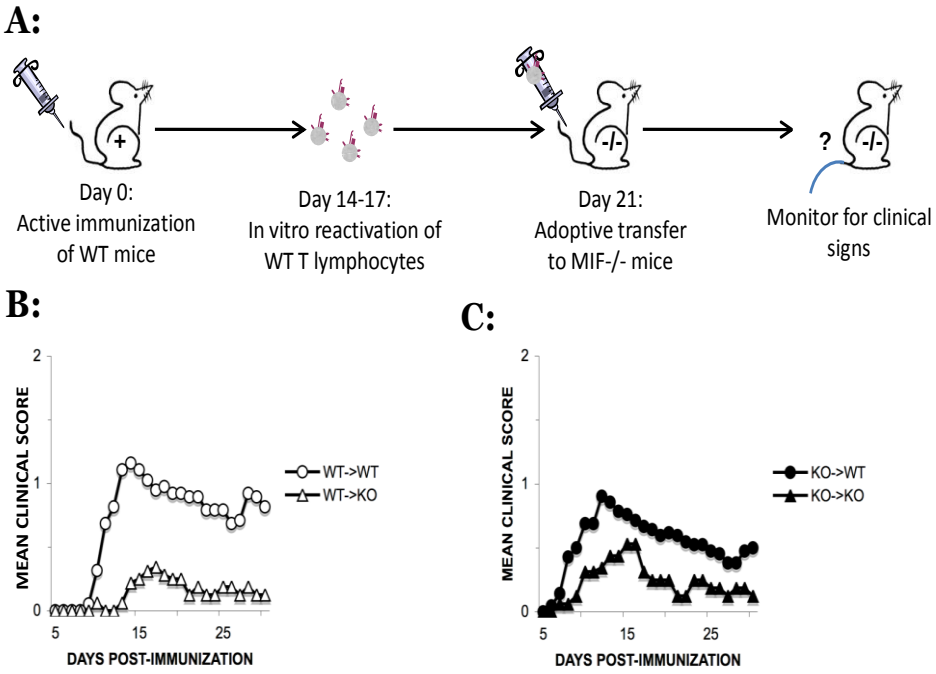


Figure 4.3: T lymphocytes alone expressing MIF are not sufficient to induce EAE

A: Following 72 hours in vitro reactivation, $10x10^6$ T lymphocytes from wild-type or MIF deficient mice were adoptively transferred into naïve recipient wild-type or MIF knockout mice.

B: Wild-type T lymphocytes were unable to induce EAE in MIF-deficient recipients.

C: MIF deficient T lymphocytes could induce EAE in wild-type recipient mice.

Data are representative of four separate experiments (n=5 per group per experiment)

*This work was done in collaboration with Aaron P. Kithcart, PhD.

	CNS	APCs	T cell	EAE
WT→ WT	MIF	MIF	MIF	YES
KO→WT	MIF	MIF	NO MIF	YES
WT→KO	NO MIF	NO MIF	MIF	NO
KO→KO	NO MIF	NO MIF	NO MIF	NO

CNS: central nervous system
APCs: antigen presenting cells

Table 4.2: MIF is required in recipient mice for EAE induction via adoptive transfer with T lymphocytes.
Expression of MIF from CNS and antigen presenting cells was required for adoptive transfer of EAE by activated leukocytes.

*This work was done in collaboration with Aaron P. Kithcart, PhD.

Chapter 5

MIF DEFICIENT MICE HAVE A MODIFIED B LYMPHOCYTE POPULATION

After determining that the relevant cellular source of MIF is bone marrow derived and eliminating T lymphocytes as the cell of origin, we continued our investigation of the lymphoid lineage of the immune system. The role of B lymphocytes in EAE and MS is unclear but has recently emerged as an area of interest with the recent success of Rituximab (Hauser S., 2008). Rituximab is a monoclonal antibody against CD20, which is a specific human B lymphocyte marker. Thus, the significant improvement of MS patients taking Rituximab argues for a role of B lymphocytes in MS pathology.

Additionally, recent findings suggest that MIF may be important for B lymphocyte maturation and development (Gore Y., 2008). MIF has been shown to be important for the maintenance of the mature B lymphocyte population active during the adaptive immune response, which is the primary form of response in the autoimmune attack of MS. In this chapter, we will first explore B lymphocyte maturation in an MIF knockout mouse compared to a wild-type mouse. Thereafter, we will explore the proliferative properties of B lymphocytes in an MIF deficient environment compared to a wild-type environment. Because it has been suggested that MIF provides survival signals to B lymphocytes, we

hypothesize that mature B lymphocytes will be fewer in number and less effective at proliferating.

5.1 MIF Deficient Mice Have Fewer Mature B Lymphocytes

We compared the B lymphocyte population in MIF knockout relative to wild-type mice. We analyzed three stages in B lymphocyte development (pro-B, immature B and mature B lymphocytes) in addition to a pan B lymphocyte marker. Figure 5.1 shows the three major stages of a B lymphocyte which we characterized. Total B lymphocyte percentages are represented as the percentage of the total live cell population that are B220 positive, which is considered a pan B lymphocyte marker. Pro-B lymphocytes were identified as B220 positive cells which were negative for IgM and IgD. Immature B lymphocytes were identified as B220 positive cells which were IgM positive and IgD negative. Mature B lymphocytes were identified as B220 positive cells which were positive for both IgM and IgD.

We compared the B lymphocyte population of naïve MIF knockout mice and naïve wild-type mice in three immune organs: the lymph nodes, the bone marrow, and the spleen. No significant differences were found in lymph nodes between an MIF deficient and a naïve wild-type mouse (Figure 5.2A).

The bone marrow showed significantly fewer B lymphocytes in the naïve MIF knockout mouse (Figure 5.2B). A larger percent of the total cell population (30.2%) was B220 positive (indicating B lymphocytes) in the wild-type mouse compared to 18.8 percent in the MIF knockout mouse ($p<0.05$).

The trend observed in the bone marrow of fewer overall B lymphocytes, more pro-B lymphocytes, and fewer mature B lymphocytes in the MIF deficient environment was much more dramatic in the spleen (Figure 5.2C). The B lymphocyte percentage in the MIF knockout mice was less than half that of the wild-type control (26.3 percent verse 55.8 percent, respectively; $p<0.05$). Even more striking was the difference in the B lymphocyte subtypes. 94.6 percent of the B lymphocyte population was positive for the pro-B lymphocyte markers in the MIF knockout compared to only 2.6 percent in the wild-type control ($p<0.001$). Interestingly, the mature B lymphocyte population of the MIF knockout mice was significantly reduced when compared to the wild-type controls (1.83% versus 91.0%, respectively; $p<0.001$).

These results support our hypothesis that MIF is involved in the survival of B lymphocytes. The MIF deficient mice had fewer mature B lymphocytes and more pro-B lymphocytes leading us to conclude MIF is involved in B lymphocyte survival.

