The Immunoregulatory and Neuroprotective roles of Dimethyl Fumarate in Multiples Sclerosis

DISSERTATION

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By

Haiyan Peng

Neuroscience Graduate Program

The Ohio State University

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Dissertation committee:

Michael Racke, MD, Advisor
Dana McTigue, PhD
Gary Wenk, PhD
Jonathan Godbout, PhD
Abstract

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the Central Nervous System (CNS). About 80% of MS patients start the disease as Relapsing Remitting MS (RRMS) that is characterized by frequent inflammatory attacks followed by spontaneous recovery. However, about 10 years after disease onset, 50% of the patients enter into secondary progressive MS (SPMS). At this stage, inflammation is limited, however, neurological decline continues. Therefore, both dysregulated immune function and neurodegeneration seem to contribute to the pathogenesis of MS.

Clinical trials showed that Dimethyl Fumarate (DMF), a novel oral treatment for MS, significantly reduced gadolinium enhancing lesions in Relapsing-Remitting MS (RRMS). The safety record, efficacy and oral availability of DMF makes it potentially more beneficial to MS patients than currently available therapies. Yet, neither the molecular mechanism nor the potential neuroprotective effect of DMF has been fully investigated.

One interesting observation from a Phase II clinical trial was a reduction in IFN-γ producing CD4+ T cells (Th1) in fumarate treated patients. Because Th1 cells are considered to be the pathogenic cells in MS, this observation may have identified one
potential mechanism of action of DMF. The first part of my graduate work was dedicated
to understanding the cellular and molecular mechanisms behind this clinical observation.
Using dendritic cell generated both in vitro and in vivo, I demonstrate that DMF inhibits
dendritic cell (DC) maturation by significantly reducing proinflammatory cytokine
production and the expression of MHC class II, CD80 and CD86. Importantly, this
immature DC phenotype generated fewer activated T cells that were characterized by
decreased IFN-γ and IL-17 production. Further molecular studies demonstrated that
DMF exerts its effects on DCs via suppression of both Nuclear Factor κB (NF-κB) and
Extracellular signal-Regulated Kinase 1 and 2 (ERK1/2) and Mitogen Stress activated
Kinase 1 (MSK1) signaling and upregulation of Heme Oxygenase-1 (HO-1). This part of
my study established a cellular and molecular mechanism by which DMF treatment led to
a reduction in Th1 population in patients with MS.

In addition to its immunoregulatory effects, DMF may have neuroprotective properties. It
was demonstrated that Nrf-2, a critical transcription factor involved in combating
oxidative stress, was activated in astrocytes after DMF treatment. However, one could
argue that the Experimental Autoimmune Encephalomyelitis (EAE) animal model used in
the previous study was problematic as this model is characterized by massive
inflammation and that DMF has immunoregulatory effects. Therefore, it remains unclear
if the beneficial effect of DMF in EAE is exclusively through its immunoregulatory
effects or neuroprotective effects or a combination of both. To exclude the effects of
DMF on inflammation, an excitotoxic model induced by microinjection of AMPA directly into mouse spinal cord was utilized in our study.

Naïve mice were fed with DMF (125mg/kg/day) for three days followed by microinjection of AMPA. Our results demonstrate that DMF pre-treatment reduces neuronal loss and improves locomotor activity as compared to vehicle-treated mice, suggesting a potential neuroprotective role of DMF. Furthermore, we also demonstrate that HO-1, an inducible enzyme downstream of Nrf-2 that also has anti-oxidative properties, was upregulated by DMF. This may suggest HO-1 as a potential target of DMF in the CNS.

Taken together, this translational research enhances our understanding of the cellular and molecular mechanisms of DMF, providing mechanistic foundation for the application of DMF in treating patients with MS. Additionally; this study helps us gain knowledge of MS pathogenesis, which may further favor the development of novel therapies in the future.
Dedicated to my family and many friends.
Acknowledgement

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Vita

June, 2000……………………………………Hebei Xinji High School, China

July, 2004……………………………………..B.S Pharmacy, Sichuan University, China

July, 2007……………………………………..M.S Pharmacology, Sichuan University, China

2007-2012……………………………………Ph.D Neuroscience, The Ohio State University

Publications


Chapter in Books


Field of study

Major Field: Neuroscience
Emphasis: Neuroimmunology
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<tr>
<td>AMPA</td>
<td>2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
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<tr>
<td>ARE</td>
<td>Anti-oxidant Response Element</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
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<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CFA</td>
<td>Complete Freud’s Adjuvant</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EDSS</td>
<td>expanded disability status score</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FoxP3</td>
<td>forkhead box P3</td>
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<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>GDNF</td>
<td>glial derived neurotrophic factor</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase-1</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intra-peritoneal</td>
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<tr>
<td>KEAP-1</td>
<td>Kelch-like ECH-associated protein 1</td>
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<tr>
<td>MBP</td>
<td>myelin basic protein</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
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<tr>
<td>MSFC</td>
<td>Multiple Sclerosis Functional Composite</td>
</tr>
<tr>
<td>NMSS</td>
<td>National Multiple Sclerosis Society</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
</tr>
<tr>
<td>OSU</td>
<td>Ohio State University</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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<tr>
<td>PPMS</td>
<td>primary progressive multiple sclerosis</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RORγt</td>
<td>RAR-related orphan receptor gamma</td>
</tr>
<tr>
<td>RRMS</td>
<td>relapsing-remitting multiple sclerosis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPMS</td>
<td>secondary progressive multiple sclerosis</td>
</tr>
<tr>
<td>Stat</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T-bet</td>
<td>t-box expressed in T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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Chapter 1: Introduction and Background

Although Robert Carswell and Jean Cruveilhier had illustrated the clinical pathology in post-mortem specimens in mid-nineteen century, it was Jean-Martin Charcot, a French neurologist, who first characterized Multiple Sclerosis (MS) as a distinct, separate disease using his anatomoclinical method. In addition, he even defined a triad (nystagmus, intention tremor, and scanning speech) for diagnosing MS, establishing MS as a specific disease entity (Kumar, Aslinia et al. 2011). Since Charcot’s scientific description of MS, our understanding of the pathogenesis of this disease in the past 140+ years has been greatly facilitated by the availability of animal models, imaging tools and molecular techniques. Yet, there is no cure; and the cause of MS remains unknown.

The idea that MS is an autoimmune disease is largely based on studies in its animal model, Experimental Autoimmune Encephalitomyelitis (EAE) (Sriram and Steiner 2005; Steinman and Zamvil 2006). Clearly, dysregulated immune responses play an essential role in MS; however, MS seems to be more complicated than just inflammation: in fact, a large body of evidence suggests that neurodegeneration is also a large component in MS, contributing to disease progression (Trapp, Peterson et al. 1998; Bjartmar, Yin et al. 1999; Peterson, Bo et al. 2001; Fisher, Lee et al. 2008; Trapp and Nave 2008). This is evidenced by the fact that current immunoregulatory medications fail to either halt the disease or slow down the brain atrophy in the progressive forms of MS.
Therefore, continuous etiological and mechanistic studies will shed insights on the cause of the disease, help identify novel therapeutic targets and develop more effective medications for the patients with MS. Additionally, a paradigm shift from immunological research to neuroprotective research has occurred in the past decade, advancing our understanding of pathology in the target organ, the Central Nervous System (CNS), and favoring the discovery of neuroprotective agents for progressive forms of MS.

1.1 Multiple Sclerosis

Multiple sclerosis is an inflammatory demyelinating disease of the Central Nervous System (CNS) (Frohman, Racke et al. 2006; Lovett-Racke, Yang et al. 2011). The pathological hallmark of MS is the presence of focal areas of inflammatory mediated demyelination and axon transection in the brain and spinal cord. As a result of CNS damage, MS patients suffer a series of symptoms including blurred vision, numbness, walking problems, bladder difficulty, memory loss and depression (Trapp and Nave 2008).

MS is the major cause of non-traumatic neurological disability in young adults in North America and Europe, affecting more than 2.5 million individuals worldwide. There is a gender bias in MS such that women are three times more likely to be affected then men (Kipp, Amor et al. 2012; Nussinovitch and Shoenfeld 2012). MS is a chronic disease that significantly affects patients’ quality of life. About 50% of patients are unable to perform work responsibilities 10 years after disease onset and 50% of them need to rely on
wheelchairs 25 years after disease onset (Sosnoff, Boes et al. 2011). About 85% of MS patients start the disease as a relapsing remitting form (RRMS) that is characterized by frequent inflammatory attacks followed by partial to complete recovery. About 20 years after the disease onset, 95% of the RRMS patients have entered into a secondary progressive (SP) state where inflammation is limited, yet, irreversible neurological decline continues (Frohman, Racke et al. 2006). This indicates the disease shifts from an active immune process to a neurodegenerative process (Trapp and Nave 2008). A third subtype of MS is primary progressive MS (PPSM) where steady neurological decline is observed at the onset of the disease without episodic relapses. These patients define about 10% of the total MS population and usually have the poorest prognosis.

The definitive cause of MS is unknown. Epidemiologic studies demonstrated that MS is particularly prevalent in temperate zones, and conversely, less common in subtropical zones and uncommon in tropical zones (Pugliatti, Sotgiu et al. 2002; Taylor, Pearson et al. 2010; Simpson, Blizzard et al. 2011). This uneven distribution indicates MS may arise from a complex etiology that involves both genetic and environmental factors and that both could make a significant contribution to causation. Twin studies demonstrated that monozygotic twins have a greater concordance rate (about 30%) than dizygotic twins (5%) for development of MS (Willer, Dyment et al. 2003). These studies indicate the involvement of genetics factors; however, the concordance is less than 100%, suggesting that genetics alone can not fully explain the disease development and that environmental factors play an important role. In fact, many studies are in support of this hypothesis. The retrospective epidemiologic study by Kurtzke in 1974 described an apparent epidemic of
MS in the Faroe Islands after the British occupation during the 1940s. He proposed that a pathogen that British troops brought in was responsible for the increased MS occurrence (Kurtzke and Hyllested 1979). The important role of environmental factors in the development of MS was further strengthened by migration studies. These studies demonstrated that the risk of MS is acquired from the geographic residence, with a cutoff of 15 years of age. For instance, the risk of developing MS in one individual, who migrates from an area of high risk to a low risk zone after age of 15, remains high (Alter, Leibowitz et al. 1966; Gale and Martyn 1995).

To better understand the cause of MS, an active search for either genetic or environmental risk factors began in the early 1970s. The first recognition of an association of MS to the genes in major histocompatibility complex (MHC) occurred in 1972. Human leukocyte antigen (HLA) class II region of the HLA-DR2 haplotype on chromosome 6p21 has been shown to be significantly associated with MS, with an estimated risk between 2 to 4 (Olerup and Hillert 1991; Sotgiu, Pugliatti et al. 2002). Other risk genes with significantly milder association such as IL-2R and IL-7R have been identified in numerous studies (Gregory, Schmidt et al. 2007; Lundmark, Duvefelt et al. 2007; Yamanouchi, Rainbow et al. 2007). In 2011, the well powered Genome wide-associate studies (GWAS) have enabled an additional 20 risk loci to be identified (Sawcer, Hellenthal et al. 2011). Although these genes may be individually interesting and collectively illuminating the roles of biological pathways, the modest contribution that these variations have to disease risk has raised concerns regarding their medical relevance (Sawcer et al. 2011).
These genetic studies provide insights into the familial cases of MS; however, they do not explain the migration data. Thus, many researchers have been attempting to determine the environmental triggers and the most commonly studied risk factor of this type is infection. There are multiple theories on how infection may induce MS. The three major hypotheses are 1) bystander activation; 2) Epitope spreading and 3) molecular mimicry (Fujinami and Oldstone 1985; McRae, Vanderlugt et al. 1995; Wucherpfennig and Strominger 1995; Vanderlugt, Neville et al. 2000; Olson, Croxford et al. 2001; McMahon, Bailey et al. 2005; Munz, Lunemann et al. 2009). Bystander activation is a T cell receptor (TCR) independent-mechanism for virus induced autoimmunity caused by virus infection. A consequence of bystander activation and local tissue damage is epitope spreading. This often occurs in the setting where persistent infection leads to tissue damage and subsequent release of endogenous autoantigens. These autoantigens then are processed and presented by antigen presenting cells (APCs), which leads to autoreactive T cell activation. The leading hypothesis, however, is molecular mimicry. The idea is that the similarity between epitopes on an infectious agent and an autoantigen is enough to cause T cells to not only respond to the infectious antigen, but to certain self-antigens. As a result, the host is injured by an activated autoimmune response.

Along with these hypotheses, significant efforts have been dedicated in the past several decades to searching for a specific pathogen associated with the onset of MS. Many viruses have been implicated including measles, Human Herpesvirus type 6, retroviruses,
and Epstein-Barr virus (EBV) (Granieri and Casetta 1997; Munch, Hvas et al. 1998; Marrie, Wolfson et al. 2000; Kakalacheva, Munz et al. 2011). Although the presence of a virus, viral antigen or viral genome has been identified in MS brains, proof of the etiologic relationship between any particular infection and MS has been elusive. Among the many potential candidates, EBV is a potentially favored cause of MS (Warner and Carp 1981; Bray, Bloomer et al. 1983). About 3-5% of EBV specific-CD4 T cells from MS patients and healthy individuals cross react with peptides derived from myelin proteins (Lunemann, Jelcic et al. 2008). In addition, virtually all MS patients are EBV seropositive (>99%) (Sumaya, Myers et al. 1980; Munch, Riisom et al. 1998; Wagner, Hennig et al. 2000). However, the argument arises as to whether other viruses also display cross reactivity and more than 85% of healthy individuals are also seropositive for EBV (Sumaya, Myers et al. 1980; Lunemann, Edwards et al. 2006). Therefore, the evidence of EBV as a risk factor is insufficient, although it remains biologically plausible.

In addition to infection, climate factors have been of great interest to many researchers because of the geographical variation in MS risk. Studies have consistently demonstrated that sun exposure is inversely correlated with MS risk (Antonovsky, Leibowitz et al. 1965; Neutel 1980). Individuals with high levels of sun exposure between the ages of 6-15 were less likely to develop MS (van der Mei, Ponsonby et al. 2003). The protective effect of sunlight is postulated to relate to enhanced vitamin D production in skin, which may have immunosuppressive properties. Administration of vitamin D was shown to significantly decrease disease severity in Experimental autoimmune Encephalomyelitis.
(EAE), the animal model of MS, indicating its potential therapeutic effects. Yet, clinical evidence with regard to the effectiveness of vitamin D therapy has been insufficient (Kimball, Ursell et al. 2007; Disanto, Chaplin et al. 2012).

1.2 Experimental Autoimmune Encephalomyelitis (EAE)

In the 1930s, Thomas Rivers from Rockefeller University, together with his colleagues, discovered an animal model, now known as EAE (Rivers, Sprunt et al. 1933). After the initial description, there was a steady progress in the use of this model. EAE was facilitated by the development of a new mineral oil based adjuvant by Jules Freund that, when combined with brain extracts, resulted in disease after only a single injection. It was further advanced when the addition of heat-inactivated Mycobacteria tuberculosis to the adjuvant (Complete Freund’s adjuvant, CFA) was found to enhance the immune response to CNS tissue. Initially this model was constructed to study paralysis associated with the rabies vaccine, not MS. However, it turned out that EAE has many similarities with MS, including demyelination, axonal transection and episodic paralysis (Steinman and Zamvil 2006). As a matter of fact, in the past 80 years, this model has assisted in our understanding of MS pathogenesis, basic mechanisms of brain inflammation, and helped develop new treatments for MS patients. Nine medications have been approved by Food and Drug Administration (FDA) so far, and three of them including glatiramer acetate (1996), mitoxantrone, and natalizumab (2007), were designed and developed by using EAE models (Steinman and Zamvil 2006). Yet, we should be aware that EAE does not entirely represent all the features of MS, although we have learned and benefited
tremendously from this model (Sriram and Steiner 2005). MS is a complicated disease with heterogeneous clinical, pathological and immunological phenotype that might be better described as a symptom complex rather than just simply one disease. We should be aware of the difference when we interpret our data and translate animal studies to clinical applications. Additionally, although EAE mimics some aspects of RRMS, it is unable to represent the progressive phase, in which inflammation is much reduced while neurological decline continues (Sriram and Steiner 2005). Therefore, this model may not be suitable for developing neuroprotective drugs. Lastly, it is difficult to examine cognitive function using EAE, since many mouse cognitive tests depend on the motor activity of the animals. Therefore, other models are needed for development of neuroprotective agents and for evaluation of cognitive function related to MS in the future.

1.3 Immune component in MS

It has been well described that a dysregulated immune system is heavily involved in the pathogenesis of MS. Infiltration of immune cells are present in the lesions within the MS brain, and can also be detected in the Cerebrospinal Fluid (CSF) (Brucklacher-Waldert, Stuerner et al. 2009; Kebir, Ifergan et al. 2009). Furthermore, the infiltrating cell number increases during relapses and reduces in the phase of remission (Kebir, Ifergan et al. 2009). In general, peripheral autoreactive T cells are activated by antigen presenting cells (APC) and differentiate into IFN-γ producing CD4\(^+\) T cells and IL-17 producing CD4\(^+\) T cells, both of which are considered to be pathogenic in MS (Yang, Weiner et al. 2009;
Lovett-Racke, Yang et al. 2011). These pathogenic cells cross the blood brain barrier via adhesion molecules and infiltrate into the CNS, where these cells can be reactivated by local antigen presenting such as microglia or infiltrating dendritic cells (DC) (Weiner 2008). As a consequence, reactivated T cells produce massive amounts of inflammatory cytokines, resulting in CNS damage. In addition, these cells also recruit other immune cells such as macrophages, B cells and CD8+ T cells to the lesion sites. Activated B cells and plasma cells may produce pathogenic autoantibodies but B cells may also contribute to the immune response by producing inflammatory cytokines and antigen presentation (Bar-Or, Fawaz et al. 2010). In concert, these mechanisms cause demyelination with subsequent axonal transection and neuronal loss (Trapp and Nave 2008). As a result, patients suffer from different symptoms from cognitive to behavioral deficits.

Current therapies mainly target the dysregulated immune system to reduce the disease activity in patients with MS (Khatri, Barkhof et al. 2011; Racke and Lovett-Racke 2011; Schaaf, Pitt et al. 2011; Goodin, Reder et al. 2012). The contribution of a dysregulated immune system to MS pathogenesis is further supported by bone marrow transplantation (BMT) in young patients with aggressive disease activity (Saccardi, Mancardi et al. 2005; Fassas and Mancardi 2008; Fassas, Kimiskidis et al. 2011; Connick, Kolappan et al. 2012). A 10-year follow up study by investigators at the University of Ottawa showed that none of their patients had a single relapse and neurological performance of a number of patients is improved compared to baseline prior to transplantation (Dr. Mark Freeman, CMSC conference, 2012). The important role of the immune system in MS pathogenesis
has been clearly established by these studies.

The physiologic function of the immune system is to protect ourselves against infectious microbes. This defense is mediated by early reaction of innate and the later responses of adaptive immunity. Innate immunity is the initial response to microbes that helps prevent, control or clear infections by the host. By expressing pattern recognition receptors (PRR) including Toll like receptors (TLRs), C-type lectins, Nod-Like Receptors (NLRs) and scavenger receptors, innate immune cells recognize specific structures of microbes that are not present on mammalian cells (Cellular and molecular immunology, Edition 6). As a result, the innate immune cells are activated, and subsequently clear microbes by cytokine secretion, phagocytosis, and direct killing. In addition, the innate immune response provides signals that can stimulate adaptive immunity to further help clear microbes.

