MicroRNAs Targeting TGFβ Signaling Underlie the Regulatory T Cell Defect in Multiple Sclerosis

DISSERTATION

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Abstract

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS), which results in a wide range of neurological disabilities. Etiological studies have implicated environmental and genetic factors in causing increased disease susceptibility. Yet, the cause of MS remains unknown, and the mechanisms that promote the disease are poorly understood. The pathophysiology that promotes the disease is hypothesized to be an immune reaction mediated by pro-inflammatory T cells reactive against CNS derived self-antigens. Interestingly, regulatory T cells (Tregs), protectors against such autoimmune responses, are defective in MS patients. While genetic studies have implicated variants of immune-related genes in disease susceptibility, these variants only account for approximately 20-60% of the genetic contributors to the disease. Therefore, a tremendous need remains for the identification of additional genetic and susceptibility factors.

To this end, our lab has been investigating microRNAs (miRNAs) in the context of MS. miRNAs regulate gene expression and thus, modify cellular pathways and disease processes. In addition to being biological regulators, miRNAs are easily quantifiable in cells, tissue, and bodily fluids, making them attractive candidates for potential markers of MS pathogenesis. The lab previously performed a miRNA profiling study on the naïve

CD4 T cells of MS patients and healthy donors, and identified 85 differentially expressed miRNAs. Pathway analysis predicted 19 of the 85 miRNAS to target and inhibit the TGFβ signaling pathway. TGFβ is a pleiotropic cytokine critical for the development and function of Tregs. Given the importance of TGFβ in regards to Tregs, we hypothesized that dysregulated miRNAs in the naïve CD4+ T cells of MS patients inhibit the TGFβ-signaling pathway, resulting in defective Tregs and enhanced susceptibility to developing MS.

Our data indicate that 1) TGFβ-signaling genes are decreased in MS patients, 2) miRNAs when overexpressed negatively regulate the TGFβ signaling pathway and reduce the capacity for Treg differentiation from naïve CD4 T cells, and 3) overexpression of miRNAs, in the context of experimental autoimmune encephalomyelitis, promote earlier onset and increased severity of disease. Taken together, our data indicate that the dysregulation of TGFβ-targeting miRNAs in naïve CD4 T cells of MS patients can inhibit TGFβ signaling, dampen Treg development, and promote CNS autoimmunity. Therefore, these miRNAs are potential candidates for markers of disease susceptibility and therapeutic targets.

Dedication

This document is dedicated to Barry C. Severin, whose support and work ethic has encouraged and inspired my academic pursuits.

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Fields of Study

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List of Abbreviations

α	alpha
ALS	amyotrophic lateral sclerosis
APC	antigen presenting cell
β	beta
BBB	blood brain barrier
CD	cluster of differentiation
CDMS	clinically definite multiple sclerosis
CFA	complete Freund's adjuvant
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebral spinal fluid
DMTs	disease-modifying therapies
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
g	gram
GA	glatiramer acetate
GWAS	genome-wide association study
hr	hour(s)

HC	healthy control
HLA	human leukocyte antigen
IFNβ	interferon beta
IL-	interleukin
i.p.	intraperitoneal
iTreg	inducible T regulatory cell
μl	microliter
mAb	monoclonal antibody
MBP	myelin basic protein
MHC	major histocompatability complex
min	minute(s)
miRNA	microRNA
ml	milliliter
MOA	mechanisms of action
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
n-	nano
nTreg	natural T regulatory cells
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PPMS	primary-progressive multiple sclerosis

pre-miRNA	precursor micro ribonucleic acid
r-	recombinant
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RRMS	relapsing-remitting multiple sclerosis
S1P	sphingosine-1-phosphate
SPMS	secondary progressive multiple sclerosis
TCR	T cell receptor
Teff	T effector
Tg	transgenic
TGFβ	transforming growth factor beta
TGFβR1	transforming growth factor beta receptor 1
TGFβR2	transforming growth factor beta receptor 2
Th	T helper
Treg	T regulatory cell
VCAM1	vascular cellular adhesion molecule 1
VLA4	very late antigen 4