5.2 B Lymphocytes Proliferate Less in the Bone Marrow of MIF Deficient Mice

After determining that MIF deficient mice have fewer mature B lymphocytes, we investigated the *in vivo* proliferative ability of B lymphocytes in a MIF deficient and wild-type environment. To explore this, we used CFSE (a fluorescent dye), which is incorporated into B lymphocytes and which fluoresces less with each successive cell division. This model is depicted in Figure 5.2A. First, we isolated B lymphocytes from wild-type donors (>90% purity). Next, we labeled the purified B lymphocytes with the CFSE dye and transferred them intravenously into either $MIF^{-/-}$x $RAG^{-/-}$ double knockout or $RAG^{-/-}$ knockout recipient mouse. In addition to the stained B lymphocytes, non-labeled wild-type T lymphocytes were transferred into the recipient mice to allow for proper EAE induction. Immediately following the cell transfer, the recipient mice were immunized with MOG (35-55). Seven days post immunization, the recipient mice were sacrificed and proliferation of the CFSE positive B lymphocytes was analyzed in the three immune organs (the lymph nodes, bone marrow, and spleen). Control mice were included which were not immunized.

B lymphocyte proliferation in the non-immunized control mice (black line in Figure 5.3B, C, and D) shows slightly less CFSE peaks corresponding to less proliferation. When comparing the proliferative ability of B lymphocytes in a MIF deficient environment (green line, Figure 5.3B, C, and D) compared to a

wild-type environment (orange line, Figure 5.3B, C, and D), a noticeable difference is apparent in the bone marrow (Figure 5.3D). The B lymphocytes proliferate less in the bone marrow of a MIF deficient mouse. This result corresponds with our previous finding that the source of relevant MIF is bone marrow derived. Thus, in a MIF deficient mouse, B lymphocyte proliferation is impaired in the bone marrow, perhaps offering the protection observed of the MIF deficient mice when immunized for EAE.

5.3 Discussion

The role of B lymphocytes in MS is currently poorly understood, yet there are many diverse hypotheses to explain the role of B lymphocytes in EAE and MS (M.K., 2008). The vast majority of these hypotheses implicate B lymphocytes in the autoimmune response through antigen presentation, auto-antibody production, or cytokine production. B lymphocytes in EAE could aid the inflammation process through recruitment of the immune cells via cytokine production and antibody tagging. MIF may be the key cytokine mediator of B lymphocytes, which causes the severe inflammation of EAE and mediates the damage to the CNS.

We have shown that the mature B lymphocyte population is modified in a MIF deficient environment. Fewer mature B lymphocytes are present in a MIF deficient mouse and the proliferative ability of these B lymphocytes is impaired.

The results argue that without MIF present in the bone marrow, B lymphocytes are unable to proliferate sufficiently. This would result in fewer mature B lymphocytes, which may be the source of protection.

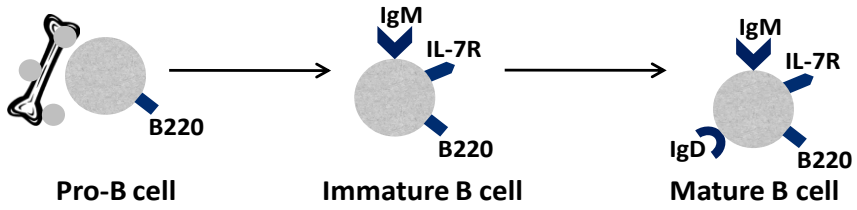


Figure 5.1: B lymphocyte maturation markers
The above cell markers were used to identify the B lymphocyte cell subtypes at the different stages of development. Pro-B lymphocytes were identified as IgM-/IgD- gated on the B220+ cell population. Immature B lymphocytes were identified as IgM+/IgD- gated on the B220+ cell population. Lastly, mature B lymphocytes were identified as IgM+/IgD+ gated on the B220+ cell population.

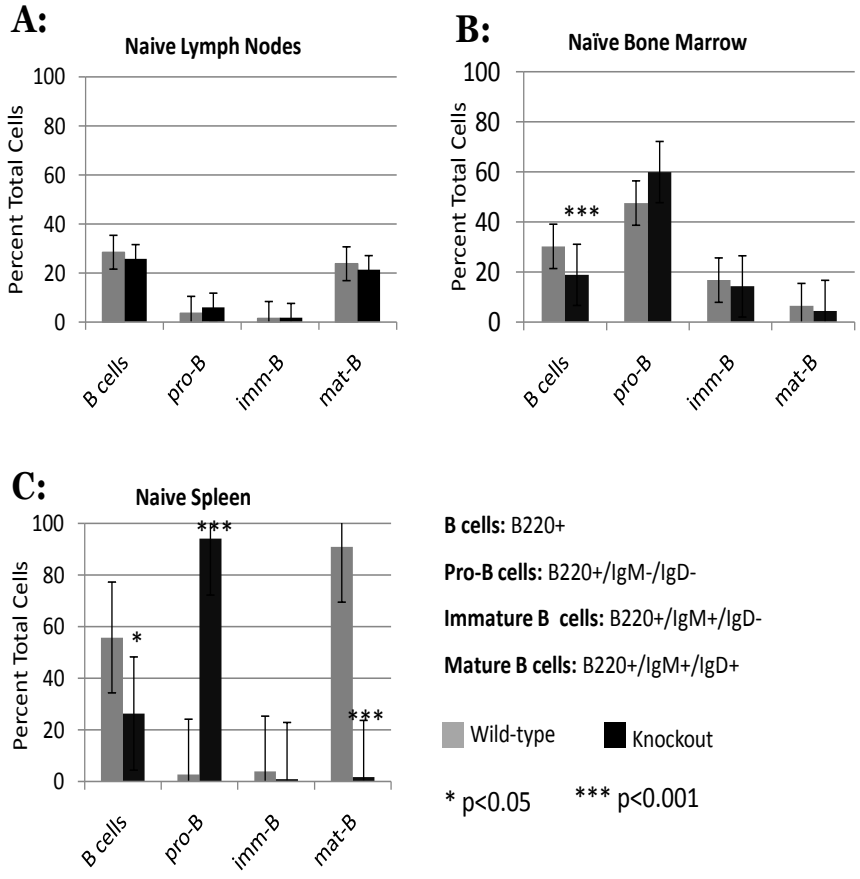


Figure 5.2: MIF deficient mice have fewer mature B lymphocytes than wild-type counterparts

A,C, and D: Lymph nodes (A), bone marrow (C), and spleens (D) were removed from naïve MIF deficient mice and wild-type mice. B cell percentages are gated on total cell population. Pro-, immature, and mature B cell numbers are gated on total B cell population. n=3 for each group.