1.3.1 Dendritic cells

Dendritic cells were originally identified and named for their stellate, tree-like shape by Steinman and Cohn in 1973 (Steinman and Cohn 1973). As critical innate immune cells, dendritic cells (DCs) are widely distributed in lymphoid tissues, mucosal epithelium and organ parenchyma. In general, there are two subpopulations of DCs, based on their cell surface markers, cytokine profile, and functional response patterns. Myeloid DCs (mDCs) are considered as classical or conventional DCs that express high levels of CD11b and CD11c (Heath and Carbone 2009). They were first identified by their ability
to stimulate strong T cell responses (Heath and Carbone 2009). The second subtype is plasmacytoid DCs (pDCs) that express intermediate levels of CD11c and express the B cell marker B220. pDCs produce large amounts of type I interferon after viral infection and are circulatory in nature and migrate to lymph nodes after activation. However, they are less efficient at priming T cell responses than mDCs (Shortman and Liu 2002).

T cell activation requires the engagement of T cell receptor to the peptide presented by MHC II and CD28 and CD80/CD86 mediated costimulatory signaling (Perrin, Lovett-Racke et al. 1999; Racke, Ratts et al. 2000; Lovett-Racke, Yang et al. 2011). In the steady state, DCs are immature and lack the capacity of driving strong T cell responses due to their low expression of MHC II and costimulatory molecules such as CD80 and CD86 (Chastain, Duncan et al. 2011). In fact, these immature DCs display a tolerogenic phenotype and are speculated to be critical to maintaining the peripheral immune tolerance, probably through several mechanisms such as generation of regulatory T cells and induction of T cell anergy/deletion (Kretschmer, Apostolou et al. 2005; Lambrecht and Hammad 2009). Activation of DCs through TLR signaling or encounter with foreign antigens leads to maturation and enhanced surface molecule expression. These modifications allow DCs to become efficient at antigen presentation, which is critical for launching the adaptive immune response (Shen, Tesar et al. 2008). In addition, cytokine production is also initiated during the maturation of DCs. This is critical as the cytokine microenvironment greatly influences naïve T cell differentiation. For instance, IL-12 instructs naïve T cells differentiate into IFN-γ producing CD4+ T cells (T cell helper1,
Th1), and IL-6 in the absence of IL-12 favors IL-17 producing CD4+ T cells (Yang, Weiner et al. 2009). Additionally, IL-23 can potentiate both Th1 and Th17 generation (Chen, Langrish et al. 2006; Gocke, Cravens et al. 2007).

In addition to the periphery, DCs can be found in certain CNS regions. Although DCs are rarely detected in brain parenchyma, they can be found in vascular-rich compartments such as meninges and choroid plexus (CP) under normal conditions (McMenamin 1999; Matyszak, Citterio et al. 2000; Serot, Bene et al. 2000). Additionally, both mDCs and pDCs can be found in the CSF of normal brains (Pashenkov, Huang et al. 2001). These studies suggest that DCs probably participate in the immune surveillance of the CNS. On the other hand, in the context of MS, studies have consistently demonstrated the substantial accumulation of DCs in the perivascular space, which strengthens the involvement of DCs in neuroinflammation and autoimmune responses (McMenamin 1999; Bailey, Schreiner et al. 2007). However, although it has been well accepted that DCs contribute to the integrity and pathology of healthy and inflamed brain, it is still debated with regard to the origin of the inflammatory response. Collective evidence suggests that CNS microglia are able to differentiate into DC-like cells in vitro in the presence of cytokines such as Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) or FMS-like tyrosine kinase 3 receptor (FLT-3L) (Santambrogio, Belyanskaya et al. 2001; Willenborg and Staykova 2003; Curtin, King et al. 2006; Zozulya, Reinke et al. 2007). It has also been suggested that bone marrow stromal cells are activated during inflammation and release active DC precursors and monocytes that are able to cross an
artificial BBB \textit{in vitro} (Zozulya, Reinke et al. 2007). However, \textit{in vivo} studies are hampered by the number of cell surface markers shared by microglia, macrophages and DCs and the low number of cells that can be isolated from CNS.

Given their important role in instructing CD4$^+$ T cell differentiation, the association of DCs to MS has been investigated. Peripheral DCs from patients with MS are more mature and polarize naïve T cells to secret higher levels of cytokines compared with DCs from healthy individuals (Kivisakk, Mahad et al. 2004; Karni, Abraham et al. 2006; Serafini, Rosicarelli et al. 2006). In addition to their key role in priming naïve T cells in the periphery, CD11c DCs are also found in the CSF and inflammatory MS lesions in the brain. This observation led scientists to study the phenotypes and functions of these DCs present in the CNS. Evidence suggests that these CD11c DCs are capable of acquiring a mature phenotype in MS lesions to stimulate autoreactive T cells locally (Serafini et al. 2006). Additionally, these DCs may migrate to cervical lymph nodes and initiate new waves of autoreactive T cells (Serafini et al. 2006). Further, the pharmacological studies of current therapies, at least in part, affect DCs function such as DC mediated polarization of effector T cell (Jung, Siglienti et al. 2004; Steinman and Banchereau 2007; Zeng, Wang et al. 2012). IFN-β treatment induces mature DC apoptosis through induction of caspase 3 and 11. Since DCs are critical for inducing effective adaptive immune response, IFN-β induced altered function of DCs could play an important role in re-establishing homeostasis, raising an additional mechanism for therapeutic effects of IFN-β (Yen and Ganea 2009). Additionally, glatiramer acetate induces type II
DCs/macrophages that are characterized by higher levels of IL-10 and TGF-β and reduced Signal Transducers and Activators of Transcription-1 (STAT1) expression (Jung, Siglienti et al. 2004). Importantly, these Type II DC/macrophages migrate into the inflamed CNS, where it fine tones the local microenvironment (Weber, Prod'homme et al. 2007). Taken together, DCs are emerging as a critical player in the priming of autoreactive T cells in the periphery and the CNS.

1.3.2 Microglia and Macrophage

Microglia cells, the resident macrophages of the CNS, are considered to be the innate immune cells of CNS and play an important role in both neuroprotection and neurodegeneration (Shields, Gilson et al. 1999; Arnett, Mason et al. 2001; Kotter, Setzu et al. 2001; Ajami, Bennett et al. 2011; Ponomarev, Veremeyko et al. 2011). In the resting state, ramified microglial cells are highly dynamic and consistently screen the microenvironment for pathologic changes. Once activated by infection or injury, these cells become activated with fewer and thicker processes. Microglia migrate toward the lesion site to clear the debris (Perry, Nicoll et al. 2010). However, they also produce a large range of cytotoxins, free radicals, neurotrophic factors and immunomodulatory molecules. Thus they might serve a role in restricting lesion size and promoting cellular repair. In addition, activated microglia display a DC-like phenotype that is characterized by enhanced expression of MHC II, CD80 and CD80. Meanwhile, production of cytokines such as IL-6, IL-12 and TNF-α is also initiated. These modifications allow microglia to activate adaptive immune cells.
Besides the resident microglia cells, peripheral monocytes can migrate into the CNS in response to injury and differentiate into mature macrophages (Simard, Soulet et al. 2006; Smith and Jeffery 2006). In fact, it is challenging to distinguish microglia from macrophages due to lack of immunohistochemical markers. In general, these cells are collectively referred as microglia and macrophages. However, it is still in debate if peripheral monocytes can transform into brain microglia.

In the context of MS, it is thought that microglia might sustain and propagate CNS inflammation through antigen presentation and cytokine secretion (Ponomarev, Maresz et al. 2007; Trapp, Wujek et al. 2007; Gray, Thomas et al. 2008; Ajami, Bennett et al. 2011). Activated microglia persist in the MS lesions in RRMS, even in progressive forms of MS where T cell number is much reduced. This may indicate a shift from adaptive immunity mediated inflammation to local inflammation maintained by microglial activation, which is likely to contribute to the continuous disease in progressive forms of MS. Along these lines, animal studies also suggest a detrimental role of microglia (Merrill, Ignarro et al. 1993; Peterson, Bo et al. 2002; Guo, Nakamura et al. 2007). Depleting microglia in the parenchyma led to milder EAE and reduced myelin and axonal damage (Huitinga, van Rooijen et al. 1990; Guo, Nakamura et al. 2007; Rasmussen, Wang et al. 2007). It was also noted that products released from activated microglia inhibit neurogenesis, while administration of minocycline, which inhibits microglial activation, restores the neurogenesis suppressed by inflammation (Ekdahl, Claasen et al. 2003).
However, recent studies suggest microglial activation may have an important role in promoting remyelination in certain circumstances and depletion of peripheral macrophages slows down remyelination (Kotter, Setzu et al. 2001; Kotter, Zhao et al. 2005). These studies demonstrated that microglia/macrophages promote CNS repair by producing neurotrophic factors including brain derived neurotrophic factors (BDNF), ciliary neurotrophic factor (CTNF) and glial cell line derived neurotrophic factor (GDNF)(Kotter, Zhao et al. 2005). Currently, many labs are trying to identify factors that can favor the beneficial effects of microglia.

Compared to innate immunity, adaptive immunity acts slower but can specifically recognize antigenic determinants and has a better capacity of clearing a specific microbe. In general, adaptive immunity can be divided into two categories, humoral mediated adaptive immunity mediated by B cells and cellular immunity mediated by T cells.

Generated in the bone marrow, B cells migrate to and further mature in the spleen. In the spleen, B cells acquire the ability to recirculate and populate all peripheral lymphoid tissues. The important role of B cells in MS pathogenesis was emphasized by the recent success of Rituximab in clinical trials (Monson, Cravens et al. 2005; Hawker, O'Connor et al. 2009; Bar-Or, Fawaz et al. 2010). Rituximab is a chimeric monoclonal antibody that targets CD20 on B cells. However, its function seems unrelated to antibody production as 1) plasma cells, or the antibody producing cells, are not affected by Rituximab because they do not express CD20 and 2) total antibody concentration remained unchanged after
Rituximab treatment (Hawker 2008). Therefore, it is speculated that Rituximab benefits MS by reducing proinflammatory cytokine production by B cells and their antigen presenting capacity to T cells (Bar-Or, Fawaz et al. 2010). Moreover, it is very interesting to note that Rituximab treatment is only effective in PPMS patients with gadolinium enhancing lesions, particularly in those who are under the age of 51 (Hawker, O'Connor et al. 2009). This may argue for a change in disease neurobiology overtime. Another explanation is that rituximab may not have access to the pathogenic cells behind an intact blood brain barrier (BBB) in these patients. The pathogenic role of B cells is further supported by additional studies. These studies demonstrate that in some SPMS patients, B cell follicles are present underneath the meninges and near large subpial cortical lesions in the brain, suggesting contribution of soluble diffuse factors secreted by B cells to the cortical lesion formation (Magliozzi, Howell et al. 2007). Interestingly, these patients present with the disease at a younger age, with more severe disease activity and faster disease progression, again suggesting the contribution of B cells in MS pathogenesis, at least in certain subpopulations of MS patients. It has become a very active research area to understand the pathogenic role of B cells in MS and search for novel therapeutic interventions that target this cell population.

The other component of adaptive immunity is cellular immunity mediated by T cells. T cell precursors are generated in the bone marrow, but develop in the thymus. During development, all the T cells go through negative selection to delete T cells that react strongly to self antigen. This is an essential step to prevent occurrence of autoimmune
diseases. T cells leave the thymus as naïve cells, meaning they have not been exposed to foreign antigens. In response to infections, APCs engulf self/foreign proteins, process them into antigens and present them to naïve T cells. As a result, T cells become activated and differentiate into specific phenotypes, depending on the particular antigen and microenvironment.

1.3.3 CD4$^+$ T cells

T cells are generally divided into two subsets CD4$^+$ and CD8$^+$ T cells. CD4 T cells help humoral immunity by promoting antigen specific antibody production. In addition, they also help CD8$^+$ T cells form memory by providing co-stimulatory signals. Because of these characteristics, CD4$^+$ T cells are also called T helper cells (Th). In contrast, CD8$^+$ T cells have their own specific functions. CD8$^+$ T cells are able to directly kill cells, such as in a viral infection, and thus are termed cytotoxic lymphocytes (CTL). Interestingly, although both subsets of T cells are present in MS lesions, CD8$^+$ T cells outnumber CD4$^+$ T cells by 3-10 fold in chronic inflammatory lesions (Babbe, Roers et al. 2000). Further, it was shown that axonal damage in the lesions correlates with the number of CD8$^+$ T cells (Bitsch, Schuchardt et al. 2000). Not only are CD8 T cells implicated in acute MS lesions, they also show oligoclonal expansion in MS brain, blood and CSF (Jacobsen, Cepok et al. 2002; Skulina, Schmidt et al. 2004). Therefore, CD8$^+$ T cells seem to be involved in MS pathogenesis. Yet, our understanding of CD8$^+$ T cells in MS has not progressed as fast as CD4$^+$ T cells. Whether these observations in regard to CD8$^+$ T cells are the cause of or the result of the disease remains unclear.
CD4+ T cells, the other subtype of T cells, have been long believed to be the key mediator in MS, mainly because of the strong evidence from both genetic studies and animal studies in EAE. It was demonstrated that the MHC II allele HLA DR2 is an independent susceptibility factor in MS, conferring a relative risk of 3 (Sawcer, Hellenthal et al. 2011). Further evidence stems from EAE, the animal model for MS, in which animals are immunized with myelin specific proteins or their encephalitogenic epitopes in Complete Freund’s adjuvant (CFA). Starting from the 1980s, many studies have consistently shown that adoptive transfer of Th1 cells isolated from EAE mice to naïve recipients causes EAE, reinforcing the importance of CD4+ T cells in initiating and maintaining the disease (Yang, Weiner et al. 2009). Therefore, CD4+ T cells have been speculated to be the primary mediator in MS.

CD4+ T cells can be divided into different subsets based on their distinct expression of cytokines and transcription factors. In the presence of IL-12 and IFN-γ, naïve T cells differentiate into T helper 1 (Th1) cells that are characterized by IFN-γ and TNF-α production and the expression of STAT1 and T-box expressed in T cells (T-bet) as transcription factors (Lovett-Racke, Rocchini et al. 2004). During this process, IFN-γ receptors on CD4+ T cells are activated by IFN-γ, resulting phosphorylation and translocation of STAT1 to the nucleus. pSTAT1 contributes to transcription of T-bet, which further facilitates STAT1 production, establishing a positive feedback loop between the two transcription factors that drive Th1 development (Lovett-Racke,
Rocchini et al. 2004; Lovett-Racke, Yang et al. 2011). This cell population is believed to be pathogenic in MS for several reasons. First of all, the presence of increased IFN-γ and TNF-α expression in MS lesions has been reported and the number of IFN-γ producing CD4+ T cells is much enhanced in the CSF of MS patients as compared to healthy individuals (Ishizu, Osoegawa et al. 2005). Second, IFN-γ treatment in MS resulted in a significant increase in exacerbations in these patients (Panitch, Hirsch et al. 1987). Third, a clinical trial with an altered peptide ligand from myelin basic protein (MBP), which was initially designed as a therapy, turned out to worsen the disease in several MS patients, which was associated with increased frequency of myelin specific T cells producing IFN-γ, suggesting that MS is mediated by myelin specific Th1 cells (Bielekova, Goodwin et al. 2000; Conlon and Steinman 2002). Last but not the least, one common mechanism of the current therapies is through dampening Th1 responses in patients.

The second subset named Th2 produces IL-4 and IL-5 and expresses GATA-binding protein 3 (GATA-3) and STAT6 as its transcription factors. This population is differentiated under the exposure of IL-4. Th2 response is important in promoting IgE and eosinophil-mast cell mediated immune reactions, which are protective against helminthic infections. It is thought that the Th2 response is protective in the context of MS as Th2 and Th1 counter-regulate each other (Kidd 2003; Guerau-de-Arellano, Smith et al. 2011; Oreja-Guevara, Ramos-Cejudo et al. 2012). Along with this idea, one therapeutic strategy translated into clinical application in MS is immune deviation, shifting a Th1 response to a Th2 response in MS patients (Racke, Bonomo et al. 1994).
For example, glatiramer acetate (copaxone), one of the first line treatments, enhances production of IL-4 and IL-10 while decreasing Th1 proinflammatory cytokine production (Skihar, Silva et al. 2009; Racke and Lovett-Racke 2011).

Th17, a third population, has been recently identified and is also considered to be pathogenic in MS. These cells produce IL-17 and express transcription factors of RAR-related orphan receptor (RORγt) and T-bet. RORγt deficient mice develop attenuated EAE, however, these mice do not have lymph nodes (Sun, Unutmaz et al. 2000; Ivanov, McKenzie et al. 2006). Thus their resistance to EAE may be because of the inability of CD4+ T cells to differentiate and proliferate in the periphery, not related to the failure of Th17 generation. In addition, IL-17 deficient mice developed mild EAE, suggesting a modest role of IL-17 in MS pathogenesis (Haak, Croxford et al. 2009). Further, studies demonstrated that encephalitogenicity of Th17 cells is determined by T-bet expression (Yang, Weiner et al. 2009). Adoptive transfer of Th17 cells generated from T-bet knockout mouse to naïve recipients does not cause EAE (Yang, Weiner et al. 2009). In contrast, generation of Th17 with IL-6 in the absence of TGF-β results in T-bet expression and the ability to induce EAE when transferred to naïve mice (Yang, Weiner et al. 2009). Clearly, Th17 cells can be encephalitogenic in the presence of T-bet. Human studies also support the pathogenic role of this population. Th17 cells are found in both MS lesions and CSF (Matusevicius, Kivisakk et al. 1999; Lock, Hermans et al. 2002; Ishizu, Osoegawa et al. 2005; Kebir, Ifergan et al. 2009). Interestingly, the number of Th17 in the CSF was significantly enhanced during an MS exacerbation, suggesting Th17
plays a role in exacerbation (Brucklacher-Waldert, Stuerner et al. 2009). Currently, the role of Th17 as a biomarker for therapy selection and a therapeutic target has been extensively studied in the field of MS research.

Another CD4+ T cell subpopulation named regulatory T (Treg) cells is also implicated in MS. These cells used to be called “suppressor T cells”, however, it used to be difficult to study these cells due to lack of surface markers. The discovery of CD25 as a marker for regulatory T cells in 1995 and the transcription factor forkhead box P3 (Foxp3) in 2003 greatly advanced Treg research (Hori, Nomura et al. 2003; Khattri, Cox et al. 2003). Mouse studies demonstrated stable expression of high amounts of Treg as CD4+CD25+ T cells, not in CD25- cells, or naïve CD4+ T cells (Fontenot, Gavin et al. 2003)(Khattri, Cox et al. 2003). Now CD4+CD25+FoxP3+ population is generally considered to be Tregs. Accumulating evidence showed that Foxp3 maintains the suppressive functions of Treg (Hori, Nomura et al. 2003). It has been found that foxp3 gene mutation in humans results in multi-organ autoimmune disorders because these individuals do not have CD4+CD25+ T regulatory cells (Gambineri, Torgerson et al. 2003). In support of this concept, EAE studies showed that CD4+CD25+ cells have a beneficial role. Tregs can suppress cytokine production by myelin specific pathogenic Th1 cells, and transfer of extra Tregs into normal mice prior to active immunization leads to attenuated disease (Kohm, Carpentier et al. 2002).