Chapter 1: Introduction

Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) that devastatingly affects approximately 2.5 million people worldwide (Hersh and Fox 2010). While the pathophysiology of the disease has not been completely uncovered, it has been characterized by inflammation, demyelination, and neurodegeneration, resulting in a wide range of neurologic symptoms. Unfortunately, MS is the leading cause of non-traumatic disability in young adults. Given the unpredictable and progressive nature of the disease, diagnosis with MS can greatly impact a patient's quality of life from an early age, putting their autonomy at risk. Currently, there is no cure for MS and available treatments are limited. Despite the ability of existing therapies to slow down disease progression, the eventual accumulation of symptoms is inevitable. Identification of individuals susceptible to developing MS may allow these patients to be proactive by submitting to routine screening and to prepare themselves for possible diagnosis. Efforts to identify factors for disease susceptibility have uncovered gender, geographical, and genetic biases. Yet, the etiology of MS is unknown and there remains no cure. Additionally, mechanisms that promote disease pathology are not well understood. This lack of knowledge has made preventing MS impossible and halting disease progression challenging. Therefore, research aimed at the

identification of novel biomarkers and therapeutic targets is critical for understanding disease activity, measuring treatment efficacy, and stopping disease progression. In order to move forward in research, it is important to first take into account the studies that serve as the foundation for modern research. The first attempts at understanding and treating MS can be dated back to the 19th century.

Multiple Sclerosis (MS)

In 1837, Robert Carswell, a Scottish pathologist, published his illustrations of lesions in the brain and spinal cords of patients *post mortem* in *Illustrations of the Elementary Forms of Disease.* While his findings predate the naming of MS and the invention of magnetic resonance imaging (MRI), his 19th century depictions of the disease provided a basis for understanding its pathogenesis and the importance of lesion formation. It was not until 1868 that MS was first described by Jean-Martin Charcot, a French neurologist, as ‘ la sclerosé en plaques,’ thus naming the disease and distinguishing MS from other known neurological ailments of the time (Charcot 1868). Charcot was the first to summarize clinical reports on MS patients and identify symptoms such as ‘nystagmus, intention tremor, and scanning speech,’ now known as Charcot’s neurological triad (Charcot 1868, Murray 2005). While Carswell and Charcot’s observations are not exclusive to MS, these findings built the foundation for modern use of the presence of CNS lesions and neurological symptoms in the diagnosis of MS patients.

Currently, 2.5 million people have been diagnosed with MS worldwide, with over 450,000 patients in the United States alone (Hersh and Fox 2010). Unfortunately, MS is the leading cause of non-traumatic disability in young adults, with diagnosis typically occurring between the ages of 20-40 (Hersh and Fox 2010). MS patients suffer from a wide range of neurological symptoms, including but not limited to pain, coordination impairment, cognitive changes, and emotional distress. The first episode of neurological symptoms experienced by a patient is known as a clinically isolated syndrome (CIS). Individuals that present with a CIS may or may not go on to develop clinically definite MS (CDMS). Standard methods for diagnosis include medical history, neurological examination, MRI, and other secondary tests, such as analysis of cerebrospinal fluid (CSF) (McDonald, Compston *et al*. 2001, Filippi, Dousset *et al*. 2002, Polman, Reingold *et al*. 2011, Gelfand 2014). In order for a patient to have CDMS, revised diagnostic criteria require that a patient demonstrates CNS specific inflammatory demyelinating lesions that are disseminated in both space and time (Polman, Reingold *et al*. 2011).

CNS lesions are typically detected by either T1-weighted gadolinium enhancing or T2-weighted MRI scans and can provide useful knowledge about the disease state. T2 lesions are representative of total lesion load, both old and new, whereas T1 lesions are more specific. Under normal conditions, gadolinium is a large dye incapable of penetrating the blood brain barrier (BBB); as such, detection of gadolinium-enhanced T1 lesions in the brain is indicative of newly formed or expanding areas of inflammation. The inflammation observed in these lesions can cause damage to the myelin sheath and

ultimately the impairment of nerve signal transduction. Symptoms are determined by the spinal or cerebral location of the lesions, and severity of the symptoms correlates with the amount of axonal damage (Trapp, Peterson *et al*. 1998, Charil, Dagher *et al*. 2007, Charil and Filippi 2007, Frischer, Bramow *et al*. 2009). While remyelination of axons can initially occur, leading to functional recovery, damage eventually accumulates, resulting in neurodegeneration and permanent neurological deficits. Periods of inflammatory attacks and symptom worsening are called relapses, where as the periods between exacerbations are called remissions.