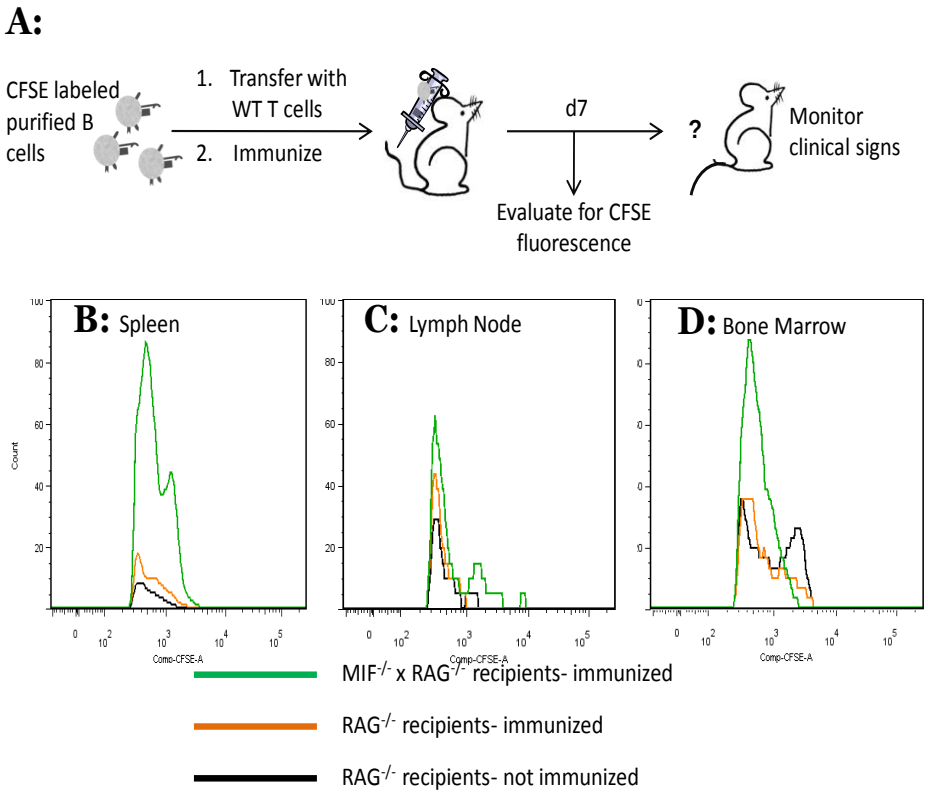


Figure 5.3: B cells proliferate less in the bone marrow of MIF knockout mice

Purified B cells (>90% purity) were CFSE labeled and transferred into MIF$^{-/-}$ x RAG$^{-/-}$ or RAG$^{-/-}$ recipients (A). The recipients were immunized with 200 ug MOG(35-55) in adjuvant. Seven days post immunization, spleens (B), lymph nodes (C), and bone marrow (D) were isolated and evaluated for CFSE fluorescence. Non-immunized mice were used as a negative control (black line). Fewer peaks corresponding to less proliferation is seen in the bone marrow of MIF knockout mice. Data are representative of two separate experiments, n=4 for each group.

Chapter 6

IN VIVO MIF PROUCTION BY B LYMPHOCYTES

We have shown that the B lymphocyte population is different in the MIF deficient environment. We next evaluated the in vivo contribution of MIF production by B lymphocytes during ongoing EAE. We hypothesized that B lymphocytes are the cell source through which MIF mediates the crucial pro-inflammatory effects during ongoing EAE. Recent studies show MIF signals through the Akt pathway (Lue H., 2007). This pathway is autocrine in nature, suggesting B lymphocytes may be responsive to the MIF they themselves produce. In this manner, B lymphocytes could represent an ideal mediator of inflammation and therapeutic target for MS. After trafficking to the site of inflammation in the CNS, it is plausible that B lymphocytes could be activated by the local cytokine milieu to produce MIF which could then act back on the B lymphocytes in an autocrine fashion for further activation or antibody production (Figure 6.1). We have previously eliminated the T lymphocytes as the relevant cellular source of MIF and are currently proposing an autocrine mechanism for MIF produced by B lymphocytes.

6.1 EAE Can Be Induced in a $RAG^{-/-}$ Mouse

In order to further investigate the *in vivo* MIF production by B lymphocytes, we utilized the $RAG^{-/-}$ mouse. To test our question regarding the autocrine nature of

MIF, we felt it important to immunize $RAG^{-/-}$ mice rather than transferring disease through an adoptive transfer model. Since $RAG^{-/-}$ mice are deficient in both B and T lymphocytes, the immunization process with MOG (35-55) had to be investigated.

T lymphocytes are crucial for EAE induction (Noseworthy J. W., 2000), thus we began our studies with the transfer of purified T lymphocytes (>95% purity) into the $RAG^{-/-}$ mouse before immunization with MOG (35-55) (Figure 6.2A). We studied the i.p. injection of T lymphocytes immediately before (data not shown) or two days prior to immunization (Figure 6.2B). Injection of the T lymphocytes into the intraperitoneal cavity immediately followed by immunization was not successful in inducing EAE. We suspect this is because the T lymphocytes were not given adequate time to traffic to the proper locations in order to be in position to mount the necessary immune response for EAE. However, the i.p. injection of the T lymphocytes two days prior to immunization was sufficient for EAE induction (Figure 6.2B). Injection of $5x10^6$ T lymphocytes resulted in the highest cumulative disease index and peak score (37.5 and 3.83, respectively; Table 6.1). Controls included immunizing $RAG^{-/-}$ mice without transferring T lymphocytes. Additionally, we included $RAG^{-/-}$ mice receiving the T lymphocyte transfer but not immunized with MOG (35-55). EAE failed to develop in either of these two control groups. However, the day of onset for all experimental groups was much later than expected for EAE (Gold, 2000). Although we could

successfully induce EAE in a $RAG^{-/-}$ mouse, we desired a more robust disease course and next investigated the intravenous injection route.

We intravenously transferred T lymphocytes into $RAG^{-/-}$ recipients and immediately immunized for EAE on the same day as the transfer (Figure 6.2C). With this protocol, the day of onset of clinical signs was observed at a much earlier time point (17.5 versus 40 days post immunization; Table 6.1). The intravenous transfer of $1x10^6$ purified T lymphocytes resulted in the most pronounced EAE course with a cumulative disease index of 73.8 and a peak score of 3.25 (Table 6.1).

To confirm the presence of T lymphocytes in the CNS, we performed staining of the CD4+ T lymphocytes in the brain and spinal cord tissue (Figure 6.3). $1x10^6$ purified T lymphocytes were transferred i.v. into a $RAG^{-/-}$ mice. Immunization with MOG (35-55) immediately followed the transfer. The infiltration of T lymphocytes in the usually immune privileged CNS tissue is a hallmark of EAE and MS pathology. Thus, the observed clinical scores are supported by the infiltration of T lymphocytes in the CNS.

After comparing both methods of EAE induction, via i.p. or i.v. injections, it became apparent that the i.v. injection of $1x10^6$ purified T lymphocytes was the most pathogenic and feasible with respect to the number of necessary donor cells needed. Since this method resulted in the reliable induction of EAE, we will use

this protocol in the following sections to investigate the autocrine MIF production by B lymphocytes.

6.2 The Most Pathogenic Ratio is 2:1 T:B Lymphocytes

After determining a method to induce EAE in a $RAG^{-/-}$ mouse, it was necessary to incorporate B lymphocytes into our protocol. In order to do this, we first determined the ideal ratio of T and B lymphocytes to induce high activation and proliferation of the lymphocytes. We purified T (>95% purity) and B (>90% purity) lymphocytes and co-cultured them in varying ratios (Figure 6.4). The co-cultures were treated with anti-CD3, a stimulus to mimic the *in vivo* activated environment during EAE. Controls included un-stimulated cells, which lacked anti-CD3 stimulation (data not shown). The cell cultures were analyzed for activation markers (Figure 6.4) and proliferation (Figure 6.5). The activation markers evaluated by flow cytometry were CD25 (Figure 6.4B) for T lymphocytes and CD80 (Figure 6.4C) for B lymphocytes. Both ratios of 1:1 and 2:1 T:B lymphocytes yielded a high percentage of activation markers (Figure 6.4).