Despite the rapidly accumulating knowledge of Treg cell involvement in immune
regulation, our understanding of the molecular mechanisms through which these cells suppress the growth and function of other T cells still remain limited. Transcriptional profiling of Treg, naïve T cells and activated T cells identified both surface molecules and secreted proteins that are critical for the function of T cells. *In vitro* studies showed that the suppressive effects of Treg on other T cells requires direct cell: cell contact, indicating the involvement of surface molecules. For example, CD25, a subunit of IL-2 receptor is highly expressed on Treg and probably can compete with effector T cells for IL-2, resulting in inhibition of their proliferation. In addition, it was demonstrated that secreted proteins such as TGF-β and IL-10 also contribute to the functions of Treg (Peng, Laouar et al. 2004; Marie, Letterio et al. 2005). IL-10 deletion in Treg reveals that IL-10 production is important for keeping the immune response in check in the environmental interfaces such as the colon and lung (Suri-Payer and Cantor 2001). Additionally, TGF-β production has been shown to regulate memory T cell function via enhancing IL-10 production (Huss, Winger et al. 2010).

It is a very interesting observation that Treg number in MS patients seems unaffected (Putheti, Pettersson et al. 2004); however, their suppressive function is impaired compared to healthy individuals (Viglietta, Baecher-Allan et al. 2004). Therefore, the dysregulated Treg function may contribute to the disease pathogenesis. Many current studies are focused on these cells in the hope of identification of dysregulated pathways and developing biomarkers and new therapies in MS.
1.4 Neurodegeneration in MS

The central role of a dysregulated immune response in MS pathogenesis has been well characterized by numerous human and animal studies and further by the successful clinical application of immunomodulatory treatments in RRMS patients. However, diminishing inflammation does not stop the disease progression (Trapp and Nave 2008; Franklin, Ffrench-Constant et al. 2012). Once these patients enter into the secondary progressive phase, current therapies have limited effectiveness if there is any. In addition, no treatment is currently approved for PPMS. Clearly, the pathogenesis of MS is much more complicated and factors other than the immunological component also present and contribute to the disease occurrence and progression. In fact, a large body of work provides solid evidence that neurodegeneration is also a large component in MS (Trapp, Peterson et al. 1998; Zhou, Trapp et al. 1998; Peterson, Bo et al. 2001; Trapp 2004; Kutzelnigg and Lassmann 2005; Papadopoulos, Dukes et al. 2009).

Axon loss has long been reported as an important component of the pathology of MS. In 1936, Putman showed 50% axonal loss in lesions studied in 11 MS patients. The development of histological staining and imaging techniques in recent years led to identification of both grey and white matter (GM and WM) pathology that are characterized by 1) demyelination, neuronal loss and microglia activation and 2) axonal swelling, axonal transection and axonal degeneration in MS lesions (Trapp, Peterson et al. 1998). These findings facilitated a paradigm shift in MS research, in which increased attention has been given to the study of neurodegeneration.
1.4.1 White matter injury-Demyelination, axonal transection and degeneration

In the late 1990s, histological demonstration of axonal swelling, axonal transection, and axonal loss in postmortem MS brains, continuous brain atrophy and reduction in neuronal markers such as N-acetyl aspartic acid (NAA) were reported. (Bjartmar, Yin et al. 1999; Trapp, Bo et al. 1999; Trapp, Ransohoff et al. 1999). Interestingly, the axonal injury seems to positively correlate with inflammation in the lesions (Ferguson, Matyszak et al. 1997; Trapp, Peterson et al. 1998; Kornek, Storch et al. 2001; Geurts, Wolswijk et al. 2003). Therefore, it is speculated that axonal injury is initiated primarily by inflammatory attacks. Although current therapies are able to manage the initial RRMS stage, there is continuous axonal degeneration in SPMS patients, who remain untreatable. This occurs despite immunotherapy, suggesting a mechanism independent of inflammation in these patients in the chronic phase of the disease. One possible explanation is loss of trophic support because of myelin loss. In fact, animal studies demonstrated that disruption of myelin proteins such as proteolipid protein (PLP) or MBP leads to chronic axonal pathology, supporting the important role of myelin in axon integrity (Lappe-Siefke, Goebbels et al. 2003). In the early phase of the disease, the acute lesions with infiltrating immune cells can produce positive signals such as cytokines, growth factors and chemokines to recruit neural progenitor cells to the areas of injury (Batchelor, Liberatore et al. 1999). In support of this idea, it has been shown that the number of progenitor cells is greater in the lesion than normal appearing areas in MS brains (Mason, Suzuki et al. 2001; Mason, Xuan et al. 2003; Kotter, Zhao et al. 2005). In the lesion areas, these
progenitor cells differentiate into premature oligodendrocytes expressing PLP or MBP and remyelinate the axons. Remyelination is beneficial in several aspects. Remyelination restores axonal function by enhancing saltatory conduction and axonal conduction velocity, which is essential for motor function. In addition, remyelination may also restore trophic support to axons and prevent further axonal damage and loss given that oligodendrocytes produce neurotrophic factors to support the integrity of axons (Yin, Crawford et al. 1998; Lappe-Siefke, Goebbels et al. 2003). Thus, an effective means of preventing axonal degeneration may be a more efficacious myelin restoration.

However, spontaneous remyelination in MS is incomplete even in the very early phase of the disease (Prineas and Connell 1979; Lassmann, Bruck et al. 1997; Patrikios, Stadelmann et al. 2006). This could be due to both intrinsic and environmental defects. Inhibitory transcription factors are dominant in these progenitor cells. In order to exit the cell cycle to differentiate into mature oligodendrocytes, their gene expression needs to be downregulated (Arnett, Fancy et al. 2004; Xin, Yue et al. 2005). In addition, the CNS in general is an inhibitory environment for remyelination due to the presence of inhibitory molecules such as LINGO-1 (Mi, Hu et al. 2007). As the disease progresses, the remyelinating capacity is further decreased for several reasons. One of non-disease related factors leading to the decline in remyelination is aging (Sim, Zhao et al. 2002). Aging is associated with a reduction in both recruitment and differentiation of progenitor cells. Additionally, phagocytotic removal of myelin debris favors remyelination and this ability is impaired in aged mice compared to young mice due to the delayed activation
and recruitment of macrophages (Zhao, Li et al. 2006). Currently, many researchers are seeking therapies that can enhance remyelination by 1) identifying pathways to overcome the intrinsic inhibitory factors in cells, 2) administration of agents to induce differentiation and 3) tissue or stem cell transplantation. In fact, anti-LINGO-1 promotes significant remyelination in the animal models and its Phase I clinical trial in RRMS patients was just completed a couple months ago (Mi, Hu et al. 2007). It looks promising that the regenerative process could be much improved in the future.

### 1.4.2 Brain atrophy

MS research has long been focused on white matter lesions, which can be readily visualized by MRI. However, white matter lesions correlate weakly with neurologic disability (Brex, Ciccarelli et al. 2002; Fisniku, Brex et al. 2008). This paradox could at least be partly explained by grey matter lesions that are characterized by demyelination and neuronal loss in cerebral cortex and deep nuclei (Fisher, Lee et al. 2008; Fisniku, Chard et al. 2008; Rudick and Fisher 2009; Rudick and Trapp 2009).

With the advancement in MRI techniques, it became possible to measure brain tissue quantitatively. Together with histological studies, researchers can examine white matter (WM) and grey matter (GM) more systematically. It turns out the normal appearing white matter (NAWM) is far from normal, and GM is also affected in MS (Peterson, Bo et al. 2001). Accelerated brain atrophy rate was detected in Clinical Isolated Syndromes (CIS) patients who convert to RRMS, but not those who did not develop further symptoms,
suggesting brain atrophy occurs early in the disease course (Calabrese, Atzori et al. 2007). To further understand brain atrophy in disease progression, a four-year longitudinal study investigated the brain atrophy rates of whole brain, GM and WM respectively in MS patients at all disease stages and characterized extensively the relationship between brain atrophy and disease disability (Fisher, Lee et al. 2008). In addition to reproduction of the previous observation that both GM and WM atrophy occurs in RRMS patients, they found that GM and whole brain, but not WM atrophy rate accelerates in patients with more advanced stages of MS. Expressed as fold increase from healthy individuals, whole brain atrophy increased progressively from 2 fold normal in CIS patients converting to RRMS to 5.8 fold normal in SPMS patients. GM atrophy increased from 3.4 fold normal in CIS patients converting to RRMS to 14 fold normal in SPMS patients. In contrast, WM atrophy was consistent and stayed at 3 fold normal across all disease subgroups. This indicates GM accounts for the increase in whole brain atrophy in patients with more advanced disease (Fisher, Lee et al. 2008). Another important finding of this study was that GM atrophy was significantly associated with neurologic disability determined by both Multiple Sclerosis Functional Composite (MSFC) and Expanded Disability Status Scale (EDSS), suggesting GM atrophy rate could potentially predict the disease progression and be used as an outcome measurement in clinical trials.

However, the association between atrophy and disability scores has been mixed as some other studies showed weak or no correlation (Chard, Griffin et al. 2002). This might not be very surprising, given that 1) different scoring systems such as EDSS and MSFC can
be affected differently based on pathology and may reflect different regions of the CNS that are affected; 2) The lesion burden that causes clinical symptoms might be not the same for all the functions 3) neuronal plasticity may, at least partially, compensate for damage to the neural network, diminishing the impact of brain damage to clinical presentation (Morgen, Sammer et al. 2006). Therefore, it would be of interest to study the correlation between specific CNS functions and brain regions. In fact, a study has shown that GM atrophy correlates with cognitive functions measured by MSFC, but not EDSS (Rudick, Lee et al. 2009). It remains to be determined how cognitive dysfunctions develop from disease onset, and how it relates to GM and WM atrophy globally and regionally.

Quantitative MRI techniques made it possible to establish the correlation between brain atrophy and disease progression. However, to closely study the pathogenesis of brain atrophy, additional techniques have been developed including histological staining, microarray and qPCR. In the last decade, many studies have carefully characterized grey matter lesions, and demonstrated that they are quite different from WM lesions in several aspects. First of all, blood brain barrier seems intact in the cortical lesions and infiltrating T and B lymphocytes were found to be sparse within the cortical lesions (Peterson, Bo et al. 2001). Second, neuronal loss and apoptosis can be observed in cortex, hippocampus and deep nuclei (Papadopoulos, Dukes et al. 2009; Hasan, Walimuni et al. 2011). Third, diffuse activation of microglia has been detected in grey matter lesions. Given the differences in GM and WM pathology, mechanisms responsible for grey matter lesions
may be different from WM lesions.

One study closely examined grey matter lesions and demonstrated that dendritic transection and neuronal death caused by apoptosis in demyelinated grey matter from patients with clinical disease ranging from 2 months to 27 years (Peterson, Bo et al. 2001). The cortical lesions could contribute to the neurological dysfunction in MS. It has been well described that memory impairment was found in about 50% of MS patients, raising the possibility that demyelination in hippocampus may cause the memory deficits (Smestad, Sandvik et al. 2010). In fact, demyelination can be found in 53-79% of postmortem MS hippocampi (Papadopoulos, Dukes et al. 2009). However, the molecular mechanism behind this observation is unknown. A recent study from Dr. Trapp’s group shed some insight on this subject. By carefully comparing demyelinated and myelinated hippocampi, they found that demyelinated hippocampi had minimal neuronal loss but significant decreases in synaptic density (Dutta, Chang et al. 2011). Further investigation suggested that in the demyelinated areas, neuronal proteins essential for axonal transportation, glutamate homeostasis and memory/learning formation are much reduced and could be responsible for the memory impairment in certain MS patients. Surprisingly, very few of the neuronal genes that are altered in demyelinated MS hippocampi were altered in the demyelinated motor cortex. Therefore, this study might provide a molecular basis for the memory decline in MS patients.

In addition to neuronal dysfunction as a typical pathology, activation of microglia has
also been observed in demyelinated grey matter. In active and chronic active cortical lesions, microglia display an activated phenotype with increased size, thicker processes and an orientation perpendicular to the pial surface (Peterson, Bo et al. 2001). Further studies demonstrated that microglia extended processes to and ensheathed the neuritic elements and neuronal cell bodies. Although those neurons that are targeted by microglia are functionally impaired, it was unclear whether this association between microglia and neurons is detrimental or protective to neurons. More recent studies in animal models provide some insights on the role of microglia activation in disease progression. Dr. Khoury’s group found persistent activation of microglia in EAE mice even after they entered into a chronic phase (Rasmussen, Wang et al. 2007). Moreover, these activated microglia are pathogenic since they correlated with phosphorylation of neurofilaments in the cortex and stripping of synaptic proteins in cortical neurons. In agreement with this finding, it has been shown that specific suppression of microglia led to milder EAE development (Ponomarev, Veremeyko et al. 2011). These studies suggest that anti-inflammatory therapies targeting microglia seem promising in the treatment of MS in the future.

It is well accepted that neurodegeneration plays an important role in MS progression. The question whether neurodegeneration or inflammation is the primary cause of MS is still in debate. Quantitative MRI showed that GM atrophy occurs early in the disease (Calabrese, Atzori et al. 2007). In addition, by staining postmortem MS brains, a large body of work has consistently demonstrated that cortical lesions are lacking in infiltrating
immune cells, suggesting a neurodegenerative mechanism independent of inflammation (Peterson, Bo et al. 2001; Rudick and Trapp 2009). However, this idea is challenged by a recent study by a research group from Mayo Clinic (Lucchinetti, Popescu et al. 2011). This study is the first report of brain biopsies specifically from early stage MS patients evaluated by histology. Cortical lesions are commonly present and nearly 40% of these early stage MS patients had cortical lesions. Histological staining revealed that foamy macrophages were found in 66% of the patients, indicating ongoing demyelination in the cortical lesions. In addition, CD3+ and CD8+ infiltrating T cells were found in 83% and 77% of the patients respectively, suggesting they contribute to the cortical lesion development. Based on their study, the authors argued that neurodegeneration occurs on an inflammatory background and may resolve gradually as disease progresses, which could explain the absence of infiltrating cells in cortical lesions by previous studies. However, one should keep in mind that MS is such a heterogeneous disease that the pathogenesis of MS individuals likely varies. Therefore, this study with a relative small sample number may only reflect certain MS populations. In addition, patients recruited in this study have a special form of MS called tumefactive form of MS in which inflammation is more severe than in other MS populations. Nevertheless, further studies are needed to better understand the relationship between inflammation and neurodegeneration.

Both GM and WM lesions are present and involved in very early disease phase and influence the disease progression. However, the pathogenesis of these lesions is not clear.
Understanding how the lesions occur and persist will help enhance our knowledge of the pathogenesis of MS and other neurodegenerative diseases.

1.4.3 Oxidative stress

Oxygen is capable of accepting electrons and has the potential to cause oxidation in other molecules. The transfer of electrons from other molecules to oxygen results in the formation of intermediates with unpaired electrons, which are named Reactive Oxygen Species (ROS) (Carlson and Rose 2006). These ROS including superoxide, hydrogen peroxide and hydroxyl radicals, can result in severe damage to lipid, protein and DNA. The human body is not unprepared for ROS generation. Cells are endowed with enzymatic and non-enzymatic (e.g glutathione, pyruvate and uric acid) anti-oxidant systems (Superoxide dismutase, SOD; glutathione peroxidase, GPx ) to consistently neutralize or convert ROS to non-toxic forms and maintain the redox homeostasis. One of the most prominent examples is glutathione, a peptide that is intracellularly synthesized from glutamate, cysteine and glycine. Although glutathione is a cofactor of GPx, it can also function as an anti-oxidant and is able to generate other antioxidants such as vitamin C and E (Nakamura, Nakamura et al. 1997). Due to its high concentration and its central role of maintaining the redox homeostasis in cells, it is the most critical of the cellular antioxidants.

Under pathogenic conditions, however, ROS production is significantly and persistently enhanced and the anti-oxidant system is not sufficient to reset the system to redox
baseline, resulting in cellular damage (Liu, Zhao et al. 2001). ROS have long been associated with MS, contributing to disease progression. A recent paper studied the markers of oxidative and nitrosative stress in RRMS and SPMS patients (Miller, Walczak et al. 2012). They found that levels of 3-nitrotyrosines (3-NT) and carbonyl groups, estimation markers of oxidative stress, were significantly enhanced in MS patients compared to healthy individuals, suggesting a pathogenic role of oxidative stress (Hill, Zollinger et al. 2004; Miller, Walczak et al. 2012). Additionally, the redox homeostasis was further disrupted by the reduced expression of the natural anti-oxidant uric acid in these patients (Miller, Walczak et al. 2012). Not only was oxidative stress enhanced in the periphery of MS patients, it is also significantly increased in MS brain, the target organ. It was demonstrated that oxidized DNA was found in oligodendrocytes that seemed to undergo apoptosis in acute MS lesions (Haider, Fischer et al. 2011). In addition, neurons stained for oxidized phospholipids frequently revealed signs of degeneration with fragmentation of their dendritic processes. Interestingly, the extent of lipid and DNA oxidation related strongly to inflammation (Haider, Fischer et al. 2011).

The evidence that ROS contributes to propagation of disease progression is further supported by animal experiments where preventative or therapeutic administration of anti-oxidants can reduce disease severity and decrease the production of ROS (Hooper, Bagasra et al. 1997; Liu, Zhu et al. 2003; Ilhan, Akyol et al. 2004). It is clear that redox homeostasis is disrupted in MS patients, and the success of anti-oxidant therapy in animal models gave rise to the possibility of anti-oxidant therapy in MS patients. However, even
with the strong rationales for anti-oxidant treatment in MS, evidence for therapeutic effects of anti-oxidants in MS is limited. Therefore, we are still at an early stage of evaluating the potentials of anti-oxidants as a therapy for MS and current research is actively seeking more potent anti-oxidants in the hope of developing more effective treatments.

1.4.4 Glutamate excitotoxicity

As an essential and the most abundant excitatory neurotransmitter in the brain, glutamate plays multiple roles maintaining normal CNS function such as vision, taste, learning and memory. Under physiological conditions, the primary source of glutamate is from presynaptic neurons (Nedergaard, Takano et al. 2002). Once released into the synapse, glutamate activates pre and post synaptic neurons and glial cells. Transporters in the outer membranes of astrocytes clear synaptic glutamate and convert it to inactive glutamine via ATP-dependent glutamine synthetase, and release glutamine into the synapse. Glutamine is then transported into neurons, where it is converted back to glutamate via glutaminase and packed into synaptic vesicles for the next release (Rahn, Slusher et al. 2012). Glutamate concentration is tightly regulated by different mechanisms, low concentration of glutamate may result in cognitive dysfunction, while on the other hand; high concentrations of glutamate may cause neuronal death and subsequent brain injury (Werner, Pitt et al. 2001; Kostandy 2012). Death or degeneration of cells in the CNS due to prolonged exposure to high synaptic glutamate is termed glutamate excitotoxicity, which has been shown to participate in many neurological disorders including stroke,

Glutamate levels were found increased in relapsing MS patients compared to healthy individuals (Stover, Pleines et al. 1997). Recently evidence of glutamate enhancement in the CNS of MS patients was further supported by MR spectroscopy (MRS). Glutamate levels were not only shown to be higher in the acute lesions, but its level was also found to increase in the NAWM (Srinivasan, Sailasuta et al. 2005). The potential role of excitotoxicity in MS was first suggested by experimental data demonstrating the beneficial effect of NMDA and AMPA/Kainate blockers in EAE and the reduction of axonal and oligodendrocyte damage in these mice (Wallstrom, Diener et al. 1996; Pitt, Werner et al. 2000; Smith, Groom et al. 2000). In addition, it was also shown that AMPA blockers did not influence T cell infiltration or proliferation in vitro, excluding their potential immunoregulatory role at the doses administered. Similarly, by immunohistochemical staining of postmortem brain tissue, studies showed that multiple steps of glutamate signaling are interrupted in MS lesions. A number of studies demonstrated that the expression of glutamate transporters including excitatory amino acid transporter 1 and 2 (EAAT1 and EAAT2) on oligodendrocyte were downregulated, suggesting an impaired ability of oligodendrocytes to take up extracellular glutamate (Pitt, Nagelmeier et al. 2003). Further studies showed that the presence of inflammatory cytokines such as TNF-α could contribute to the reduced expression of these transporters on human oligodendrocytes (Pitt, Nagelmeier et al. 2003). Not only was the number of
glutamate transporters reduced, but the function of these transporters was also impaired (Pitt, Nagelmeier et al. 2003). In the same study, it was shown that the ability of oligodendrocytes to take up glutamate was reduced by 75% in the presence of TNF-α. Extracellular glutamate may be further affected by the reduced expression of glutamine synthetase (GS) and glutamate dehydrogenase (GDH), two key enzymes for glutamate metabolism in MS lesions (Werner, Pitt et al. 2001).