Based on patterns of relapses and remissions, MS patients can be separated into three main categories, relapsing-remitting (RRMS), secondary-progressive (SPMS), and primary progressive (PPMS). PPMS is the most rare form of the three categories, occurring in only 10-15% of patients, and is characterized by steady worsening of disease from the time of onset without remission. RRMS is the most common form of MS and is defined by acute attacks of inflammation resulting in relapses followed by complete or incomplete remissions. This phase is followed by SPMS, which is defined by a steady worsening in disease, potentially with brief incomplete remissions. Of the 85% of people initially diagnosed with RRMS, almost all eventually advance to the progressive phase of the disease. Given the unpredictable and progressive nature of the disease, diagnosis with MS can greatly affect a patient's quality of life from an early age. Both relapses and progression occur spontaneously, forcing patients to live a life full of fluctuating function and uncertainty. Currently, there is no cure for MS. Available treatments are limited, with

only one treatment available for SPMS and none available for PPMS. Studies have demonstrated that early use of therapies in patients presenting with a CIS have a delayed conversion to CDMS (Jacobs, Beck *et al*. 2000, Comi, Filippi *et al*. 2001, Kappos, Polman *et al*. 2006, Comi, Martinelli *et al*. 2009, Miller, Wolinsky *et al*. 2014). Therefore, diagnosis of patients early during the disease process while treatments are available is essential for delaying progression of the disease. However, diagnosis is challenging, as there is no single test for MS and the cause is unknown. Therefore, understanding the mechanisms that underline disease susceptibility and activity is necessary for identifying novel therapeutic targets, stopping disease progression, and potentially preventing disease all together. To this end, numerous studies have attempted to uncover the main contributors to the disease; initial research focused primarily on widespread epidemiologic and genetic analyses of MS patients.

Epidemiological studies analyzing the familial, geographic, and gender distribution of MS have indicated that there are both genetic and environmental components to disease susceptibility. Familial studies have established ~25% concordance between monozygotic twins and ~5% concordance among dizygotic twins, demonstrating that MS has incomplete penetrance (Mackay and Myrianthopoulos 1966, Ebers, Bulman *et al*. 1986, Willer, Dyment *et al*. 2003). Therefore, genetic predisposition only partially explains MS etiology. In the past 30 years, variations within the *human leukocyte antigen* (*HLA*) class II locus have been most frequently identified as MS associated risk alleles (Haegert and Marrosu 1994). However, HLA associated risk only accounts for

approximately 20-60% of genetic susceptibility for MS (Haines, Terwedow *et al*. 1998). In addition to classic HLA risk alleles, genome-wide association studies (GWAS) have identified variants of immune-related alleles, such as *interleukin 2 receptor alpha chain* (*IL-2Rα)* and *interleukin 7 receptor alpha chain* (*IL-7Rα*), as potential risk factors (Baranzini, Galwey *et al*. 2009, De Jager, Jia *et al*. 2009, Beecham, Patsopoulos *et al*. 2013). Importantly, genetic studies have implicated genes of the immune system as being important in MS, providing insight into the pathogenesis of the disease. However, these studies have had limited success in identifying genetic markers with high sensitivity. Taken together, these data suggest MS is multi-factorial and not solely influenced by genetic factors.

Historically, population studies have shown that MS is more frequently observed in post-pubescent individuals (Chitnis 2013), women (Greer and McCombe 2011, Wallin, Culpepper *et al*. 2012), Caucasians of European decent (Kurtzke, Beebe *et al*. 1979, Rosati 2001), and in areas farthest from the equator. However, newer studies demonstrating a shift in ethnic bias indicate that individuals of African descent have equal incidence of MS compared to Caucasians, which has been attributed to darker skin tones limiting sunlight uptake (Kurland and Reed 1964, Evans, Beland *et al*. 2013, Langer-Gould, Brara *et al*. 2013). Additionally, the bias based on latitudinal location also suggests that sunlight (Simpson, Blizzard *et al*. 2011), in addition to other environmental factors such as pathogen exposure (Gale and Martyn 1995), may be important. Migration studies analyzing the effects of changes in latitudinal location indicate that the effect of