In addition to the activation markers, we evaluated the cultures for proliferation. The T lymphocytes were stained with the fluorescent dye CFSE, which decreases in intensity with each successive cell division. At 48 hours, the 2:1 T:B lymphocyte data expressed the greatest shift corresponding to the greatest extent of proliferation (Figure 6.5). This data, taken with the above activation marker

data, shows that the most potentially pathogenic ratio for our EAE studies is the 2:1 T:B lymphocyte ratio. Interestingly, this is the approximate observed ratio of lymphocytes in the human spleen (Peterson L.K., 2008).

6.3 MIF Present in B Lymphocytes Alone is not Sufficient to Induce EAE.

After establishing a method to reliably induce EAE in a $RAG^{-/-}$ mouse and determining the optimum lymphocyte ratio, we narrowed our focus to the *in vivo* autocrine MIF production by B lymphocytes. To create a mouse model where the only source of MIF is B lymphocyte derived, we first purified T lymphocytes from an $MIF^{-/-}$ donor mouse and B lymphocytes from a wild-type donor mouse (Figure 6.6A). The purified cells were next transferred via i.v. injections into an $MIF^{-/-}$ x $RAG^{-/-}$ recipient mouse and this mouse was immunized for EAE with MOG (35-55). The only source of MIF in this mouse model was from the B lymphocytes. Control groups were also immunized (Figure 6.6).

We found that the ability of B lymphocytes to produce MIF was not sufficient for EAE induction. Mice with MIF present in the recipient mouse experienced more severe EAE regardless of the ability of B lymphocytes to produce MIF (Figure 6.6B). This allowed us to conclude that MIF production by B lymphocytes is not the relevant source of MIF in EAE.

6.4 MIF Production by B and T Lymphocytes Together is not Sufficient for EAE Progression

To confirm that MIF produced by T lymphocytes does not act on B lymphocytes thereby representing the relevant source of MIF in EAE, we developed a second mouse model in which the ability to produce MIF was kept constant between the donor B and T lymphocytes (Figure 6.7A). With this model, the MIF produced by the T lymphocytes could be the necessary MIF for the B lymphocytes or vice versa. However, these results too showed that the MIF knockout recipients exhibited a milder course of EAE (Figure 4B). Thus, we can conclude that relevant source of MIF in EAE is not lymphocyte derived.

6.5 Discussion

MIF has been shown to activate the Akt pathway which has an autocrine mechanism. We show that MIF production by B lymphocytes alone is not sufficient to restore EAE susceptibility in an MIF deficient mouse. We have also shown previously that T lymphocytes are not the relevant source of MIF by themselves or in combination with B lymphocytes.

One potential caveat to this study is that B lymphocytes are not critical to the induction of EAE with the MOG (35-55) peptide and therefore the role of MIF production by B lymphocytes may be masked in our model. However if this were the case, we would not expect to observe the protection seen in the MIF

deficient mice. When naïve MIF deficient mice are immunized with MOG (35-55) peptide, susceptibility to EAE is greatly reduced. Thus, if our model were masking the MIF contribution of B lymphocytes, we would expect to observe this masking in the MIF deficient mouse as well. We do not think immunizing with MOG (35-55) is masking the role of B lymphocytes with respect to MIF production.

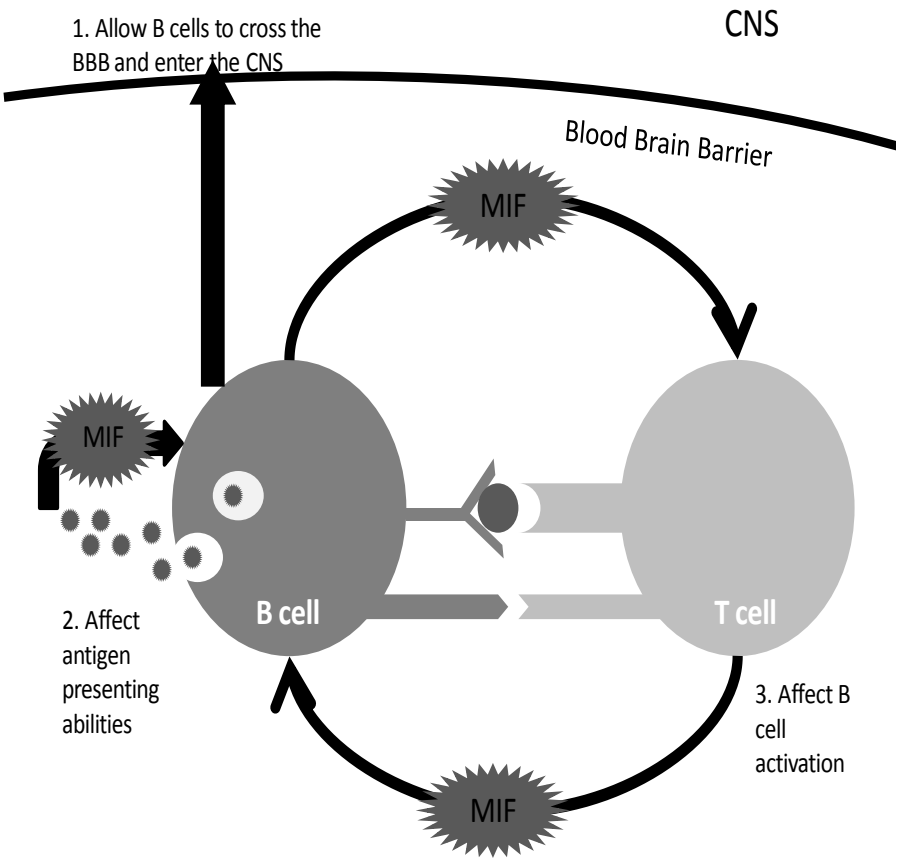


Figure 6.1: Possible Roles of B Lymphocytes after MIF activation
Though we have previously shown that MIF produced by T lymphocytes is not sufficient to induce EAE, we propose MIF acts in an autocrine manner to affect B lymphocytes. Possible results of B lymphocyte activation by MIF is enhanced antigen presentation enhanced trafficking into the central nervous system (CNS), or enhanced T lymphocyte activation.