In addition to disrupted glutamate homeostasis in oligodendrocytes, astrocytes may contribute to extracellular glutamate by releasing glutamate at the synapse (Struzynska 2009). Under normal physiological conditions, astrocytes play an essential role in clearing synaptic glutamate, preventing excitotoxicity. However, in the MS lesions, instead of taking up glutamate and converting it to glutamine, astrocytes release glutamate through several mechanisms: 1) reversed uptake by glutamate transporters initiated by high intracellular Na and glutamate (Szałkowski, Barbour et al. 1990); 2) glutamate exchange via cystine-glutamate transporter (Warr, Takahashi et al. 1999); 3) calcium-dependent glutamate exocytosis initiated by high extracellular potassium or high intracellular sodium and glutamate (Parpura, Basarsky et al. 1994). Similarly, it has been suggested that microglia and infiltrating macrophages secrete glutamate through cystine-glutamate antiporter mechanisms (Kigerl, Ankeny et al. 2012). In fact, a recent study demonstrated that the expression of cystine-glutamate antiporter on monocytes and microglia is enhanced in MS lesions and in the CNS of EAE mice, suggesting its contribution to excitotoxicity (Pampliega, Domercq et al. 2011). Additionally, the
presence of proinflammatory cytokines in the CNS potentiates glutamate mediated toxicity. It has been consistently demonstrated that activated microglia release significant amounts of TNF-α, which enhances glutamate secretion from astrocytes and potentiates the susceptibility of oligodendrocytes to excitotoxicity (Bezzi, Domercq et al. 2001; Miller, Sun et al. 2005).

In conclusion, multiple points of glutamate homeostasis are dysregulated in the CNS of MS patients, resulting in enhanced glutamate levels. Excessive activation of glutamate receptors causes overloading of calcium in mitochondria, which generates ROS including NO and peroxynitrite. The combination of oxidative stress and high levels of calcium results in opening of mitochondria transitional pores, causing cell death via necrosis or apoptosis.

1.5 Current therapy of MS

IFN-β

Since viral infection had been postulated as the cause of MS, the anti-viral capacity of IFN-β led to its first clinical trial in RRMS (Jacobs, Cookfair et al. 1996; 2001). Currently, there are three formulation of IFN-β. Two preparation of IFN-β-1a are available under the names of Avonex and Rebif. Refib is given subcutaneously three times of a week while Avonex is applied intramuscularly once a week. IFN-β-1b is under the name of Extravia and Betaferon that are administered via daily injection. Although the precise mechanisms of action of IFN-β have not been fully understood, this therapy
may modulate immune responses from different aspects. It was demonstrated that IFN-β impairs the maturation of DCs by reducing the expression of MHC II and costimulatory molecules, and subsequently influences antigen presentation (Jiang, Milo et al. 1995; Genc, Dona et al. 1997). In addition, IFN-β reduces DC migration capacity towards draining lymph nodes for antigen presentation via suppression of C-C chemokine receptor type 7 (CCR7) (Yen, Kong et al. 2010). Additionally, IFN-β treatment reduces serum MMP-9 levels. Since MMP-9 has been implicated in disruption of BBB and is upregulated in the serum of MS patient prior to the appearance of new Gd enhancing lesions, it is speculated that IFN-β may help maintain the integrity of BBB, preventing infiltrating pathogenic cells into CNS (Trojano, Avolio et al. 1999; Waubant, Goodkin et al. 1999). Future study should help clarify the mechanisms in IFN-β treatment, allowing physician to better select the best treatment for each individual patient.

**Glatiramer acetate**

In addition to IFN-beta, Glatiramer acetate (GA, copaxone) represents another common first line therapy for MS that is administered by daily subcutaneous injection. GA is a synthetic mixture of glutamate, alanine, tyrosine and lysine, in a molar ratio of 1.5:3.6:4.6:1. It was originally designed to mimic structure of MBP, the presumed target antigen in MS (Racke and Lovett-Racke 2011). Initially, GA was synthesized to cause EAE. However, surprisingly, it attenuated EAE, the animal model of MS (Teitelbaum, Meshorer et al. 1971). This observation greatly attracted the interest of many MS researchers to investigate its mechanisms of action. GA may alter the disease activity
through several mechanisms. GA induces immune deviation, favoring a shift of Th1 to Th2 differentiation by promoting type II macrophages and anti-inflammatory cytokines such as IL-10 (Aharoni, Teitelbaum et al. 1997; Weber, Prod'homme et al. 2007). Additionally, studies have shown that GA reactive T cell home to CNS and produce neurotrophic factors locally to facilitate remyelination (Ziemssen, Kumpfel et al. 2002). Moreover, not only does GA influence CD4+ T cell populations, a number of studies have suggested that GA treatment leads to an expansion of regulatory CD8+ cells in MS patients, providing additional mechanisms of GA (Karandikar, Crawford et al. 2002). Because of its long history of tolerability and safety, it will likely remain an important option for the treatment of MS patients for years to come.

**Natalizumab**

Natalizumab is a recombinant humanized monoclonal antibody that binds to the α4 subunit of the α4β1 integrin and interferences with the α4 mediated binding to its natural ligands of the vascular cell adhesion molecule 1 (VCAM1). The efficacy of Natalizumab in EAE led to initiation of clinical trials in the treatment of MS (Deloire, Touil et al. 2004). In 2004, Natalizumab was approved by FDA for RRMS and now is the most effective treatment for RRMS (Havrdova, Galetta et al. 2009). One of the risks of Natalizumab treatment is progressive multi-focal leucoencephalopathy (PML), which is caused by reactivation of JC virus that could result in severe disability or death (Bloomgren, Richman et al. 2012). To determine the benefit to risk ratio, a new two step ELISA kit to measure anti-JC virus antibody in the serum and plasma has been
developed by the company and seems to be promising to predict the risk of developing PML (Gorelik, Lerner et al. 2010). Other adverse effects include hypersensitive reaction and hepatotoxicity. Accordingly, patients have to be monitored during Natalizumab infusion and their liver enzymes need to be measured regularly.

**Fingolimod**

Fingolimod (Gilenya®), the first oral treatment for RRMS, was approved by FDA and European Medical Association (EMA) in 2010 (Comi, O’Connor et al. 2010; Kappos, Radue et al. 2010; Khatri, Barkhof et al. 2011). This medication is given once daily in capsule form. The mechanism of Fingolimod is not known with certainty. However, the predominant view is that Fingolimod inhibits the egress of lymphocytes from secondary lymphoid tissue through suppression of sphingosine-1-phosphate (S1P) receptors potentially reducing the trafficking of pathogenic cells to the CNS (Horga and Montalban 2008). In support, a marked reduction of CD4+ T and B cells in the periphery blood was observed in Phase II and III clinical trials (Kappos, Antel et al. 2006; Kappos, Radue et al. 2010). Moreover, due to its lipophilic nature, Fingolimod can diffuse to the CNS and may further benefit the local microenvironment since S1P can be found in CNS cells including astrocytes, Oligodendrocytes and neurons (Glickman, Malek et al. 1999; Jaillard, Harrison et al. 2005; Choi, Gardell et al. 2011; Cohen and Chun 2011). However, more evidence is needed to make a defined conclusion of its neuroprotective effects. Side effects of Fingolimod is related to cardiovascular system including hypertension and heart failure. Because deaths have been reported among patients treated with Fingolimod,
in April of 2012, FDA modified the prescribing information for Fingolimod. It defines who should avoid taking Fingolimod based on pre-existing conditions and careful heart monitor and other recommended testing should be performed on patients before the first dosage (Cohen and Chun 2011).

**Teriflunomide**

Telifluromide (Aubagio®), approved in Sept of 2012, added a second daily oral treatment for RRMS. Telifluromide is the active form of leflunomide, a therapy approved for RA patients. Because of its efficiency in treating RA patients, the clinical application of Leflunomide has investigated in other inflammatory diseases (Bartlett, Dimitrijevic et al. 1991). Phase III clinical trials have demonstrated that Telifluromide treatment was superior to placebo on a range of endpoints by MRI including Gd-enhancing lesions reduction, annualized relapse rate and disability progression (Miller, O'Connor et al. 2012). As an inhibitor of the dihydroorotate dehydrogenase (DHODH), the rate limiting enzyme in the de novo pyrimidine synthesis, it was shown that Telifluromide impairs DNA synthesis, especially in blasting lymphocytes (Fairbanks, Bofill et al. 1995). Additionally, it was shown that COX-2 is also a primary molecular target of Telifluromide, and in fact Telifluromide suppresses the formation of PGE2, the enzymatic product of COX-2 (Hamilton, Vojnovic et al. 1999). The side effects of this treatment include abnormal live tests, diarrhea and nausea and hair thinning (O'Connor, Wolinsky et al. 2011). Although an understanding of long-term safety regarding to rare adverse effects e.g. PML will await future trials with longer patient number for long-term
treatment, its to-date safety experiences could be supplemented by its pro-drug leflunomide, a treatment for rheumatic arthritis in 1998. Two PML cases related to leflunomide have been documented since its approval (Palazzo and Yahia 2012). With the capability of monitoring the plasma anti-JC virus antibody, PML may be well managed in the patients who decide to take Telifluromide.

**Summary**

Since 1993 when IFN-β, the first available MS therapy, was approved for RRMS, a number of other effective interventions have become available for patients with MS. Currently there are nine medications (Oct, 2012) for treatment in RRMS (Table 1). This is an excellent achievement for MS research and MS clinical management. First of all, the benefit of these medications usually comes with unwanted adverse effects, some of which could be life-threatening including risk for progressive multifocal leucoencephalopathy (PML) (Kleinschmidt-DeMasters, Miravalle et al. 2012) and cardiovascular failure related to Natalizumab and Fingolimod treatment respectively (Lindsey, Haden-Pinneri et al. 2012). Secondly, patients do not respond to current therapies equally. It has demonstrated that about a third of patients fail to respond the IFN-β therapy probably due to the production of antibodies (Comabella, Lunemann et al. 2009). Thirdly, seven out of nine medications are administered by either i.v or i.m routes, which causes extra pain and inconvenience for patients already suffering from MS. For instance, local skin reaction around the injection sites is observed in some Copaxone treated patients (Sanchez-Lopez, Rodriguez del Rio et al. 2010). Last but most
importantly, these medications fail to slow down continuous brain atrophy in progressive forms of MS. Therefore, a new challenge is to develop safe and neuroprotective medications that can be administered orally. One of the promising candidates is Dimethyl fumarate (DMF).

1.6 Dimethyl fumarate (DMF)

Dimethyl fumarate, together with monomethyl fumarate (MMF) was registered as Fumaderm® for the treatment of psoriasis, an inflammatory skin disease in 1994 (Mrowietz and Asadullah 2005). Since psoriasis is regarded as an immune disorder that shares similarities with other inflammatory diseases such as MS, the therapeutic effects of DMF in MS has been investigated (Prinz 2001). Interestingly, both Phase II and III clinical trials have demonstrated that oral administration of DMF significantly reduces gadolinium lesions in RRMS patients and slows down disease relapse rate (Schimrigk, Brune et al. 2006; Fox, Miller et al. 2012; Gold, Kappos et al. 2012; Kappos, Gold et al. 2012; Perumal and Khan 2012; Saidha, Eckstein et al. 2012). Importantly, the safety record from both MS and psoriasis patients looks excellent. Mild side effects including GT tract reaction (~36% of the patients), flushing (~35% of the patients) and diarrheas were found in patients in the first month, but decreased thereafter. Flushing seems caused by MMF, the main metabolite of DMF. As an agonist for nicotinic acid receptor, MMF promotes secretion of prostaglandin E2 (PGE2) by langerhans cells and keratinocytes, leading to cutaneous vasodilation (e.g flushing). However, this unwanted effect may be alleviated by co-administration of aspirin together with DMF (DX72, 4th cooperative
meeting of ACTRIMS and CMSC). Currently, this medication is under FDA evaluation for the treatment of RRMS. With its excellent safety profile, efficacy and oral availability, DMF is very likely to become a new addition to the current MS therapies.

With its excellent therapeutic effects in psoriasis and the recent success in MS treatment, however, the molecular mechanisms of DMF have not been fully understood and novel aspects are emerging. Previous studies have demonstrated that DMF treatment results in reduced chemokine expression on endothelial cell and subsequently prevents immune cell infiltration to the targeted organ (Nelson, Carlson et al. 1999). Additionally, a number of studies also suggested that DMF treatment leads to reduced T cell number in the blood stream via both apoptosis and reduced NF-κB signaling (Treumer, Zhu et al. 2003). More recently, accumulating evidence demonstrated that Nrf-2 (nuclear factor E2 (erythroid-derived 2)-related factor), a critical transcription factor involved in combating oxidative stress, was activated after DMF treatment (Linker, Lee et al. 2011; Scannevin, Chollate et al. 2012). Given that oxidative stress has been implicated in the MS brain, it becomes of interest to many research labs to address the question to what degree DMF has primary neuroprotective properties.

My dissertation work is dedicated to understanding the mode of actions of DMF in MS. Our hypothesis is that DMF benefit MS from both immunoregulatory and neuroprotective aspects. The first aim of my study is to determine the effects of DMF on DC maturation and subsequent DC mediated T cell responses. Additionally, the other aim
of my graduate work is to determine the potential neuroprotective effects of DMF in an excitotoxic model induced by microinjection of AMPA into the spinal cord. I believe that this translational study will not only benefit future drug development, but will also enhance our understanding of the molecular pathways that are critical to the pathophysiology of MS.
Figure 1.1 Antigen presentation and T cell differentiation
<table>
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<th>Therapy</th>
<th>Mechanisms</th>
<th>Adverse effects</th>
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<tr>
<td>IFN-β</td>
<td>Immune deviation with enhanced IL-10 and reduction of T cell number</td>
<td>Flu symptoms</td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>Immune deviation, inhibition of antigen presentation and enhancement of Treg</td>
<td>Pain and swelling at injection site</td>
</tr>
<tr>
<td>Fingolimod</td>
<td>a sphingosine 1-phosphate receptor modulator, sequestering lymphocytes in lymph nodes,</td>
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<td>Natalizumab</td>
<td>Inhibition of leukocytes migration to the CNS</td>
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<td>Teriflunomide</td>
<td>Blockade of proliferation and functioning of activated T and B cells</td>
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</tr>
<tr>
<td>Mitoxantrone</td>
<td>Inhibition of T and B cell proliferation, impair antigen presentation and proinflammatory cytokine production</td>
<td>Hair loss, nausea and diarrhea</td>
</tr>
</tbody>
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Table 1.2 Current Therapy for Multiple Sclerosis

By October, 2012
Chapter 2:

**Dimethyl fumarate inhibits dendritic cells maturation via suppression of NF-κB and ERK1/2-MSK1 and upregulation of Heme Oxygenase-1**

**Introduction**

In the steady state, immature DCs lack the capacity to initiate a strong T cell response due to low expression of MHC class II, CD80 and CD86. Activation of DCs through Toll like receptor signaling or encounter with antigen leads to maturation and increased expression of MHC class II, CD80 and CD86. These modifications allow DCs to become efficient at presenting antigen to naïve T cells, which is critical for launching the adaptive immune response (Chastain, Duncan et al. 2011). Cytokine production is also initiated during the maturation of DCs. This is important as unique cytokine micro-environments influence naïve T cell differentiation. IL-12 instructs T cells towards IFN-γ producing CD4+ cells (Th1), while IL-6 in the absence of IL-12 favors IL-17 producing cell (Th17) differentiation (Yang, Weiner et al. 2009; Lovett-Racke, Yang et al. 2011). Additionally, IL-23 induces the expansion of Th17 cells and potentiates Th1 cell differentiation (Cua, Sherlock et al. 2003; Gocke, Cravens et al. 2007). Given the important role of DCs in instructing CD4+ T cell differentiation, the association of DCs with MS has been investigated. Peripheral DCs from patients with MS are more mature and polarize naive T
cells to secrete higher levels of cytokines compared with DCs from healthy individuals (Karni, Abraham et al. 2006). In addition to their key role in priming naïve T cells in the periphery, CD11c+ DCs are also found in the cerebrospinal fluid (CSF) and inflammatory MS lesions in the brain (Kivisakk, Mahad et al. 2004). This observation attracted the interest of many scientists to study the phenotypes and functions of DCs retained in the CNS. Evidence suggests that these CD11c+ DCs are capable of acquiring a mature phenotype in MS lesions to stimulate autoreactive T cells locally. In addition, these DCs may migrate to cervical draining lymph nodes and initiate new waves of autoreactive T cells (Serafini, Rosicarelli et al. 2006). Based on these studies, DCs are emerging as a critical player in the priming of autoreactive T cells in the periphery and the CNS.

Phase II and III clinical trials have demonstrated that oral administration of DMF significantly reduces gadolinium-enhancing lesions, annual relapse rate and disease progression in Relapsing-Remitting MS (RRMS) (Schimrigk, Brune et al. 2006; Kappos, Gold et al. 2008). The safety record, efficacy and oral availability of DMF make it potentially more beneficial to MS patients than currently available therapies. Although immunological studies show that DMF treatment leads to a reduction in IFN-γ producing CD4+ T cells in both MS and Psoriasis patients, the cellular and molecular mechanisms of DMF are not fully understood (Schimrigk, Brune et al. 2006). Understanding the mechanisms by which DMF exerts its therapeutic effects will not only benefit drug development, but will also enhance our understanding of the molecular pathways that are critical to the pathophysiology of MS.
In the present study, we focus on the effect of DMF on DC maturation and subsequent DC-mediated T cell responses since DCs are the key element in the interface of innate and adaptive immune responses. We show that DMF inhibits maturation of DCs by significantly reducing proinflammatory cytokine production and expression of MHC class II, CD80 and CD86. These immature DCs generate fewer activated T cells, namely IFN-γ and IL-17 producing CD4+ T cells. Further, our molecular studies demonstrate that DMF hinders the maturation of DCs via 1) suppression of both p65 and Extracellular signal-Regulated Kinase 1 and 2 (ERK1/2)- Mitogen Stress activated Kinase 1 (MSK1) signaling and 2) upregulation of Heme Oxygenase-1 (HO-1).