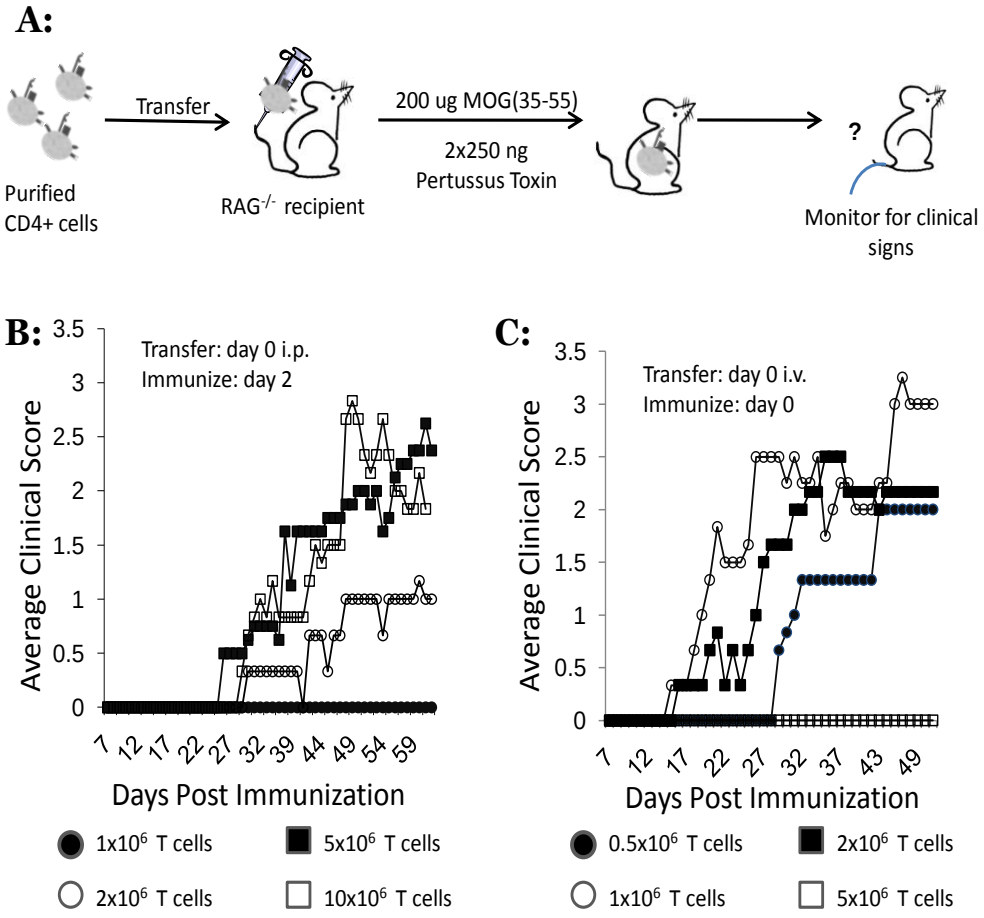
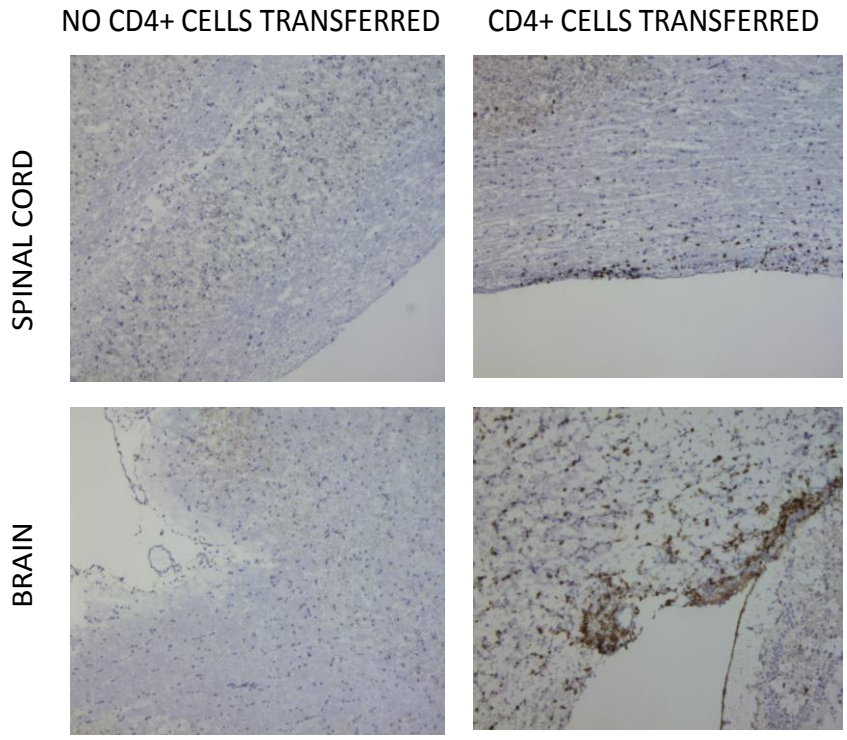


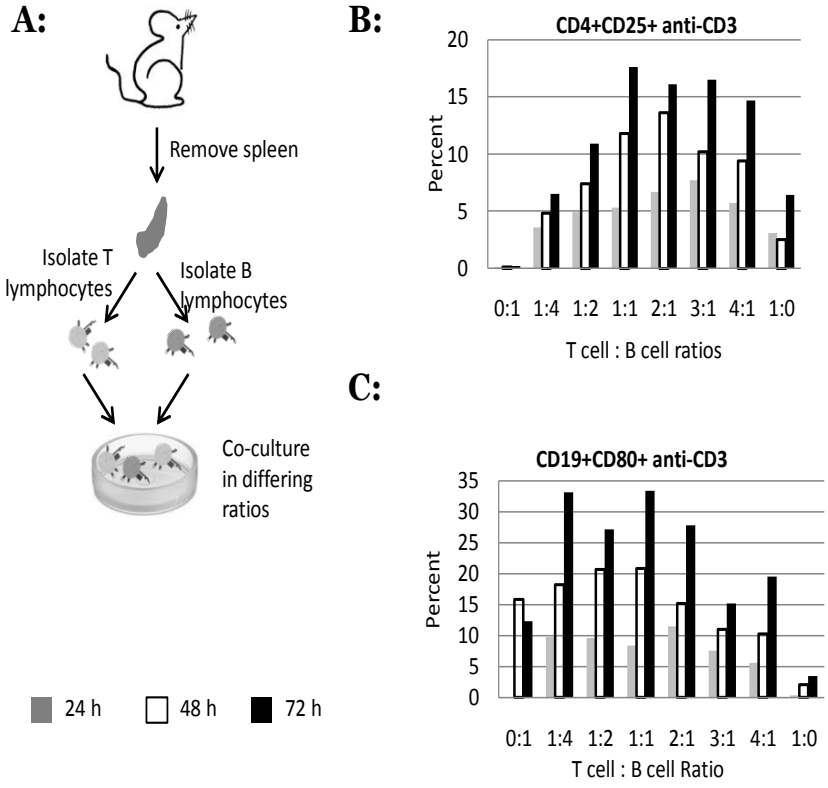
Figure 6.2: EAE can be induced in a RAG$^{-/-}$ mouse.
A: EAE induction in a RAG knock out. T lymphocytes (>95% purity) were transferred either i.p. (B) or i.v. (C) into RAG$^{-/-}$ mice 2 days prior to or immediately before active immunization with MOG (35-55) peptide.
B: CD4+ T lymphocytes (>95% purity) were transferred i.p. into RAG$^{-/-}$ mice 2 days prior to active immunization with MOG (35-55) peptide. The day of onset was late compared to a wild-type active immunization (data not shown). n=4 for each group.
C: CD4+ T lymphocytes (>95% purity) were transferred i.v. into RAG$^{-/-}$ mice immediately prior to active immunization with MOG (35-55) peptide. 1.0x10^6 transferred CD4+ T lymphocytes exhibited typical day of onset and disease course. n=3 for each group.

# CD4+ wild-type cells	INCIDENCE	ONSET[a]	CDI[b]	PEAK SCORE[c]
$0.5x10^6$ iv	33%	29 ± 13.7	ND	ND
$1.0x10^6$ ip	0%	ND	ND	ND
$1.0x10^6$ iv	67%	17.5 ± 8.50	73.8 ± 12.8	3.25 ± 0.75
$2.0x10^6$ ip	50%	35.5 ± 18.50	9.7 ± 6.8	1.75 ± 0.75
$2.0x10^6$ iv	67%	20.5 ± 10.33	51.8 ± 39.6	3.75 ± 0.25
$5.0x10^6$ ip	75%	40 ± 20.35	37.5 ± 28.2	3.83 ± 0.24
$5.0x10^6$ iv	0%	ND	ND	ND
$10.0x10^6$ ip	100%	36 ± 7.93	23 ± 18.7	3.0 ± 0.71

Table 6.1: EAE can be induced in a RAG$^{-/-}$ mouse.
$1.0x10^6$ CD4+ T lymphocytes (>95% purity) transferred intravenously into RAG$^{-/-}$ mice immediately prior to active immunization with MOG (35-55) peptide have increased incidence, severity of EAE, and earlier day of onset of disease.
[a] Day of onset was calculated as the mean of the first day of clinical scores among mice that developed EAE, ±SD.
[b] Cumulative disease index (CDI) was calculated as the sum of clinical scores day 7-50 per animal and averaged within each group, ±SD
[c] Peak score was measured over the duration of disease per animal and averaged, ±SD.
ND= not determined due to low incidence.

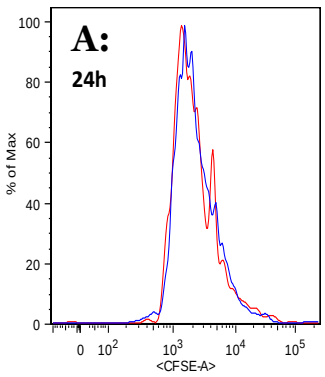


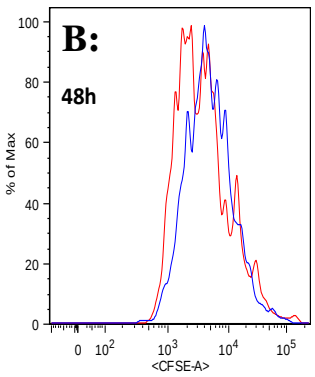
<u>Figure 6.3</u>: Transferred T lymphocytes are appropriately observed in the central nervous system
CD4+ staining confirms the presence of T lymphocytes in the CNS. CNS tissue was isolated on day 26 post active immunization and i.v. transfer of $1x10^6$ CD4+ T lymphocytes.

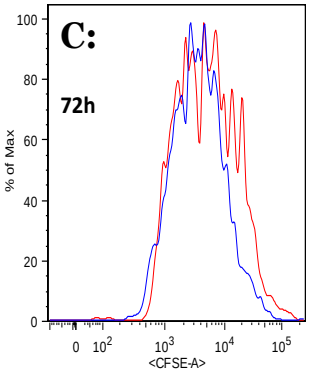


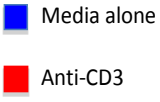
<u>Figure 6.4</u>: 2:1 T:B cell ratio is most pathogenic ratio for lymphocyte activation markers.

Purified B and T lymphocytes were co-cultured for 24, 48, and 72 hours in differing ratios and stimulated with media alone (B, D) or anti-CD3 supplemented media (C,E). The presence of CD4+CD25+ (activated T lymphocytes) and CD19+CD80+ (activated B lymphocytes) cells were evaluated via flow cytometry. A ratio of 2: 1 T : B cell resulted in the overall greatest pathogenic phenotype as evidenced by the high percentage of activated T and B lymphocytes. n=3 for each group









D:

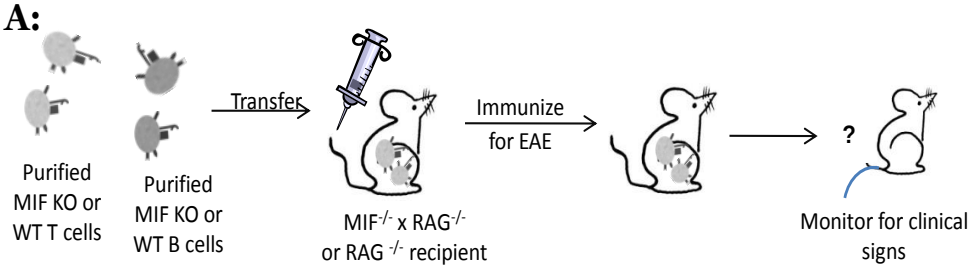
1:1 T:B	24h	48h	72h
Media Alone	3	5	6
Anti-CD3 stimulated	4	6	6

E:

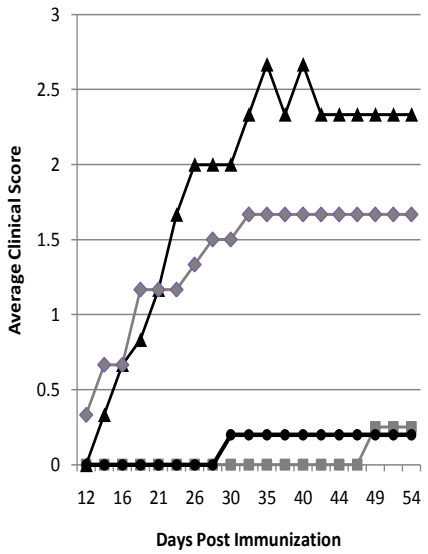
1:1 T:B	24h	48h	72h
Media Alone	5	6	6
Anti-CD3 stimulated	5	9	4

Figure 6.5: 48h is most pathogenic time point for 2:1 T:B ratio. Histograms representing CFSE peaks at 24 (A), 48 (B), and 72 (C) hours from the 2:1 T cell: B cell group. Anti-CD3 stimulated media shows greater shifts, more distinct peaks, and a greater number of peaks corresponding to a greater degree of proliferation. More peaks were observed in the 2:1 T:B cell ratio when compared to the other cell ratios (data not shown.)

A:



B:



Shape	Donor B cells	Donor T cells	Recip-eint	Inci-dence	CDI[a]	Peak Score[b]
▲	YES MIF	NO MIF	YES MIF	4/6	34.7	2.7
◆	NO MIF	YES MIF	YES MIF	4/6	26.2	2.5
●	NO MIF	YES MIF	NO MIF	2/5	2.2*	0.2*
■	YES MIF	NO MIF	NO MIF	1/5	0.7*	0.2*

Figure 6.6: MIF present in B lymphocytes alone can not induce EAE in a MIF knockout recipient.

A: Purified T lymphocytes (>95% purity) and purified B lymphocytes (>90% purity) of opposite donors (MIF knockout or wild-type) were in intravenously transferred into MIF$^{-/-}$ x RAG$^{-/-}$ or RAG$^{-/-}$ recipients. Immediately following transfer, the mice were immunized for EAE. Clinical scores were observed daily for days 10 through 42.