Methods

Bone marrow derived dendritic cells (BMDC) generated in-vitro

BMDCs were generated according to standard procedures. In brief, bone marrow cells were removed from the femurs and tibias of B10.PL or C57BL/6 mice and cultured in: RPMI1640 (Invitrogen) supplemented with L-glutamine and penicillin/streptomycin at 2mM, βME (5x10^{-5} M), 10% FBS, and 2% J558L media. J558L cells (kindly provided by Dr. Miguel Stadecker) secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) which is needed to promote differentiation of bone marrow cells into DC. Cells were cultured at 2 × 10^6 cells/well for 10 days with 5% CO2 and 95% air at 37 °C. Fresh media supplemented with 2% J558L media was added to the cell culture on day 3, 6 and 8. Cells were harvested on day 10 for in vitro assays. For mitogen-activated protein
kinase (MAPK) and phospho-histone-3 analysis, BMDCs were harvested on day 10 and serum starved for 3-4 hours before LPS or DMF addition. This procedure allows us to 1) exclude the effects of the serum factors (e.g. growth factors) that may consistently activate MAPK and p-Histone-3 and 2) distinguish between specific agonist-induced signaling and that mediated by DMF.

*Dendritic cells generated in vivo*

DCs were expanded *in vivo* by subcutaneously (s.c) administering $4 \times 10^6$ B16 melanoma cells, which over-express murine Flt3L, to C57BL/6 female mice. DCs were isolated 3-4 weeks later. Whole spleens were processed into a single cell suspension, and CD11c$^+$ DCs were then purified using CD11c magnetic bead positive selection (Miltenyi Biotec, Auburn, CA) on an AUTOMACS Separator (Miltenyi). Purity of DCs was verified by flow cytometry to be greater than 95% (Supplemental Figure 1).

*Dendritic cell and MOG$_{35-55}$ specific T cell co-culture*

*In vivo* generated DCs were purified and plated onto 24-well plate at $1 \times 10^6$ cells/well. Cells were stimulated with LPS (100ng/ml, Sigma Aldrich) in the presence or absence of DMF (70μM, Sigma Aldrich) for 24 hours. Supernatants were then removed and DCs were washed twice in warm PBS. The myelin oligodendrocyte glycoprotein (MOG)-specific 2D2 T cell receptor (TcR) transgenic mouse generated by Dr. Vijay Kuchroo (Bettelli, Pagany et al. 2003) was a generous gift from Dr. Caroline Whitacre. MOG$_{35-55}$ specific T cells ($4 \times 10^6$/well) were added to the conditioned DC culture in the presence of
MOG<sub>35-55</sub> (2μg/ml). T cell activation and cytokine production were determined by flow cytometry and ELISA, respectively.

**Thymidine incorporation assay**

DCs generated *in vivo* was purified were plated on a 96-well plate at 1x10<sup>5</sup> cells/well. Cells were stimulated with LPS (100ng/ml) in the presence or absence of DMF (70μM) for 24 hours. Supernatants were then removed and DCs were washed twice in warm PBS. MOG<sub>35-55</sub> specific T cells (4x10<sup>5</sup> cells/well) were added to the conditioned DCs in 96-well plates for 48 hours. Cells were pulsed with 50 μl of media containing 0.5 μCi of [³H]-thymidine for 18 hours. Cells were then harvested with the FilterMate Harvester (PerkinElmer, Shelton, CT) onto Unifilter plates (PerkinElmer) and incubated overnight at room temperature to dry. A total of 30 μl Microscint 20 (PerkinElmer) were added to each well, and plates were sealed. [³H]-thymidine incorporation was read using a TopCount NXT machine (PerkinElmer).

**Cytokine ELISA**

Supernatants were collected for each culture condition and analyzed for IL-12p70, IL-6, IFN-γ, and IL-17 as previously described. ELISA was performed using purified anti-mouse capture antibodies and biotinylated rat anti-mouse detection antibodies (BD Biosciences, San Jose, CA, USA). Cytokine concentrations were calculated by generating a standard curve using recombinant proteins (R&D Systems) and analyzed using SoftMax Pro Software (Molecular Devices, Sunnyvale, CA, USA).
Flow cytometric analysis was performed to evaluate CD44 expression on T cells as previously described. In the coculture, GolgiPlug (1 mg/mL; BD Biosciences) was used to block cytokine secretion for the last 4 hours prior to staining. In addition, the expression of CD11c, CD80, CD86 and MHC class II on DCs were also examined under different conditions. About $10^5$ live cell events were acquired on a FACSCanto II (BD Biosciences) and analyzed using FlowJo software (Tree Star). The following antibodies were purchased from BD Biosciences: FITC-conjugated anti-CD44, PerCP-conjugated anti-CD4, APC-conjugated CD11c, FITC-conjugated MHC class II, PE-conjugated CD86 and V450-conjugated anti-CD80.

Immunohistochemistry

BMDCs were plated onto coverslips at 50,000 cells/cover slip and stimulated by LPS (100 ng/ml) in the presence or absence of DMF (70μM). At 5 and 60 minutes after stimulation, cells were washed by 0.1M PBS and fixed by 2% paraformaldehyde (PFA) for 30 minutes at 4°C. Cells were then stained by primary monoclonal rabbit-anti-mouse p65 antibody (1:500, Cell signaling). The location and distribution of p65 was visualized by incubation with Alexa Fluor 568 (1:500, Invitrogen)-conjugated goat anti-rabbit secondary antibody. DAPI (4′,6-diamidino-2-phenylindole, Sigma) was used to stain cell nuclei. To analyze the p65 distribution and reduce human bias, an automated scanning program was developed using Metamorph Image Analysis system. The intensity of p65
labeling in whole cell and nucleus were respectively collected by the program. Nuclear p65 distribution is expressed by the ratio of nuclear p65 and whole cell p65.

**Western blot**

Whole cell protein was extracted using 1% Triton X-100 lysis buffer (20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM Na Orthovanadate and 1:100 Protease Inhibitor (Sigma Aldrich) for phospho-p65, total p65 and IкBα and HO-1 detection. For MAPKs detection, whole cell protein was extracted using 1xSDS buffer with 1mM PMSF, 1mM Na Orthovanadate and 1:100 Protease Inhibitor. For cytoplasmic and nuclear protein isolation for C-terminal and N-terminal HO-1 detection (cell signaling), NE-PER nuclear and cytoplasmic extraction kit was purchased from Thermo scientific. Whole-cell lysates were electrophoretically separated on Criterion Tris-HCl Gel (10%) gel (Bio-Rad), transferred to nitrocellulose membranes (GE healthcare life sciences), and then probed with the antibody of interest. Detections were performed using HRP-conjugated secondary Abs, followed by development with ECL Western blotting substrate (Cell signaling), as previously described. The density of the bands was determined by using an AlphaImager (Alpha Innotech Corp.) p65 antibody, and p\text{ser}^{536} - p65 antibody, IкBα antibody, p\text{Thr}^{581} - MSK1 antibody, phospho-ERK_{1/2} antibody, phospho-JNK antibody, and phospho-p38 antibody were purchased from Cell Signaling. HO-1 antibody and N-terminal antibodies were purchased from Stressgen. C-terminal HO-1 antibody was from Santa cruz. p\text{ser}^{276} - p65 antibody was from Abcam. β-actin was from Sigma. U0126 was from Cell signaling and H89 was purchased from
EMD Millipore.

**Transfection with siRNA.**

The HO-1 and nonsense (NS) siRNA (Dharmacon, Lafayette, CO) target sequences were for 5-AAGCCACACACGAUCAUUGUAAdTdT-3 (sense) and 5-UUACAUAGUGCUUGUGGCUCdTdT-3 and 5- CGAACGAGUACCGUACACUdTdT (sense) and 5-AGU GUACCGGUACUCGUUCGdTdT-3 (antisense). siRNA transfection was performed in serum-free transfecting medium. To form complexes, the TransIt TKO buffer (Mirus, Madison, WI) and siRNA (HO-1 or control; Thermo Fisher Scientific) were each diluted in transfecting medium incubated at room temperature and then added to the cells as suggested by the manufacturer. After 24 h, medium was removed, and mRNA or protein analysis was performed 24–72 h after transfection.

**Luciferase assay**

The p19 luciferase construct was a gift from Y. Chen (University of Pennsylvania, Philadelphia, PA). RAW246.7 cells were plated at 1 million/well in 6-well plate and transfected with 1.5ug pGL3-Il23p19 reporter plasmid (bp −1180/+110 promoter fragment). After transfection with Lipofectamine reagent, cells were treated with the indicated concentrations of DMF and activated with 100ng/ml LPS. Or cells were rested over night followed by NS siRNA or HO-1 siRNA transfection (see (Ghoreschi, Bruck et al. 2011) for 20h. The cells were harvested with reporter lysis buffer (Promega) 6 hours after the addition of LPS or DMF and assayed for luciferase activity (Guerau-de-Arellano,
Smith et al. 2011). Luciferase readouts first normalized with total protein, then normalized with NS siRNA DMSO control condition.

**Statistical analysis**
GraphPad Prism software (GraphPad, La Jolla, CA) was used to perform all statistical analyses. All statistical analyses were performed using a two-tailed Student t test. A 0.05 p value was considered significant.

**Results**

DMF inhibits DC maturation
As a promising novel medication, the beneficial effects of DMF correlate with a reduction in IFN-γ⁺ CD4⁺ T cells in patients (Schimrigk, Brune et al. 2006; Ghoreschi, Bruck et al. 2011). Given the important role of DCs in instructing T cell differentiation, we sought to determine whether DMF impairs DC maturation and subsequent DC-mediated T cell responses. BMDCs were stimulated by LPS, a TLR4 agonist, in the presence or absence of DMF and cytokine secretion was determined by ELISA at 6 and 24 hours. DMF treatment significantly inhibited production of IL-12 and IL-6, proinflammatory cytokines that mediate Th1 and Th17 differentiation (Fig. 2.1A). The inhibitory effect of DMF was dose-dependent, with maximal inhibition achieved at the 70μM concentration. MTT assay indicated no toxicity of DMF at the dosages used (data not shown). Our cytokine data is in agreement with previous work from both our lab and others.
To further characterize DC responses to DMF, the expression of DC maturation markers was evaluated. Although the method for GM-CSF differentiated DCs is reproducible, easy and inexpensive, GM-CSF differentiated DCs display an inflammatory phenotype. Therefore, for the DC maturation and co-culture studies, we utilized DCs generated in vivo that are phenotypically equivalent to steady-state DCs in vivo. Our results suggest that LPS stimulation led to enhanced expression of MHC class I$^{\text{high}}$, CD80 and CD86$^{\text{high}}$ while addition of DMF completely abrogated the up-regulation of these surface molecules (Fig. 2.1B). Taken together, these data indicate that DMF inhibits DC maturation by suppressing both pro-inflammatory cytokine production and the expression of cell surface molecules required for effective antigen presentation to T cells.

**DMF-treated DCs are less effective antigen presenting cells**

As potent antigen presenting cells, DCs present peptides on MHC molecules to naive T cells and instruct T cell activation and differentiation. Since DMF treatment inhibited DC maturation and the production of cytokines that mediate the differentiation of Th1 and Th17 cells, we next wanted to test if DMF subsequently influenced DC-mediated T cell activation. *In vivo*-generated DCs were stimulated by LPS in the presence (LPS/DMF-DCs) or absence (LPS-DCs) of DMF for 24 hours, washed to remove DMF and LPS, then co-cultured with T cell receptor transgenic T cells specific for MOG$_{35-55}$ in the presence of MOG$_{35-55}$ peptide for 48-72 hours. This experimental set-up allowed us to rule out direct effects of DMF on T cells, since only DCs are exposed to DMF. T cell
activation and proliferation were evaluated by flow cytometry and $^3$H-Thymidine assay, respectively. As demonstrated in Fig. 2.2A, T cells generated by LPS/DMF-DCs had a markedly lower percentage of CD4$^+$ T cells expressing CD44, a marker of effector and memory T cells, compared to those generated by LPS-DCs. To determine if this reduction in cellular activation also resulted in decreased cytokine production, namely the hallmark Th1 and Th17 cytokines IFN-$\gamma$ and IL-17, cytokine production was evaluated by ELISA. Consistently, both IFN-$\gamma$ and IL-17 in the LPS/DMF-DC+MOG$_{35-55}$ specific T cell co-culture were significantly reduced (Fig. 2.2B). In addition, T cells generated by LPS-DCs had a high proliferation, while T cells from LPS/DMF-DCs co-culture had a significantly decreased proliferation (Fig. 2.2C). Thus, DMF inhibits the maturation and function of DCs, as well as the subsequent DC-mediated T cell response, resulting in reduced activation, cytokine production and proliferation of MOG$_{35-55}$-specific T cells.

**DMF inhibits the NF-$\kappa$B pathway in DCs**

To explore the molecular mechanisms through which DMF impairs the maturation of DCs, the NF-$\kappa$B pathway was analyzed since it is critical for TLR-induced DC maturation (Fig. 2.9, point 1). In the resting state, p65 is sequestered by I$\kappa$B$\alpha$ in the cytoplasm. Upon stimulation with agents such as LPS, I$\kappa$B$\alpha$ is rapidly phosphorylated and degraded. As a consequence, p65 is freed and translocates to the nucleus, where it binds to promoters of inflammatory genes and drives gene transcription. During this process, p65 is also phosphorylated at different sites to allow for optimal transcriptional activity (Chen and Greene 2004). To test if DMF treatment influences p65 activity,
BMDCs were stimulated by LPS in the presence or absence of DMF. Cells were fixed at 5 min and 60 min after LPS stimulation. Total p65 distribution was then evaluated by histology at each time point. As shown in Fig. 2.3A, the majority of p65 resides in the cytoplasm at 5 min after LPS activation; and as expected, a robust translocation of p65 to the nucleus was observed at 60 min. In contrast, DMF treatment retains p65 in the cytoplasm, resulting in significantly reduced p65 translocation to the nucleus (Fig. 2.3A-B).

It has been well described that degradation of IκBα plays a critical role in altering the dynamic balance of p65 between cytoplasm and nucleus, favoring nuclear entry of p65. To determine if decreased degradation of IκBα was responsible for the reduced translocation of p65 to the nucleus after DMF treatment, IκBα protein levels were determined by western blot. As demonstrated in Fig. 2.3C, DMF does not prevent IκBα degradation, suggesting that DMF does not inhibit p65 liberation. Phosphorylation of p65 enhances its nuclear stability and its capacity to recruit other co-activators to the promoter. To determine if DMF inhibits phosphorylation of p65 at specific serine residues, western blotting was performed on whole protein extracts using antibodies specific for phospho-ser276 and -ser536. As demonstrated in Fig. 2.3C-D, DMF reduces p65 phosphorylation, which may result in reduced capacity of p65 nuclear localization and decreased p65 transcriptional activity. These analyses of p65 localization and phosphorylation provide molecular evidence that DMF modulates signaling mechanisms that lead to DC maturation and cytokine production.
**DMF suppresses ERK<sub>1/2</sub>-MSK1 signaling**

In addition to NF-κB activation, mitogen-activated protein kinase (MAPK) family signaling is also involved and plays different roles in DC maturation. Suppression of p38 or Extracellular signal-Regulated Kinase 1 and 2 (ERK<sub>1/2</sub>) activation by inhibitors prevents DC maturation while activation of c-Jun N-terminal kinase (JNK) may limit DC maturation. To determine if DMF alters MAPK signaling (Fig. 7, point 2), BMDCs were serum starved for 3-4 hours and then treated with DMF 1 hour prior to LPS stimulation. U0126, an ERK<sub>1/2</sub> inhibitor, was included as a positive control. Whole cell proteins were extracted at 5, 10, 20 and 30 minutes after LPS stimulation and MAPK activity were determined by western blot. Figure 2.4 demonstrates that DMF treatment specifically inhibited phosphorylation of ERK<sub>1/2</sub> (Fig. 2.4A-B) but did not influence phosho-p38 or phospho-JNK activity (Fig. 2.4C). Previous studies suggest that MSK1, a downstream kinase of ERK<sub>1/2</sub>, positively regulates p65 signaling pathways. MSK1 enhances p65 transcriptional capacity by promoting phosphorylation of p65 at serine 276 (Fig. 9, point 3). In addition, MSK1 phosphorylates histone-3 at serine 10, which in turn promotes a permissive chromatin structure for transcriptional activity induced by p65 (Fig. 9, point 4). To address this issue, MSK1 activity was determined by western blot. As shown in Fig. 4A-B, LPS stimulation results in enhanced phosphorylation of MSK1 and this enhancement was impaired by DMF treatment.
DMF mediated ERK$_{1/2}$-MSK1 reduction contributes to the compromised NF-$\kappa$B activity

Although the MAPK and NF-$\kappa$B pathways are independent, they can interact via MSK1, where MSK1 phosphorylates p65 and enhances its transcriptional activity (Fig. 9, point 3). To dissect the contribution of ERK$_{1/2}$-MSK1 signaling reduction to NF-$\kappa$B inhibition caused by DMF, the MSK1 inhibitor H89 was used. The effect of DMF-mediated MSK1 reduction on different levels of the p65 pathway was examined and compared to that of H89 (Fig. 2.5). BMDCs were stimulated by LPS (500ng/ml), LPS+DMF or LPS+H89 (10μM) and whole cell proteins were analyzed for p$_{\text{ser10}}$-H3 and p$_{\text{ser276}}$-p65. As expected, H89 did not affect I$\kappa$B$\alpha$ degradation. Interestingly, H89 successfully mimicked the inhibitory effect of DMF on p$_{\text{ser276}}$-p65 and p$_{\text{ser10}}$-H3, suggesting that DMF-induced-MSK1 reduction contributes to the compromised NF-$\kappa$B activity.

H89 mimics the inhibitory effect of DMF on DC maturation

To further determine if DMF-induced MSK1 reduction is functionally important, the effect of H89 on DC maturation was determined. We hypothesized that H89 is able to mimic the inhibitory effect of DMF on DC maturation. To test this, purified DCs generated in vivo were stimulated by LPS, LPS+H89 or LPS+DMF for 24 hours. The expression of MHC class II, CD80 and CD86 was evaluated by flow cytometric analysis (Fig. 2.6A). To determine if the MHC class II$^{\text{high}}$, CD80 and CD86$^{\text{high}}$ cells are the same population, cells were first gated on MHC class II$^{\text{high}}$, then CD80 and CD86$^{\text{high}}$ (Fig. 2.6B). In the untreated condition, about 45% of MHC class II$^{\text{high}}$ cells are CD86$^{\text{high}}$ and only about 5% are CD80 positive. In contrast, after LPS stimulation, the percentage of
MHC class II$^{\text{high}}$ cells increased, and virtually all MHC class II$^{\text{high}}$ cells were also CD86$^{\text{high}}$ and about 73% of MHC class II$^{\text{high}}$ CD86$^{\text{high}}$ cells were also CD80 positive. This demonstrates that LPS induces a mature population expressing MHC class II$^{\text{high}}$ CD86$^{\text{high}}$ and CD80, which is expected to be highly efficient at driving T cell responses. Importantly, DMF completely blocked the development of this mature population. H89 partially mimicked the effect of DMF on DC maturation by decreasing the expression of MHC class II$^{\text{high}}$ from 38% to 8%, CD80 by 31% to 4% and CD86$^{\text{high}}$ from 44% to 11% (Figure. 2.6A). In addition, cytokine production was evaluated in different conditions. DMF reduced the secretion of both IL-12 (undetectable) and IL-6 (95% reduction). Similarly, H89 treatment led to a 85% reduction in IL-12 and a 60% reduction in IL-6 (Figure. 2.6C) Taken together, our data suggest that MSK1 reduction is critical for the inhibitory effect of DMF on DC maturation.

**DMF enhances HO-1 expression**

Several lines of studies demonstrated that DMF treatment leads to HO-1 induction in immune cells (Lehmann, Listopad et al. 2007; Cross, Cook et al. 2011; Linker, Lee et al. 2011; Scannevin, Chollate et al. 2012). This is a very interesting observation given that HO-1 has been suggested to have a protective property in EAE model (Chora, Fontoura et al. 2007).