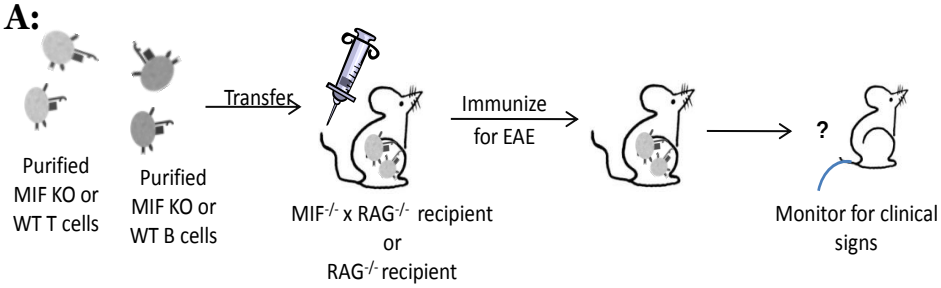
B: Clinical scores of the different groups shown in (C)**.** Regardless of MIF production by B lymphocytes, recipients with MIF in the environment were more susceptible to EAE.

[a] Cumulative disease index (CDI) was calculated as the sum of clinical scores during experiment (days 12-54) per animal and averaged within each group.

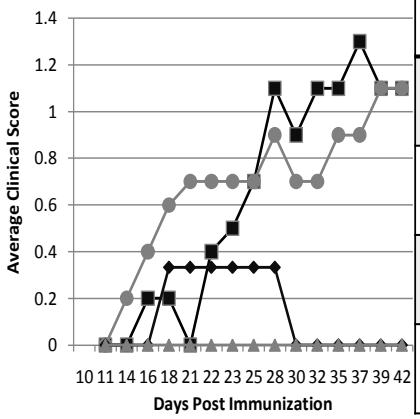
[b] Peak score was measured over the duration of the experiment (days 12-54) per animal and averaged within each group.

* $p<0.05$ for groups compared to groups wild-type recipients.

A:



B:



Shape	Donor B cells	Donor T cells	Recip-eint	Inci-dence	CDI[a]	Peak Score[b]
■	NO MIF	NO MIF	YES MIF	3/5	9.7	1.3
●	YES MIF	YES MIF	YES MIF	3/5	10.0	1.1
◆	YES MIF	YES MIF	NO MIF	1/5	2.0*	0.3*
▲	NO MIF	NO MIF	NO MIF	0/5	0.0*	0.0*

Figure 6.7: MIF present in both T and B lymphocytes can not induce EAE in a MIF knockout recipient.

A: Purified T lymphocytes (>95% purity) and purified B lymphocytes (>90% purity) of the same donor were in intravenously transferred into MIF$^{-/-}$ x RAG$^{-/-}$ or RAG$^{-/-}$ recipients. Immediately following transfer, the mice were immunized for EAE. Clinical scores were observed daily for days 10 through 42.

B: Clinical scores of the different groups shown in (C). Regardless of the lymphocyte phenotype, recipients with MIF in the environment were more susceptible to EAE.

[a] Cumulative disease index (CDI) was calculated as the sum of clinical scores during experiment (days 11-42) per animal and averaged within each group.

[b] Peak score was measured over the duration of the experiment (days 11-42) per animal and averaged within each group.

* $p<0.05$ for groups compared to groups with wild-type recipients.

Chapter 7

DISCUSSION AND CONCLUSIONS

Previous findings in our laboratory have shown that MIF is important for the progression of EAE. MIF deficient mice exhibit less severe clinical signs during the induction phase of EAE with little progression thereafter and some mice recovering completely. The protection seen in the MIF deficient mice led us to explore the efficacy of MIF inhibitors. MIF would be a novel therapeutic target since a specific cytokine is yet to be identified as a mediator in MS pathology. The majority of the current therapies focus on managing the inflammatory response and nearly all are immunosuppressive drugs. Additionally, none of the current therapies are approved for oral administration. Thus, the oral administration of a specific MIF inhibitor would represent a significant therapeutic advancement.

We investigated the efficacy of several small molecule inhibitors of MIF, as well as the dosing requirements and route of administration. We demonstrated that the pro-inflammatory and autoimmune disease-enhancing effects of MIF are reversible with a small molecule MIF inhibitor. Several different MIF inhibitors were shown to be effective at treating EAE by reducing the clinical scores relative to the vehicle controls and other stereoisomers. We observed the inhibitor to be effective at doses as low as 1.0 mg/kg when administered orally. Taken together,

the inhibitor studies pinpoint MIF as a viable therapeutic target for the treatment of MS. The reversible effects show that the reduction of MIF levels can be effective after onset of disease, representing a clinically relevant time point when patients would first see a neurologist for their symptoms. Additionally, the low dose greatly adds to the safety of the inhibitors and may also suggest a level of specificity with which MIF mediates the progression of disease. Lastly, we observed that the inhibitor was effective when administered orally, representing a novel characteristic for an MS therapy. However, a more specific MIF inhibitor targeted to a defined and critical cell type may effectively treat MS and reduce side effects from treatment. Global inhibition of MIF may weaken the immune system, causing increased susceptibility to infectious agents. MIF may have an important immune function in other systems as it may be required for immune surveillance.

The next goal was to identify the relevant source of MIF in EAE. The ubiquitous expression of MIF taken together with the low dosing requirement led us to hypothesize that the source of biologically relevant MIF in EAE is a specific immune cell. Through a series of bone marrow chimera experiments, we showed that the source of the relevant MIF is bone marrow derived. This finding allowed us to exclude the nervous system as the contributor, a viable candidate due to the intricate interaction of the immune system with the nervous system in MS. Narrowing our focus to the immune compartment, we hypothesized that T

lymphocytes represented the most likely candidate to produce the relevant MIF. T lymphocytes were one of the first described cell types in MIF production and are critical in MS pathology. Thus, we performed a series of adoptive transfer experiments to determine if MIF production by T lymphocytes alone was sufficient to induce disease. We found that it alone was not sufficient. Activated T lymphocytes from a wild-type donor mouse were unable to restore EAE susceptibility in a MIF knockout mouse, showing that MIF production by T lymphocytes is not critical for EAE pathology. However, the recipient environment of the mouse proved important. If MIF was absent from the recipient, the mouse was less susceptible to EAE.

Following the elimination of T lymphocytes as the relevant source of MIF, we explored B lymphocytes, another bone marrow derived cell, as potentially relevant in the progression of EAE. We first evaluated the B lymphocyte population in MIF deficient mice, hypothesizing that if MIF is important for B lymphocyte function we would observe a difference in the B lymphocyte population of an MIF deficient mouse relative to a wild-type mouse. MIF deficient mice have fewer mature B lymphocytes and more immature B lymphocytes when compared to wild-type mice. This difference was most pronounced in the spleen, where the final stages of B lymphocyte maturation occur. The difference in B lymphocyte numbers suggests either a critical survival or maturation mechanism is absent in an MIF deficient environment. Since the

relevant MIF is bone marrow derived and B lymphocyte maturation occurs in the bone marrow, we hypothesize a critical maturation signal is absent during the early stages of B lymphocyte development.