To test the effects of DMF on HO-1 expression in our experimental setting, BMDCs were stimulated with LPS (100ng/ml) in the presence or absence of DMF (70μM). Whole cell
proteins were extracted at 3, 4, 5, 6, 8, 21, 25 hours after the addition of DMF and HO-1 expression was determined by western blot in each condition (Figure 2.7A). Our results demonstrated that DMF addition substantially enhanced HO-1 protein starting from 3 hours and peaked around 8 hours. To further confirm the HO-1 upregulation is specifically associated with DMF treatment, BMDCs were treated with different conditions including media, DMSO, DMF, LPS, LPS+DMSO and LPS+DMF, and whole cell protein were isolated at 6 hours. Our results confirmed that HO-1 was enriched only in the presence of DMF (Figure 2.7B).

*DMF inhibits IL-23 production through upregulating HO-1*

Our collaborator has shown that IL-23, a cytokine can potentiate both encephalitogenic Th1 and Th17 differentiation (Gocke, Cravens et al. 2007; McGeachy, Chen et al. 2009), was significantly reduced by DMF treatment of DCs. Further, in the same study, HO-1 siRNA application successfully restored IL-23 production in DMF-treated DCs (Ghoreschi, Bruck et al. 2011). This observation is quite surprising as HO-1 is not a classical transcription factor. Yet, it has been recently demonstrated that HO-1 can be cleaved into C-terminal HO-1 and N-terminal HO-1 in the cytoplasm and the cleaved form of HO-1 (N-HO-1) is capable of migrating to the nucleus, where it interferes with transcriptional activity of NF-κB (Lin, Weis et al. 2007). Therefore, it is possible that cleaved HO-1 may be indirectly involved in the transcriptional activity of NF-κB. In fact, in agreement with those studies, we and Dr. Rocken’s lab have shown that HO-1 induced by DMF can be cleaved and enter into the nucleus (Figure 2.8A).
To better understand the molecular mechanism through which HO-1 influences IL-23 production, our collaborator analyzed whether DMF-induced HO-1 associates with NF-κB binding sites within the IL-23p19 promoter by chromatin immunoprecipitation (CHIP) (Ghoreschi, Bruck et al. 2011). Indeed, their results demonstrated that specific enrichment of three defined regions of the IL-23 promoter was detected only in DMF-treated DCs but not in control DCs. These regions contain binding sites for either AP-1 or NF-κB (RelA and c-Rel). To test if this association of HO-1 to the NF-κB binding sites is functional, luciferase assay was utilized. The RAW cell line was transfected with an IL-23p19 luciferase promoter construct before addition of LPS. Interestingly, treatment of these cells with DMF led to a dose-dependent inhibition of the LPS-induced luciferase activity (Figure 2.8B). Moreover, application of HO-1 siRNA restored the IL-23p19 luciferase activity, indicating that DMF induced HO-1 enhancement impairs IL-23 transcriptional activity (Figure 2.8C).

**Discussion**

Our data show that the expression of DC maturation markers including MHC class II<sup>high</sup>, CD80 and CD86<sup>high</sup> is substantially inhibited by DMF. In addition, IL-12, IL-23 and IL-6 production are significantly reduced. These cytokines are essential for Th1 and Th17 cell differentiation. TLR4 mediated NF-κB activation involves both myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways (Kawai, Takeuchi et al. 2001; Covert, Leung et al. 2005; Doyle and O'Neill 2006) (Figure. 2.9). Although
proinflammatory cytokine production is mainly via MyD88-dependent signaling, these cytokines are still inducible in MyD88/TNF Receptor-associated factor-6 (TRAF6) double knockout mice, indicating the involvement of MyD88-independent pathway in cytokine production after TLR4 stimulation. Thus, it is possible that the inhibitory effect of DMF on DC maturation is via both pathways. Additionally, DMF may also block other TLRs signaling because the MyD88 dependent pathway is involved in all TLRs signaling (with the exception of TLR3), and MyD88 independent pathway is involved in TLR3/TLR4 signaling (Doyle and O'Neill 2006).

Our data further demonstrate that these immature DCs have impaired capacity in driving T cell activation. T cells generated by LPS/DMF-DCs activate and proliferate less and produce decreased levels of Th1 and Th17 hallmark cytokines. This is in agreement with the clinical observation that DMF treatment leads to pathogenic Th1 (IFN-γ^+^CD4^+^ T cell) cell reduction (Schimrigk, Brune et al. 2006). Thus, our study provides an important mechanism by which DMF suppresses T cell activation and proliferation. Clinical data in regard to the effect of DMF on Th17 cells, another pathogenic cell population, is not yet available. However, IL-17 production in the LPS/DMF-DCs+MOG_{35-55}-specific T cell co-culture was also significantly reduced, suggesting that Th17 differentiation is affected by DMF treatment as well. It would be of interest to determine whether the Th17 population in MS patients is decreased during the course of DMF treatment. It should be noted that some studies suggest that DMF leads to direct enhancement in CD4^+^ T cell apoptotic rate (Treumer, Zhu et al. 2003). However, the increases in apoptotic rate were
not sustained, and by 12 weeks of treatment the rate of apoptosis had returned to baseline levels, yet the IFN-γ producing CD4+ T cell count remains low during the entire treatment course (Schimrigk, Brune et al. 2006; Ghoreschi, Bruck et al. 2011). Because of its immunosuppressive effects, concerns may arise on potential adverse effects of DMF treatment. Yet, based on the MS clinical trial data and experiences from psoriasis patients, there is no evidence of increased risk of opportunistic infection or malignancy in DMF treated individuals.

To enhance our understanding of the effects of DMF on DCs, we began to explore the molecular targets of DMF in DCs. Previous studies showed that both NF-κB and MAPK activation contribute to LPS-mediated DC maturation (Handley, Thakker et al. 2005; Mou, Lin et al. 2011). In the resting state, p65 is bound to IκBα in the cytosol. Upon LPS stimulation, IκBα is phosphorylated, ubiquitinated and degraded by the proteosome machinery, releasing p65 to translocate to the nucleus and transactivate genes (Chen and Greene 2004; Bakkar and Guttridge 2010) (Figure 2.9, point 1). In addition, site-specific phosphorylation also occurs to increase p65 transcriptional activity either by enhancing binding to coactivators and basal transcription factors or by increasing p65 nuclear localization and stability (Chen and Greene 2004). Phosphorylation at the same site can be achieved by multiple kinases, often in a cell specific manner. For instance, ser276 of p65 is phosphorylated by protein kinase A (PKA) and MSK1, whereas phosphorylation at ser536 is mediated by different IκB Kinases (IKKs) (Reber, Vermeulen et al. 2009). Both phosphorylation of ser536 and ser276 play critical roles in regulating p65 activity (Chen
In the current study, we demonstrate that DMF inhibits p65 signaling by decreasing its phosphorylation and its entry to the nucleus. However, reduced p65 nuclear localization is independent of IκBα as DMF does not prevent its degradation. Our finding is in agreement with others where in TNF-α stimulated endothelial cells, DMF inhibits translocation of p65 to the nucleus without inhibiting IκBα degradation (Loewe, Holnthoner et al. 2002). What causes reduced nuclear localization of p65 by DMF treatment? One possible explanation might be the reduced phosphorylation of p65 caused by DMF. Phosphorylation of p65 at serine 276 was suggested to promote acetylization and retention of p65 in the nucleus. In addition, evidence also suggests that p^{ser536}-p65 does not exist in the classical NF-κB complex of p50 and p65. Instead of being controlled by IκB, its nuclear localization is probably facilitated by another protein that is not involved in the classical NF-κB pathway (Sasaki, Barberi et al. 2005). In our study, the fact that the reduction in p^{ser536}-p65 is independent of IκBα degradation indicates that DMF regulates phospho-p65 nuclear translocation through an alternative mechanism. We hope to address this question once these phospho-p65 nuclear translocation regulators are identified.

MSK1, a downstream protein of ERK_{1/2} or p38, potentiates p65 transcriptional activity (Figure 2.9, point 3). Initially identified as a nuclear kinase, MSK1 was later shown to be present in the cytosol as well (Vermeulen, De Wilde et al. 2003; Beck, Vanden Berghe et
al. 2008). MSK1 phosphorylates p65 at serine 276 and this phosphorylation was much reduced in MSK1/2 deficient mouse embryonic fibroblasts (Chen, Williams et al. 2005). It has been well documented that once phosphorylated at serine 276, p65 undergoes a conformational change, allowing its enhanced capacity of recruiting other co-factors to the gene promoters (Vermeulen, De Wilde et al. 2003). In addition, through phosphorylation of Histone 3 at serine 10, MSK1 promotes local relaxation of DNA and enhances the accessibility of p65 to gene promoters including IL-6, IL-12p40 and IL-8. In fact, by chromatin immunoprecipitation (ChIP) analysis, MSK1 and its substrates including both p\textsuperscript{ser276}-p65 and p\textsuperscript{ser10}-Histone3 were shown to localize to the endogenous NF-κB containing IL-6 promoter upon TNF-α stimulation (Vermeulen, De Wilde et al. 2003; Arthur 2008).

In our study, we demonstrate that DMF inhibits MSK1 activation through its upstream protein ERK\textsubscript{1/2} (Figure 2.9, point 2). Our observation is in conflict with a study published by Gesser et al where DMF inhibits MSK1 without affecting p38 or ERK\textsubscript{1/2} (Gesser, Johansen et al. 2007). However, different cell types and different stimuli may explain the differences we observed. Further, the importance of MSK1 as a target of DMF was supported by the mimicry experiments. We showed that without decreasing IκBα degradation, H89, a MSK1 inhibitor, reduces phosphorylation of p65 at serine 276 and histone-3 at serine 10. Moreover, this reduction in p\textsuperscript{ser276}-p65 and p\textsuperscript{ser10}-histone-3 is functionally important because H89 successfully inhibits DC maturation by reducing IL-6/IL-12 and MHC class II\textsuperscript{high}, CD80 and CD86\textsuperscript{high} expression. It should be noted that H89
partially mimics the function of DMF, which indicates the involvement of other phospho-p65 as (p\text{ser536}-p65) targets by DMF.

HO-1 was discovered four decades ago as the rate-limiting enzyme in heme degradation by converting heme to the bile pigments and generating carbon monoxide, biliverdin and ferrous iron (Cuadrado and Rojo 2008). Beyond its intrinsic role as a metabolic enzyme, HO-1 is now recognized as a ubiquitous inducible cellular stress protein which exerts a major role in cellular defense mechanisms. In addition to its anti-oxidative role, accumulated evidence shows that HO-1 is able to directly modulate the immune response (Cuadrado and Rojo 2008; Matsushima, Tanaka et al. 2009; Remy, Blancou et al. 2009; Ferenbach, Ramdas et al. 2010; Ghoreschi, Bruck et al. 2011). Under LPS stimulation, splenocytes from HO1/− mice showed higher proinflammatory cytokine production including IL-6, IL-1, TNF alpha and IFN-γ, indicating that deficiency of HO-1 tends to generate an exaggerated inflammatory response (Tracz, Juncos et al. 2007). Additionally, HO-1 knockout mice developed more severe EAE, suggesting a possible, protective role for HO-1 in MS (Chora, Fontoura et al. 2007).

Previous studies have shown that HO-1 was enhanced by DMF treatment in human monocytes, and HO-1 siRNA partially restored IL-12/IL-23 p40 expression (Lehmann, Listopad et al. 2007). In agreement, our collaborator found that HO-1 siRNA application restored IL-23 p19 and partially restored IL-12 p40 (Ghoreschi, Bruck et al. 2011). In lines with others, I also found that in DCs, conditions that induce HO-1 result in
translocation of a truncated, N-terminal part of HO-1 into the nucleus. Simultaneously, our collaborator found that in DCs treated with DMF, nuclear HO-1 physically associates with AP-1– and NF-κB–binding sites of the promoter region of IL-23p19 (Ghoreschi, Bruck et al. 2011). This is of biological significance, as nuclear HO-1 interferes with the transcriptional activity of AP-1 and NF-κB. Therefore, DMF induced HO-1 contributes to IL-23 reduction through upregulating HO-1, which is able to enter into the nucleus and suppresses the promoter activity of IL-23 (Figure 2.9, point 5).

HO-1 is largely regulated by the Nrf-2 signaling pathway, and the increased expression is perhaps due to activation of Nrf-2 as a number of papers have demonstrated that DMF treatment turns on Nrf-2 in astrocytes and subsequently promotes anti-oxidants production including HO-1 (Linker, Lee et al. 2011; Scannevin, Chollate et al. 2012). In the resting state, Nrf-2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap-1). In response to stimuli such as UV lights or oxidative stress, Nrf2 is dissociated from Keap1 and quickly translocates into the nucleus, where it binds to Antioxidant Response Element (ARE) and subsequent promote antioxidant production such as glutathione and HO-1 (Shen and Kong 2009). Therefore, we speculate that HO-1 upregulation is driven by Nrf-2 signaling pathway. Surprisingly, we did not find enhancement of Nrf2 mRNA after DMF treatment (data not shown). Yet, it does not exclude the Nrf2 activation because it is possible that the new Nrf2 protein synthesis is not needed as basal Nrf2 level is sufficient for nuclear translocation and driving HO-1 transcription. In this case, message level of Nrf2 might remain unchanged.
In conclusion, we demonstrate that DMF inhibits DC maturation and function, and subsequently suppresses Th1 and Th17 cell differentiation. Further, our molecular data demonstrate that DMF exerts its effect at multiple steps including 1) suppression of both p65 and ERK$_{1/2}$-MSK1 signaling and 2) upregulation of HO-1. Our study provides an important mechanism for the action of DMF in modulating inflammation and provides insight into pathways that are effective targets for therapeutic intervention in inflammatory diseases with unknown etiology such as multiple sclerosis.
Figure. 2.1 DMF inhibits dendritic cell maturation. (A) BMDCs were stimulated by LPS (100ng/ml) in the presence or absence of different dosages of DMF (17μM, 35μM and 70μM). Cytokine secretion was determined by ELISA at 6 and 24 hours. (B) DCs were generated in vivo, purified and stimulated by LPS (100ng/ml) in the presence or absence of DMF (70μM) for 24 hours. MHC class II, CD80 and CD86 were then stained for flow cytometric analysis. Numbers indicate the percentage of MHC class II^{high}, CD80 positive or CD86^{high}. *p<0.01. Data are derived from one experiment representative of three independent experiments.
Figure 2.1 DMF inhibits dendritic cell maturation.
Figure 2.2 LPS/DMF-DCs generate less T cell activation and proliferation characterized by decreased IFN-γ and IL-17 production. Purified DCs were stimulated by LPS (100ng/ml) in the presence or absence of DMF (70μM) for 24 hours, supernatant was then removed and DCs were washed twice by warm PBS. MOG35-55-specific T cells were then added to the conditioned DC culture in the presence of MOG35-55 peptides (2μg/ml). (A) T cell activation was determined by flow cytometric analysis of CD44, an effector T cell marker, at both 48 and 72 hours after co-culture was set up. The number above the gate indicates the percentage of activated CD4+CD44+ T cells. (B) Cytokine production including IFN-γ and IL-17 in the co-culture system supernatant was evaluated by ELISA. (C) Proliferation was assessed by [3H] thymidine incorporation. Cells were pulsed at 48 hours with [3H] thymidine and harvested 18 hours later. [3H] Thymidine incorporation was measured using a TopCount NXT (PerkinELmer). *p<0.01. Data are derived from one experiment, which is representative of at least three independent experiments.
Figure 2.3 DMF inhibits p65 phosphorylation and nuclear translocation. (A-B) BMDCs were stimulated by LPS (100ng/ml) in the presence or absence of DMF (70μM). (A) Cells were fixed by 2% PFA at 5 and 60 minutes after LPS stimulation and then labeled with p65 and DAPI, revealing increased cytoplasmic retention of p65 in DMF conditions. The immunohistochemical results are quantified in (B), which shows the ratio of nuclear p65 over whole cell p65 in LPS (black bar) and LPS+DMF (white bar) conditions. Data are derived from one experiment representative of two independent experiments. (C) Time course of phospho-p65 and IκBα expression in LPS and LPS+DMF conditions. BMDCs were stimulated by LPS (500ng/ml) in the presence or absence of DMF (70μM). Whole cell protein was then extracted at 0, 5, 20, 30, 40 and 60 minutes and phospho-p65, p65, IκBα and β-actin levels were determined by western blot and quantified in (D) as the ratio of phospho-p65/p65 or IκBα/ β-actin. Data are derived from one experiment representative of three independent experiments.
Figure 2.3 DMF inhibits p65 phosphorylation and nuclear translocation.
Figure 2.4 DMF inhibits ERK$_{1/2}$-MSK1 signaling. Serum starved-BMDCs were treated with DMF (70μM) or ERK$_{1/2}$ inhibitor U0126 (10μM) 1 hour before the addition of LPS (100ng/ml). Whole cell protein was then extracted at 5, 10, 20, 30 minutes and p-ERK$_{1/2}$, ERK$_{1/2}$, p-JNK, JNK, p-p38, p38, p-MSK1 and β-actin were determined by western blot. (A) Time course of expression of p-ERK$_{1/2}$ and p-MSK1. (B) Quantification of western blot images. Values are expressed as the ratio of p-ERK$_{1/2}$/ERK$_{1/2}$ and p-MSK1/ β-actin. (C) Time course of expression of p-JNK, JNK, p-p38 and p38 in each condition. Data are derived from one experiment representative of three independent experiments.
Figure 2. 4 DMF inhibits ERK\(_{1/2}\)-MSK1 signaling.
Figure 2.5 DMF-mediated-MSK1 reduction interferes with p65 signaling. (A) Serum starved-BMDCs were treated with DMF (70μM) or MSK1 inhibitor H89 (10μM) 1 hour before the addition of LPS (100ng/ml or 500ng/ml). Whole cell protein was then extracted at 5, 20, 30 40 minutes and p_{ser10} Histone-3, Histone-3, p_{ser276} p65, p65, IκBα and β-actin were determined by western blot. (B) Quantification of western blot images. Values are expressed as the ratio of p_{ser276} p65/p65, p_{ser10} Histone-3/Histone-3 and IκBα/β-actin. Data are derived from one experiment representative of three independent experiments.
Figure 2.6 DMF-mediated MSK1 reduction is essential for the inhibitory effect of DMF on DC maturation.

Purified DCs were stimulated by LPS (100ng/ml), LPS+H89 (10μM) or LPS+DMF (70μM) for 24 hours. Untreated condition was included as control. (A) MHC class II$^{\text{high}}$, CD80 and CD86$^{\text{high}}$ expression were determined by flow cytometry. (B) Cells were first gated on MHC class II$^{\text{high}}$ and then gated on CD80 and CD86$^{\text{high}}$. (C) Cytokine secretion in each condition was evaluated by ELISA. *$p<0.01$. Data are derived from one experiment representative of two independent experiments.
Figure 2.7 DMF markedly enhances HO-1 expression in BMDCs. (A) BMDCs were stimulated by LPS (100ng/ml) and IFN-γ (10ng/ml) in the presence or the absence of DMF (70μM). Whole cell proteins were isolated at different time points after the addition of LPS or DMF. (B) To confirm the enhancement of HO-1 is specific associated with DMF, BMDCs were treated with six different conditions including media only, DMSO only, DMF only, LPS+IFN-γ, LPS+IFN-γ+DMSO and LPS+IFN-γ+DMF. Whole cell were isolated at 6 hours after stimulation. (C) Quantification of western blot images from (B). Values are expressed as the ratio of HO-1/β-actin. Data are derived from one experiment representative of two independent experiments.
Figure 2.8 HO-1 induced by DMF was cleaved and migrates into the nucleus, where it interferes the binding capacity of p65 to the promoter region of IL-23p19*. (A) BMDCs were stimulated by LPS (100ng/ml) and IFN-γ (10ng/ml) in the presence or the absence of DMF (70μM). Cytoplasmic and nuclear proteins were isolated separately and cleaved HO-1 (both N-terminal and C-terminal parts) was determined by western blot. (B) RAW246.7 cells were transfected with a reporter construct containing the IL-23p19 promoter and treated with the indicated doses of DMF. Luciferase activity was determined after stimulation with 100ng/ml LPS for 6 hours. Data from one representative of two independent experiments are shown. Error bars represent SEM. (C) RAW246.7 cells were transfected with a reporter construct containing the IL-23p19 promoter followed by transfection with NS siRNA or HO-1 siRNA for 20 hours. Then cells were treated with DMF (17μM). Luciferase activity was measured after stimulation with 100ng/ml LPS for 6 hours. Error bars represent SEM.

*This conclusion is based on data generated in our lab and our collaborator’s lab (Ghoreschi, Bruck et al. 2011).
Figure 2.8 HO-1 induced by DMF was cleaved and migrates into the nucleus, where it interferes the binding capacity of p65 to the promoter region of IL-23p19.
**Figure 2.9 Proposed model for DMF mediated impairment of DC maturation.**

All TLR signaling (except TLR3) events recruit the adaptor MyD88, followed by IRAK family of protein kinases, leading to TRAF6 activation followed by NF-κB activation. In our study, we show that DMF suppresses p65 phosphorylation and nuclear localization (Point 1). p65 activation is further reduced by two additional signaling. First of all, DMF mediated MSK1 reduction (Point 2) results in decreased phosphorylation of p65 at serine 276 (Point 3) and histone-3 at serine 10 (Point 4), leading to impaired NF-κB transcriptional activity. In addition, DMF markedly enhances HO-1 expression, which can be cleaved and translocates into the nucleus, where it further interferes the binding activity of p65 to the promoter of IL-23 p19 (Point 5).
Figure 2.9 Proposed model for DMF mediated impairment of DC maturation.
Figure 2.10 (Supplemental figures) Purity of *in-vivo* generated DC. Whole splenocytes were labeled with CD11c microbeads and separated by AUTOMACS. Purity of cells before and after column was determined by flow cytometry.
Supplemental Figure 2.11 The inhibitory effect of DMF on IL-12 and IL-6 is independent of HO-1 enhancement. BMDCs were plated at 8 million/ well in 6- well plate for overnight and transfected with nonsense siRNA (NS) or HO-1 siRNA for 20 hours. Cells were then treated with LPS (100ng/ml) +IFNγ (10ng/ml) in the presence or absence of DMF for 24 hours. Supernatants were collected for cytokine analysis. Data represents one of three independent experiments. Error bars represents SEM.
Chapter 3: The neuroprotective study of DMF

Introduction

A number of clinical studies have demonstrated a strong correlation between brain atrophy and disease progression in both RRMS and SPMS patients, emphasizing the involvement of neurodegeneration in MS (Sailer, Fischl et al. 2003; Sastre-Garriga, Ingle et al. 2005; Bieniek, Altmann et al. 2006; Portaccio, Amato et al. 2006; Fisher, Lee et al. 2008; Fisniku, Chard et al. 2008; Gauthier, Berger et al. 2009; Geurts, Stys et al. 2009; Rudick, Lee et al. 2009). The triggers of neurodegeneration are not clear, although in the RRMS phase, dysregulated inflammation seems to play a dominant role. Yet, inhibition of inflammation does not stop disease progression, giving support to the notion that additional mediators independent of inflammation are present. One of the accepted mechanisms is glutamate mediated toxicity (Matute, Alberdi et al. 2001; Werner, Pitt et al. 2001; Pampliega, Domercq et al. 2011).

As an essential and most abundant excitatory neurotransmitter in the brain, glutamate plays multiple roles maintaining normal CNS function. Glutamate concentration is tightly regulated by several different mechanisms. Low concentration of glutamate may result in cognitive dysfunction, while high concentration of glutamate may cause neuronal death and subsequent brain injury (Takahashi, Giuliani et al. 2003; Kostandy 2012). Death or
degeneration of cells in the CNS due to prolonged exposure of high synaptic glutamate is termed glutamate excitotoxicity, which has been shown to participate in many neurological disorders including stroke, spinal cord injury and multiple sclerosis. (Stover, Pleines et al. 1997; Arundine and Tymianski 2003; Vallejo-Illarramendi, Domercq et al. 2006; Strijbis, Inkster et al. 2012).

Glutamate levels were found to be increased in relapsing MS patients compared to health individuals (Stover, Pleines et al. 1997). Evidence of glutamate enhancement in the CNS of MS patients was further supported by a MR spectroscopy (MRS) study (Srinivasan, Sailasuta et al. 2005). Not only were glutamate levels shown to be higher in the acute lesions, but glutamate was also found to be increased in the normal appearing white matter (NAWM) (Srinivasan, Sailasuta et al. 2005). A possible pathogenic role of dysregulated glutamate in MS was further supported by animal studies. Administration of NMDA and AMPA/Kainate blockers attenuated EAE, which was associated with a reduction of axonal and oligodendrocyte damage in these mice (Wallstrom, Diener et al. 1996; Pitt, Werner et al. 2000; Smith, Groom et al. 2000). Therefore, modulation of glutamate toxicity may provide significant therapeutic benefit in MS.

Phase II and Phase III clinical trials demonstrated significant beneficial effects of DMF in RRMS patients (Schimrigk, Brune et al. 2006; Fox, Miller et al. 2012; Gold, Kappos et al. 2012). Currently under FDA evaluation, DMF is likely to become a new addition to current MS therapies. Previous studies from our lab and other groups have demonstrated
the immunoregulatory effects of DMF as well as its main metabolite MMF in both EAE models and clinical trials, pointing out one aspect of the mode of action of DMF (Schilling, Goelz et al. 2006; Ghoreschi, Bruck et al. 2011). Moreover, it has become of interest to investigate if DMF has primary neuroprotective effects based on two interesting observations. First of all, it was shown that after initial depletion of GSH, DMF ultimately significantly enhanced intracellular levels of GSH in endothelial cells (Nelson, Carlson et al. 1999). Additionally, a number of studies have identified that Nrf2, a transcription factor involved in combating oxidative stress, is activated in astrocytes after DMF or MMF treatment (Lin, Lisi et al. 2011; Scannevin, Chollate et al. 2012). This raises the possibility that DMF has primary neuroprotective properties. Moreover, MMF, the main metabolite can be readily detected in the CSF of naïve rats that were given DMF, strengthening the likelihood that DMF can influence the CNS microenvironment and subsequently benefit MS (Linker, Lee et al. 2011). Yet, one could argue that the EAE model used in the previous study was problematic given that this animal model is characterized by massive inflammation (Racke, Bonomo et al. 1994; Lovett-Racke, Rocchini et al. 2004) and that DMF also has immunoregulatory effects (Schilling, Goelz et al. 2006; Wilms, Sievers et al. 2010; Cross, Cook et al. 2011; Peng, Gueru de-Arellano et al. 2012). Therefore, it remains unclear whether the beneficial effects of DMF observed in EAE were through its anti-inflammatory, primary neuroprotective effects or a combination of both.

To exclude the effects of DMF on inflammation, an excitotoxic model induced by AMPA
Microinjection was utilized in our study (Pitt, Gonzales et al. 2010). Microinjection of AMPA, a glutamate analog, leads to acute neuronal injury as determined by histology and behavioral testing. Interestingly, our current data (Study I) demonstrated pre-treatment of DMF showed a tendency to reduce neuronal damage caused by excitotoxicity, indicating potential neuroprotective effects of DMF. In agreement, DMF pretreatment significantly enhances locomotor function of injured mice, suggesting that the proactive effects of DMF translate to functional consequences. Further, feeding DMF to naïve mice led to HO-1 upregulation, suggesting an appealing molecular target of DMF in the CNS compartment.

Methods

Study I

DMF administration
DMF was dissolved in ethanol (5mg/ml), and this solution was used to evenly coat the surface of mouse food chow (200mg DMF/100g food). Food was then air-dried at room temperature before feeding. Food was weighed each day to calculate DMF consumption and fresh food was supplemented every three days.

Unilateral antibody microinjections
Adult female C57BL/6 mice were anesthetized with ketamine and xylazine (80 mg/kg and 10 mg/kg, respectively, i.p.). Equal volumes and concentrations of AMPA solution (PH=7.2) were microinjected into the right ventral horn of DMF pretreated
(125mg/kg/day for 3 days) or naïve mice (Pitt, Gonzales et al. 2010). Using a sterile technique, a laminectomy was performed (with partial dural reflection) at the T11–12 vertebral level. To ensure accuracy and to minimize pipette-mediated injury caused by respiratory movement, the spinal column was secured via the spinous processes adjacent to the laminectomy site using Adson forceps fixed in a spinal frame. Sterile glass micropipettes (pulled to an external diameter of 25–30 μm and prefilled with AMPA solution) were positioned approximately 0.4 mm lateral to the spinal midline using a hydraulic micropositioner (David Kopf Instruments). From the meningeal surface, pipettes were lowered 0.8 mm into the ventral horn of the underlying gray matter. Using a PicoPump (World Precision Instruments), AMPA was injected over 15 minutes. Pipettes remained in place for 2 additional minutes to allow the injectate to dissipate into the parenchyma (Ankeny, Guan et al. 2009). To facilitate localization of the injection sites, the adjacent dura was marked with sterile charcoal. After surgery, muscles and skin were sutured, and mice were hydrated with physiological saline (2 ml, subcutaneously).

**Behavioral analysis**

Locomotor recovery was compared using the Basso Mouse Scale (BMS) locomotor rating scale, specifically designed for use in SCI mice. The BMS is a 10-point scale, based on operational definitions of hind limb movement, with additional emphasis placed on evaluating trunk stability. Briefly, individual mice were simultaneously observed by 2 investigators for a 4-minute testing period, during which hind limb movements, trunk/tail stability, and forelimb-hindlimb coordination were assessed then graded according to
published methods (Basso, Fisher et al. 2006). The subscore component of the BMS scores individual aspects of fine locomotor control in the hind limbs (e.g., paw rotation at initial contact) and trunk/tail (e.g., ability to maintain the tail in an upright position during locomotion), i.e., aspects of locomotion that if they were to change alone, would not necessarily change the overall BMS score (Basso, Fisher et al. 2006). For this reason, subscoring is a useful adjunct to the BMS scale and helps reveal more subtle changes in behavioral recovery. Behavioral analyses were performed using a two-tailed Student t test. A 0.05p value was considered significant.

*Tissue processing*

At the time of sacrifice, mice were deeply anesthetized and then perfused transcardially with PBS followed by 250 ml of 4% paraformaldehyde in PBS. Spinal cords were removed, post-fixed for 2 h at 4°C, and placed in 0.2M phosphate buffer (PB) overnight. Tissue was cryoprotected in 30% sucrose at 4°C for 48 hours. For tissue embedding, spinal cords were frozen on dry ice and cut into 6 mm blocks centered on the injection site. After submersion in OCT compound (Electron Microscopy Sciences, Hatfield, PA), blocks were frozen and crosssections were cut at 10 μm on a cryostat and mounted onto slides. Tissue was stored at -20°C until used (Schonberg, Goldstein et al. 2012).

*Histology and immunohistochemistry*

Adjacent sets of sections encompassing the lesions were stained with eriochrome cyanine (EC) plus anti-mouse 200-kDa neurofilament (chicken anti–neurofilament heavy chain
[anti-NFH], see below), as described previously (Ankeny, Guan et al. 2009). When primary antibodies were derived from mice, nonspecific staining was blocked using a cocktail of bovine serum albumin and complete horse and mouse serum for 1 hour at room temperature. After blocking solution was removed, sections were overlaid with preconjugated primary and secondary antibody cocktail (e.g., containing mouse anti-neurofilament plus biotinylated horse anti-mouse IgG diluted in blocking solution). This antibody cocktail was incubated for 18 hours at room temperature or 4°C. In some cases, cell nuclei were revealed using DAPI.

*Motor neuron count*

Sections spanning the lesion site were stained with cresyl violet to visualize neurons. The total number of ventral horn motor neurons in each section was quantified in every third section by a blinded observer (sections were spaced 100 μm apart), beginning 2.7 mm rostral to epicenter and spanning to 2.7 mm caudal to epicenter. Sections were viewed using Nomarski optics to visualize tissue topography, and motor neurons were identified based on location in ventral horn, size and morphology. For each distance, group means were calculated and compared using two-way repeated measures ANOVA followed by Bonferroni post-hoc comparisons (McTigue, Tripathi et al. 2007).

*Results*

*DMF pretreatment reduces neuronal damage caused by excitotoxic injury*
To determine the protective effect of DMF on neuronal loss caused by the excitotoxic injury, mice were pre-fed with DMF (125mg/kg) for three days followed by AMPA injection into the spinal cord. Mice were then sacrificed at 24 h and the spinal cord was processed and cut into cross sections. Neurons in each cross section were visualized by Nissl staining. To evaluate the severity of injury in each group, the number of neurons located in the ventral horn was counted. Analysis of neuronal counts revealed that the pre-DMF treatment group had more motor neurons caudal to the epicenter compared to control mice, indicating a possible protective effect of DMF pretreatment (Fig 3.2). The observation that DMF seems more effective in protecting neurons caudal to the epicenter is very interesting and perhaps is caused by the quicker dilution of AMPA towards the lumbar section (caudal) since lumbar has a greater diameter compared to that of thoracic section.

**DMF pretreatment enhances the locomotor activity of injured mice**

To further determine if pre-treatment of DMF can improve the locomotor activity of the injured animal, two behavioral tests were performed in our study. The activity box allows us to characterize the overall motor activity while BMS is able to characterize the behavior in greater detail (Basso, Fisher et al. 2006). Our results demonstrate that pre-treatment of DMF enhances overall motor activity of injured mice, with increased total distance and increased horizontal and ambulatory activity recorded over a period of 10 minutes (Fig 3.3). This is in support of the histologic analysis. In contrast, although a moderate enhancement was observed in BMS score, it did not reach a statistically
significant difference between the two groups (Fig 3.4). The different sensitivity of the two behavioral analyses may count for this discrepancy and perhaps DMF mediated behavioral improvement was sufficient to be determined by activity box but not by BMS testers.

HO-1 was upregulated by DMF feeding

To enhance our mechanistic understanding of DMF, we sought out to explore the molecular and cellular effects of DMF in the CNS compartment. Both our lab and other groups have demonstrated that HO-1, an oxidative enzyme downstream of nrf-2, was induced by DMF treatment in peripheral immune cells (Lehmann, Listopad et al. 2007; Cross, Cook et al. 2011; Ghoreschi, Bruck et al. 2011; Peng, Guerau-de-Arellano et al. 2012). Interestingly, in addition to its immunoregulatory role, HO-1 has been suggested to have neuroprotective properties in different neurodegenerative models (Min, Yang et al. 2006; Espada, Ortega et al. 2010; Huang and Chuang 2010; Zhan, Wang et al. 2012). Therefore, HO-1 became an appealing molecular target of DMF in the CNS to us.

To determine if DMF can influence the CNS behind the intact BBB, naïve mice were fed with DMF for 3 days or 14 days and then HO-1 expression was determined by qPCR and ELISA. Our results indicated that DMF feeding can markedly enhance HO-1 expression in the CNS compartment (Fig 3.5). The fact that HO-1 upregulation was observed in naïve mice fed with DMF suggests that DMF/MMF is likely to cross the intact BBB. This is not surprising considering their lipophilic nature. In fact, our observation is in
agreement with the more recent findings of other groups where MMF, the effective metabolite of DMF, was detected in brain tissues and CSF, with about 8% of the peripheral MMF concentration (Linker, Lee et al. 2011; Poster 11, ACTRIMS). Taken together, HO-1, an inducible enzyme that could be induced by activation of Nrf2, may serve a potential target of DMF in the CNS compartment.

Discussion
This work was initiated to study the neuroprotective effects of DMF in a less inflammatory model of CNS injury. Our work (Study I) suggests that DMF pre-treatment reduces neuronal loss caudal to the injection site (epi) (Fig 3.2). No anatomical improvements were observed in the epicenter, which suggests that the damage created at the impact site was too severe to be rescued by DMF treatment. In contrast, the severity of damage at the lesion periphery may be milder, which allows for a better therapeutic opportunity in which intervention is possible. A similar phenomenon has been noted in other SCI studies using minocycline and peroxisome proliferator-activated receptors (PPAR) gamma. In these studies, the functional recovery was correlated with tissue sparing in the lesional pole but not in the epicenter (Teng, Choi et al. 2004; McTigue, Tripathi et al. 2007).

DMF seems to be more effective in protecting neurons caudal to the epicenter. A number of reasons may account for this observation. First of all, a greater number of neurons are distributed in the lumbar enlargement (caudal to the injection site) compared to Thoracic
segment. This specialized anatomy may give DMF a better chance to exert its protective effects. More importantly, given that the diameter of the lumbar enlargement (caudal to the epicenter) is greater compared to Thoracic level, caudal and rostral diffuse rate might be different, leading to a milder injury caudally due to dilution of AMPA.

To further determine if DMF mediated neuronal protection can be translated to clinical functional improvement; behavioral performance of injured mice was evaluated by two behavioral testing including BMS and activity box (Fig. 3.3 and Fig 3.4). In agreement with histological data, behavioral data suggest that compared to nontreated injured mice, DMF pretreated mice exhibited enhanced locomotor activity. Therefore, results from study I indicate that DMF has primary neuroprotective effects.

With the promising results in Study I, however, we do notice that this neuron-injury model seems to be too severe as both hind limbs (7 out of 8 animals) were paralyzed after unilateral microinjection of AMPA. To extend the possibility of therapeutic intervention, we decided to take one step back to optimize the AMPA dosage again in the hope of creating an injury model with a relatively confined lesion and moderate behavior impairment. After adjusting both the volume and the concentration of AMPA, the concentration of 4mM with a volume of 160nl will be utilized for further experiments because this dosage of AMPA causes moderate behavioral impairment that gradually recover within the first week (data not shown).
To enhance our understanding of the mechanistic action of DMF, we sought to explore the molecular targets of DMF in the CNS. Other groups have identified Nrf-2, a critical transcription factor involved in the anti-oxidative pathway, to be activated in the CNS, raising the possibility of Nrf-2 as a critical target of DMF. Nrf-2 activation leads to enhanced production of a variety of downstream molecules such as GSH and HO-1 (van Muiswinkel and Kuiperij 2005; Nguyen, Nioi et al. 2009). In fact, a recent study has elegantly shown that DMF induced HO-1 reduces neurotoxin release from HIV infected macrophages (Cross, Cook et al. 2011). In line with these studies, we demonstrate that DMF feeding to naïve mice significantly enhanced HO-1 expression in the CNS compartment.