Although rapid B lymphocyte proliferation is characteristic of the bone marrow, it may be inhibited if the B lymphocytes are not progressing through the maturation process. Thus, the absence of MIF may inhibit B lymphocyte maturation, and the increasing population of pro-B lymphocytes may cause a reduction in proliferation, allowing the pro-B lymphocytes time to mature. Yet, without MIF present in the environment, these B lymphocytes may not fully mature. With the reduced proliferation, fewer B lymphocytes enter the secondary lymphoid organs, including the spleen. Fewer mature B lymphocytes taken together with fewer overall B lymphocytes in the spleen suggests that not only are the pro-B lymphocytes not properly developing, they are also not surviving. This suggests that MIF also plays a role in the survival of B lymphocytes. The same reduction B lymphocyte proliferation may not be observed in the spleen, because the few mature B lymphocytes that are available may proliferate adequately in response to antigen. Thus, the difference in the spleen is not the ability to proliferate, but that significantly fewer B lymphocytes are available to respond to antigen presentation. The impaired proliferation of B lymphocytes in the bone marrow of MIF deficient mice shows that there may be fewer B lymphocytes

initially, accentuating the impact of the reduced survival rate observed in the spleen.

Next, we investigated whether MIF produced by B lymphocytes alone is sufficient to restore EAE susceptibility in MIF knockout mice. We hypothesized that MIF acts by an autocrine mechanism via B lymphocytes to mediate the inflammatory damage seen in EAE and MS. If MIF does act in an autocrine manner as we hypothesized, then B lymphocytes could produce the MIF needed for their survival. Thus, B lymphocytes from a wild-type environment would be predicted to restore EAE susceptibility in a MIF deficient mouse. Through a series of transfers and immunizations, we determined that B lymphocyte production of MIF alone is not sufficient for the progression of EAE. Mice with MIF deficient environments and MIF deficient T lymphocytes, but with MIF producing B lymphocytes, remained protected from EAE.

We performed another series of experiments to develop mouse models where MIF was produced by both T and B lymphocytes. This model allowed us to investigate whether MIF production by T lymphocytes was able to affect B lymphocytes and restore EAE susceptibility in an otherwise MIF deficient mouse. Although previous findings with the adoptive transfer experiments excluded MIF production by T lymphocytes alone as the source of the relevant MIF, this model instead, explores the contribution of T lymphocyte produced

MIF to the maturation and proliferation of B lymphocytes. We observed that regardless of MIF production by either B or T lymphocytes, mice with an MIF deficient background remained protected from EAE immunization. This allowed us to eliminate the lymphoid lineage of bone marrow derived cells as the relevant source of MIF in EAE.

Considering the above results, our data suggests a role of MIF with respect to B lymphocytes in EAE and MS. The recent studies with Rituximab therapy show that the depletion of B lymphocytes is significantly beneficial in the treatment of MS (Hauser S., 2008). Additionally, we have previously reported that the absence of MIF is protective during the progression of EAE. Our results have shown that the absence of MIF results in fewer mature B lymphocytes and less proliferation of the resulting B lymphocyte population. These observations, when taken with the success of B lymphocyte depletion therapies, argue for an important function of MIF in the development of B lymphocytes. Although we have shown that B lymphocytes do not mediate inflammation through the autocrine production of MIF or via MIF production by T lymphocytes, the effect of MIF on B lymphocytes cannot eliminated as an important factor in EAE progression.

Another cell may produce the necessary MIF which then acts on B lymphocytes to mediate the inflammatory response. The relevant cellular source of MIF may

be a cell that enters the CNS after the BBB is compromised. Or more likely, due to the bone marrow derivation of the relevant cellular source, the pathogenic MIF may be produced by a resident CNS cell such as a bone marrow derived dendritic cell. The production of MIF by this cellular source may act on B lymphocytes, providing them with the adequate survival signals.

Additionally, the relevant source of MIF may be another bone marrow derived cell other than the B lymphocyte. Unpublished observations from our lab show that microglia are modified in an MIF deficient environment. Microglia are bone marrow derived cells and are the resident APCs in the CNS. In the activated state, they can present antigen and phagocytose foreign material. Primary microglial cultures, when exposed to exogenous MIF, elaborate pro-inflammatory cytokines, such as IL-6 and iNOS. Corroborating this observation, the injection of MIF into the spinal cord of wild-type mice causes the microglia to adopt an activated morphology. When microglia are not activated and in the ramified state, they can move freely about neural tissue but cannot present antigens or produce pro-inflammatory cytokines. However after injection with MIF, the microglia show an activated ameboid morphology, indicating they can now present antigen, exert cytotoxic effects, and secrete pro-inflammatory cytokines. Thus, the production of MIF may be activating microglia to present myelin to T-lymphocytes and secreting pro-inflammatory cytokines to recruit additional immune cells to the CNS. The antigen presenting abilities of B lymphocytes are

more specific and able to process and break down whole protein. MIF activated microglia may initiate the immune response in EAE and MS but then recruit B lymphocytes to the site of damage, where they can more effectively activate the adaptive immune response.

A second hypothesis for the role of MIF in B lymphocyte maturation involves the ability of MIF to upregulate toll-like receptor 4 (TLR4) (Roger, 2001). Natural agonists following infection could engage TLRs during the onset of MS. Stimulation of TLR4 agonists results in MIF production and once MIF is produced, it can mediate the upregulation of TLR4 (Roger, 2001). Recently, TLR4 has been shown to be an accessory stimulus in B cell development by complementing the B lymphocyte-activating factor (BAFF) pathway (Hayashi, 2010). Ultimately, TLR4 has been implicated in stimulating B cell maturation (Hayashi, 2010). Since MIF is a potent upregulator of TLR4 and MS and EAE involve TLR4 agonists, MIF may act via TLR4 to help the BAFF pathway which results in B lymphocyte maturation. In the MIF deficient mouse, TLR4 may not be up regulated and thus, B lymphocytes do not receive the appropriate maturation signals.

MIF may also mediate B lymphocyte activity through the vascular cell adhesion molecule, VCAM-1. MIF deficient mice down-regulate VCAM-1, an important cellular adhesion molecule involved in the diapedesis of lymphocytes into CNS

tissue (Denkinger, 2003). Interestingly, VCAM-1 is also intricately involved in early B lymphocyte maturation. VCAM-1 is expressed on bone marrow stromal cells and binds with the VLA-4 molecule on the surface of pro-B lymphocytes to induce expression of the IL-7 receptor. Expression of IL-7R is an intermediate step between pro-B lymphocytes and pre-B lymphocytes. The absence of MIF and down-regulation of VCAM-1 may prevent pro-B lymphocytes from properly attaching to the bone marrow stromal cells and receiving the adequate maturation signals.

More research is needed in this area to explore the myeloid lineage of bone marrow derived cells to determine the specific source of the pathogenic MIF in EAE and MS. The difference in TLR4 regulation should be explored in the MIF deficient environment. We would expect TLR4 to be down-regulated in an MIF deficient mouse relative to a wild-type mouse. Another future direction would be to compare the B lymphocyte population between an MIF deficient mouse and wild-type mouse after immunization. The results thus far have compared B lymphocyte populations only in naïve mice. We would expect these differences to be apparent after immunization and possibly, more pronounced. Lastly, MIF production by other bone marrow derived cells should be explored to determine their contribution to B lymphocyte maturation and survival in EAE.

Answering the above questions has important implications for elucidating the effector functions of B lymphocytes which require MIF for maturation. Additionally, they may provide direction to better understand the role of MIF in inflammation. This would lead to the development of MIF inhibitors to modify B lymphocytes in a variety of inflammatory diseases, autoimmune or not.

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