This is a very interesting observation for two reasons. First of all, our data indicate that DMF/MMF may be able to physically penetrate the intact BBB and subsequently influence the microenvironment of CNS. In fact, a couple of more recent studies by other groups successfully detected MMF in CSF and brain tissue in naïve rats that received DMF treatment, confirming the capacity of DMF/MMF to diffuse into the CNS compartment (Linker, Lee et al. 2011). Second of all, HO-1 has been demonstrated to have neuroprotective functions in a number of neurodegenerative disease models (Hung, Liou et al. 2008; Espada, Ortega et al. 2010; Huang and Chuang 2010; Jazwa and Cuadrado 2010; Zhang, Wang et al. 2012). For instance, it was demonstrated in vitro that neurons over expressing HO-1 were more resistant to oxidative stress compared to wild type neurons (Panahian, Yoshiura et al. 1999). Further, a recent study suggested that gene
delivery of HO-1 to rat substantia nigra could rescue neurons in a Parkinson’s disease model. In the same study, it was shown that HO-1 delivery enhances the expression of neurotrophic factors in the target region, which may, at least partially, be responsible for the observed neuroprotective effects (Hung, Liou et al. 2008). Therefore, the fact that HO-1 was upregulated by DMF treatment in our study raised the possibility that DMF may exert its protective effects though induction of HO-1 in the CNS.

Future studies will focus on identification of the cellular location of HO-1 and determination if blocking HO-1 results in diminished protective effects of DMF. Additionally, we also wanted to test if DMF is beneficial in a long-term injury model. This long-term model will also allow us to evaluate if DMF can promote repair in the CNS. I believe this work will provide important insights on the protective mechanisms of DMF and further help its optimal clinical application in the future.

**Future directions**

**The potential neuroprotective effects of DMF in a chronic neuronal-injury model**

As discussed, our first study (Study I) indicates that DMF pretreatment could reduce neuronal damage caused by acute excitotoxicity and lead to enhanced locomotor activity. To confirm this observation, two more studies (Study II and Study III, Figure 3.8) will be conducted with optimized AMPA microinjection dosage (4mM, 160nl). Study II is to confirm the results of study I while the goal of study III is to determine the potential
neuroprotective effects of DMF in a chronic injury model. This is important as it may further allow us to evaluate the capacity of DMF to promote CNS repair. Animals will be pretreated with DMF for 3 days prior to AMPA microinjection and through the end of experiments. Locomotor activity will be tested on day 1, 3, 5, 7, 10, 14, 21 days after injury (Figure 3.8). Tissue damage will be analyzed by histology in both DMF-treated and non-treated animals at the end of study.

**To test if knocking down HO-1 diminishes the protective effects of DMF**

Previous work by other groups has shown that HO-1 induction exerts neuroprotective effects in the CNS in different disease models. Interestingly, in our study, HO-1 protein expression was upregulated by DMF feeding. This motivated us to pursue further if HO-1 is a critical target of DMF. To test this idea, HO-1 knock down strategy will be applied and the protective effects of DMF will be evaluated afterwards.

**Conditioned Media transfer**

The goal of this experiment is to determine if the effect of DMF is direct on neurons or through microglial cells. To determine the direct effects, primary neuronal culture will be treated with DMF or MMF followed by glutamate addition to the cell culture. The neuronal viability will be determined by MAP-2 ELISA 24 hours after glutamate stimulation. This assay will help us understand if DMF can enhance the cellular defense in neurons. In addition, primary microglial cells will be stimulated by LPS (10ng/ml) in
the presence or absence of DMF for 24 hours and supernatant from each condition will be transferred to naive primary neuronal cultures. Neuronal viability will be determined by MAP-2 ELISA 24 hours after the conditioned media transfer. This experiment will help elucidate the indirect protective effects of DMF on neurons.

**Cellular identification of HO-1 after DMF treatment**

The goal of this experiment is to identify the cellular distribution of HO-1. HO-1 was shown to be upregulated in the CNS compartment after DMF feeding in our study, however, the cellular distribution of HO-1 remains unclear. To better understand the target cells of DMF, HO-1 expression will be analyzed by western blot and immunohistochemistry in primary CNS cell cultures. To test if HO-1 is an essential molecular target in these cells, knocking down strategy will be again applied to determine if the protective effects of DMF on individual cell type will be diminished.
**Figure 3.1 Illustration of AMPA injection model.** AMPA solution (concentration: 3mM and volume: 300nl) was injected unilaterally into right ventral horn at T12 vertebral level of naïve mouse. Histologic analysis was performed 24 h after injection. As indicated, axons were stained with neurofilament in red and neurons were visualized by Nissl staining in green. Neurons were well preserved in the area 3mm distal to the injection site (right), while severe neuron loss can be observed by Nissl staining adjacent to the injection site (left). *Arrows indicate neurons.*
Figure 3.2 Mice received DMF had more motor neurons caudal to the injury site (epi). Ventral horn from spinal cord sections 2.7 mm caudal or rostral to the epicenter immunolabeled with Nissl for neurons from control and DMF group. Motor neuron counts from both contralateral and ipsilateral to the injection site revealed that sections from the DMF treated group contained more motor neurons caudal to the epicenter. Data are expressed as mean±SEM. $p>0.05$. 
Figure 3.3 DMF pretreatment led to enhanced motor activity. 24 hours after AMPA microinjection, motor activity of mice (n=4) from both control and DMF pre-treated mice was determined by activity box for a period of 10 minutes. Activity counts or distance were recorded automatically and analyzed by software (fusion, 1.10). Data are expressed as mean±SEM. *p<0.01 vs. control.
Figure 3.4 Locomotor scores were greater in mice receiving DMF prior to AMPA injection. Locomotor activity of vehicle and DMF treated mice was evaluated by two experienced testers for 4 minutes in the open field 24 hours post microinjection. Data are expressed as mean±SEM. No statistically significant difference was found between the two groups. $p=0.3437$.  

![Graph showing Basso Mouse Scale (BMS) for control and DMF treated mice at DPI 1]
Figure 3.5 HO-1 is upregulated by DMF treatment in naïve mice. Naïve mice were fed with DMF either 3 days or 14 days. Mice were then anesthetized and perfused with cold 0.2 M PBS (PH=7.4). Protein and mRNA then were isolated from brain and spinal cord compartments separately. HO-1 mRNA and protein expression were analyzed by PCR and ELISA. **p<0.01 vs control.
Figure 3.6 Proposed model of the neuroprotective effects of DMF/MMF. In the resting state, Nrf-2, as a critical transcription factor for combating oxidative stress, is sequestered by Keap1 in the cytoplasm. In response to DMF or MMF treatment, Nrf-2 is released by keap1 and translocates into the nucleus where it binds the ARE element within the HO-1 promoter, resulting enhanced HO-1 induction (Cuadrado and Rojo 2008). HO-1 then catalyzes cellular Heme into three products including carbon monoxide (CO), free iron and billiverdin. It is speculated that these three products mediate the profound cytoprotective effects of HO-1 activity. CO has been shown to protect cardiovascular
endothelium cell against apoptotic changes induced by oxidative stress (Parfenova, Basuroy et al. 2006; Zimmermann, Leffler et al. 2007). Another product of HO-1 activity is biliverdin, which is converted to bilirubin by biliverdin reductase (BVR). Billiverdin acts as an excellent scavenger for both H$_2$O$_2$ and NO, protecting cells against oxidative injury (Dore, Takahashi et al. 1999; Kaur, Hughes et al. 2003). However, the consequence of iron released from HO activity remains controversial (Dennery, Sridhar et al. 1997; Lee, Andersen et al. 2006).
**Figure 3.7 (Supplemental Figure) Axons are preserved in AMPA injection model.**

To determine the axonal and myelin integrity, EC-NF staining from epicenter of spinal cord was conducted. The histology indicates the preservation of axons and myelin the AMPA injection model, at 24 hours post injection.

**Figure 3.8 (Supplemental Figure) Experimental design of future work.**
Chapter 4: Conclusion

My research has focused on understanding the mode of actions of DMF, a novel oral therapy for RRMS, from both immunoregulatory and neuroprotective perspectives. This work is meaningful at several levels. First of all, understanding the mode of action of DMF could help us gain knowledge on the pathogenesis of MS and further contribute to new therapy development. The first part of my work emphasized the immunogenic role of DCs in MS and suppression of DC maturation via DMF leads to reduced generation of pathogenic T cells. This is in line with many other studies demonstrating that DCs from MS patients display a more mature phenotype (Karni, Abraham et al. 2006) and that DCs are actually a shared cellular target of several other MS therapies such as IFN-β and glatiramer acetate (Jiang, Milo et al. 1995; Weber, Prod'homme et al. 2007; Racke and Lovett-Racke 2011). Although the current FDA-approved immunomodulators slow disease progress in RRMS, these therapies do not stop disease and, there is no effective therapy for progressive forms of MS. Therefore, a big challenge is to develop a safe, effective neuroprotective therapy for MS because accumulating evidence suggests that MS is more complicated than just inflammation, and that neurodegeneration also plays a significant role in the disease progression (Franklin, Ffrench-Constant et al. 2012). Therefore, the neuroprotective study of my graduate work could provide rationales for the clinical application of DMF in SPMS and PPMS.
Fumaderm®, with DMF as its main effective component, has been used for the treatment of psoriasis patients in Germany for almost twenty years (Mrowietz and Asadullah 2005; Reich, Thaci et al. 2009). On the basis of an encounter between a German dermatologist and a neurologist over two patients with psoriasis whose multiple sclerosis stabilized after treatment with oral fumarate, the drug, which has immunosuppressant properties, was studied in multiple sclerosis (Schimrigk, Brune et al. 2006; Ropper 2012). This pilot trial demonstrated that fumarate treatment benefited RRMS by significantly reducing Gd enhancing lesions of CNS. Importantly, this oral treatment was well tolerated and only mild adverse effects such as GI tract reaction and flushing were observed in patients. And these side effects decreased continuously in all the patients after 6 weeks (Schimrigk, Brune et al. 2006). The excellent safety record in both psoriasis and MS patients, the efficacy and the oral availability of Fumaderm led to the initiation of larger trials later on to determine the efficacy and safety of DMF (BG-12 as the brand name), an improved formulation of Fumaderm (Fox, Miller et al. 2012; Gold, Kappos et al. 2012).

One clinical observation in the first trial was very interesting to us, where a reduction in CD4⁺IFN-γ⁺ cell population was found in fumarate treated MS patients (Schimrigk, Brune et al. 2006). Because Th1 cells have long been considered to be pathogenic cells, this observation may have identified one mechanism through which DMF benefits RRMS patients. A goal of my research was to understand the cellular and molecular mechanism behind this clinical observation.
Given the critical role of antigen presenting cells in T cell activation and differentiation, we sought to analyze the effects of DMF on DCs, the professional antigen presenting cells and determine if DMF directly modifies DCs such that they altered T cell priming (Steinman 2012). Our results demonstrated that DC maturation was impaired in the presence of DMF. This prompted us to set up the DC+T cell co-culture system to further determine if the phenotypic change in DCs had functional consequences on T cell differentiation. Indeed, DMF-treated-DCs displayed a reduced capacity to induce T cell differentiation. Interestingly, not only the generation of Th1 cell was significantly reduced, which is in correlation with the clinical observation, but the development of Th17 cells, another pathogenic population, was also impaired. At the time of the pilot clinical trial (2006), the pathogenic role of Th17 population in MS had not been fully appreciated. Not surprisingly, the number of Th17 cell was not characterized in that clinical study. Yet, our data suggest that DMF treated DCs have a reduced capacity to generate both Th1 and Th17. It will be of interest to analyze the phenotypes of CD4+ T cells (both Th1 and Th17) in both DEFINE and CONFIRM phase III trial of DMF in MS patients. One could argue that DMF induced T cell apoptosis also contributes to the reduction of pathogenic T cells the trials (Treumer, Zhu et al. 2003). However, it was demonstrated that the apoptotic rate of CD4+ T cells was not sustained, in fact, it came down to basal levels 12 weeks after the initial dosing of DMF (Schimrigk, Brune et al. 2006). This would actually strengthen our finding that sustained low cell count of pathogenic T cell after DMF treatment could be largely caused by impaired antigen presenting capacity of DCs.
To enhance our understanding of the molecular targets of DMF, we began to explore the mechanisms by which DMF alters DCs. This is significant because identification of molecular targets could favor the development of novel therapies in the future. Activation of two signaling pathways has been implicated in DC maturation including both MAPK and NF-κB (Cao, Lu et al. 2008; Buttari, Profumo et al. 2011). In fact, several lines of studies in the context of psoriasis suggested that both pathways were impaired after DMF treatment (Loewe, Holnthoner et al. 2002; Gesser, Johansen et al. 2007). However, these studies were conducted in different cell types such as endothelial cell or keratinocytes. Because both pathways could be cell type specific and stimuli specific, it was essential to test the effects of DMF in DCs specifically. We showed that multiple steps of NF-κB activation were altered by DMF. The translocation of p65, an NF-κB subunit, to the nucleus was significantly reduced in the presence of DMF (Figure 2.3). Further, phosphorylation of p65 at serine 276 and serine 536 was also impaired (Figure 2.4). This is interesting because current therapies also affect p65 activity (Yasui, Hideshima et al. 2005; Eggert, Goertsches et al. 2008). For instance, glucocorticoid administration, a common clinical practice to reduce the acute inflammation in MS patients, suppresses NF-κB activity in monocytes, the DC precursors found in blood (Joyce, Steer et al. 1997).

In addition to NF-κB, MAPK (JNK, p38 and ERK1/2), another critical signaling for DC maturation was also investigated in our system. Our results suggest that DMF specifically inhibits ERK1/2 activation but did not influence JNK or p38 (Figure 2.4). Further, MSK1,
a downstream protein of ERK1/2 that positively regulates p65 signaling, was also suppressed in the presence of DMF (Figure 2.4). Therefore, p65 activity was further impaired via DMF mediated reduction in ERK1/2-MSK1 signaling. Moreover, our lab in collaboration with Dr. Rocken Martin’s collaborator has shown that DMF induces HO-1 in DCs, which is cleaved and enters into the nucleus (Figure 2.6). By CHIP and luciferase assays, we and our collaborator showed that cleaved HO-1 interferes with p65 binding capacity to the promoter of p19, resulting in reduced IL-23 production (Figure 2.8; Ghoreschi, Bruck et al. 2011). Taken together, by fine-tuning different signaling pathways, DMF suppresses overall p65 activity, leading to impaired maturation of DCs.

By studying cellular and molecular mechanisms of DMF, not only this part of my graduate work provides the mechanistic foundation of DMF for its treatment in RRMS patients, but it also enhances our understanding of MS pathogenesis. As developing effective therapies could not happen without understanding the pathogenesis of the disease, my study could help develop new therapies that have superior effectiveness and reduced adverse effects in the future.

The second part of my graduate work switched from immunology to neuroscience for two reasons. First of all, although inflammation clearly plays an essential role in MS pathogenesis, stopping inflammation does not halt disease progression and there is no effective therapy for progressive forms of MS. One idea is that neurodegeneration becomes a dominant factor propagating disease progression in the progressive phase
(Franklin, Ffrench-Constant et al. 2012). Therefore, seeking an effective neuroprotective therapy becomes a new challenge for the MS field. Second of all, there is evidence in support of the potential neuroprotective role of DMF. About a decade ago, a number of studies showed that DMF addition enhances intracellular and extracellular levels of glutathione, the most abundant and essential anti-oxidative molecule (Duffy, So et al. 1998; Nelson, Carlson et al. 1999). This is very interesting because significantly higher levels of oxidative stress have been suggested to occur in the CNS of MS patients and anti-oxidant treatments have been tested in EAE (Liu, Zhu et al. 2003; Ilhan, Akyol et al. 2004). In lines with these studies, more recent work has indicated that DMF mediated activation of nrf-2 seems to be responsible for enhanced production of anti-oxidative molecules including GSH (Linker, Lee et al. 2011). Interestingly, as a critical transcription factor for combating oxidative insult, Nrf2 is naturally upregulated in the MS brains compared to healthy brains, suggesting that a self-defense mechanism against oxidation has been initiated in response to CNS damage (Linker, Lee et al. 2011). However, self-activated Nrf2 may not be sufficient to protect the host against insults and perhaps therapies offering an additional boost of this pathway will be beneficial. Therefore, it became of interest to determine whether DMF has primary neuroprotective effects that may benefit MS patients.

To test this idea, one critical question is whether DMF is able to penetrate the BBB. To help understand this question, naïve mice were fed with DMF and I evaluated the brain and spinal cord for the expression of HO-1, a downstream molecule of Nrf-2 activation.
Indeed, HO-1 expression was significantly enhanced in the CNS compartment of DMF treated-mice, suggesting DMF is able to influence the CNS microenvironment in healthy mice. In fact, a couple of research groups recently detected MMF in both brain tissue and CSF in rodents that received DMF treatment, confirming the capacity of DMF/MMF to enter into the CNS (Linker, Lee et al. 2011).

Another critical question is how to choose a proper animal model to test the primary neuroprotective effects of DMF. It should be noted that DMF has immunoregulatory properties and one could argue that the beneficial effect observed in EAE is exclusively through its immunomodulatory effects (Schilling, Goelz et al. 2006; Linker, Lee et al. 2011). Therefore, to dissect its potential primary neuroprotective effects from its immunoregulatory properties, it was necessary to identify a relevant animal model with limited inflammation. In our study, the excitotoxic model induced by AMPA microinjection directly into the spinal cord leads to relatively confined lesions and behavior impairment in animals, which allowed us to evaluate the potential beneficial effects of DMF by comparing locomotor activity and tissue sparing. Importantly, this model is relevant to MS because it has been shown that excitotoxicity contributes to MS pathogenesis.

Our results from Study I showed that DMF pre-treatment reduces neuronal loss and enhances locomotor activity, indicating potential neuroprotective effects of DMF. One may argue that it is more meaningful to conduct therapeutic treatment instead of pre-
treatment. However, it should be noted that DMF is likely to be given twice daily to MS patients after its approval and pre-treatment could mimic the treatment paradigm during the remitting phase of the disease. In addition, as discussed earlier, HO-1 is upregulated in the CNS in naïve mice that received DMF, suggesting HO-1 as a possible target of DMF. Future studies (Study II and Study III) will confirm the results from Study I and further determine the potential long-term beneficial effects of DMF in this model. Additionally, future experiments will be aimed at identifying the location of HO-1 upregulation and if it is an essential target of DMF in the CNS.

Although my dedication to the neuroprotective study of DMF began relatively recent, our data so far looks promising and supportive of my hypothesis. By completing the proposed future experiments, this part of my graduate work helps enhance our understanding of the mechanisms of DMF in the CNS compartment, and further provides rationales for the clinical application of DMF for progressive forms of MS, where neurodegeneration plays a dominant role. Additionally, besides in MS, activation of Nrf2 and HO-1 has been demonstrated to be beneficial in other neurodegenerative disease models such as Huntington’ disease, Parkinson’s disease, Alzheimer’s disease and stroke (Ramsey, Glass et al. 2007; Zhao, Sun et al. 2007; Vargas, Johnson et al. 2008; Chen, Vargas et al. 2009; Kanninen, Heikkinen et al. 2009; Innamorato, Jazwa et al. 2010; Sakata, Niizuma et al. 2012). Given the great complexity of the CNS, it is unlikely there will be one therapy that can treat all neurodegenerative diseases, yet by studying a shared important signaling pathway in different diseases, for instance, the Nrf2-HO-1 axis; we may hasten the
process of identifying critical molecular targets for therapeutic interventions in CNS disorders.
References


Peng, H., M. Guerau-de-Arellano, et al. (2012). "Dimethyl fumarate inhibits dendritic cell maturation via nuclear factor kappaB (NF-kappaB) and extracellular signal-
regulated kinase 1 and 2 (ERK1/2) and mitogen stress-activated kinase 1 (MSK1) signaling." _J Biol Chem_ **287**(33): 28017-28026.