relocation on disease predisposition is age dependent (Alter, Leibowitz *et al*. 1966, Dean 1967, Dean and Elian 1997). Individuals that move as children assume the susceptibility of their adopted country; whereas adults that migrate retained the susceptibility rate of their native country. These findings suggest that exposure to certain environmental stimuli during childhood, a period critical in the development of the immune system, can affect an individual's predisposition to developing MS. Understanding how these stimuli potentiate their effects could provide valuable insight into the immune mechanisms underlying the initiation of MS, which in turn would further the investigation for preventative and therapeutic treatments for the disease.

Currently, there are two popular hypotheses for how exposure to infectious agents in the environment can inadvertently trigger the immune responses observed in MS. The first hypothesis, molecular mimicry, suggests that pathogens have antigenic elements that mimic CNS antigens and can cross react with autoreactive T cells. The alternative hypothesis, bystander effect, proposes that during the normal course of clearing an infection there is non-specific activation of autoreactive T cells. Both of these hypotheses implicate pathogens as being the initiating triggers in susceptible individuals. For instance, viruses such as Epstein-Barr virus (Handel, Williamson *et al*. 2010) and human herpes virus 6 (Chapenko, Millers *et al*. 2003) have been associated with MS, but none have been proven to be causal agents of the disease. While etiologic studies have identified some potential contributing factors of MS, there is tremendous need for understanding how and where disease is initiated. An ongoing question in MS is whether

inflammatory events in the periphery or neurodegenerative events in the CNS drive the initiation of this indisputably immune-mediated disease. Two primary hypotheses, outside-in and inside-out, have been posed to address this question (Stys, Zamponi *et al*. 2012). The outside-in hypothesis suggests that an immune-activating event, such as molecular mimicry and bystander activation, occurs in the periphery that leads to immune-targeting of the CNS. Evidence supporting this hypothesis has demonstrated the association of immune-related genes and mechanisms with MS (Sawcer, Hellenthal *et al*. 2011). Recently, an alternative hypothesis has been proposed, based on clinical observations, that suggests a primary neurodegenerative event in the CNS releases myelin antigens, resulting in activation of the immune system and a secondary autoimmune response (Hauser and Oksenberg 2006, Trapp and Nave 2008, Dutta and Trapp 2011). Further investigation of these hypotheses is crucial, as elucidating the mechanisms that drive MS is essential for identifying therapeutic targets that can modulate early in disease. However, the anatomical location of MS makes in-depth investigation of patients challenging, highlighting the importance of an *in vivo* model. The animal model known as experimental autoimmune encephalomyelitis (EAE) has been crucial in dissecting the components that contribute to various stages in CNS autoimmunity.

Experimental Autoimmune Encephalomyelitis (EAE)

The development of EAE, previously called experimental allergic encephalomyelitis, as a model for MS was pivotal in providing a controlled method for identifying *in vivo* mechanisms of CNS demyelination and autoimmunity.The EAE model was initially

developed by Rivers, Sprunt, and Berry in 1933, in an attempt to understand why some individuals develop post-rabies vaccination encephalomyelitis (Rivers, Sprunt *et al*. 1933, Rivers and Schwentker 1935). Monkeys that received repeated intramuscular injections of aqueous emulsion and rabbit brain extracts, experienced paralysis caused by CNS lesions (Rivers and Schwentker 1935). EAE has many similarities to MS, such as the presence of inflammatory lesions. Over the past 40 years, various EAE models have been developed, each mimicking specific components of MS pathogenesis. When EAE models are correctly applied, they can be useful tools for identifying immune components and testing MS therapeutics.

Active and passive inductions are the two widely accepted methods for producing EAE in mice. Active induction of EAE involves the immunization of rodents with CNS-specific immunogens, such as myelin oligodendrocyte glycoprotein (MOG) or myelin basic protein (MBP), emulsified in complete Freund's adjuvant (CFA), resulting in the initiation of an immune response against self-antigens found in the brain and spinal cord (Stromnes and Goverman 2006). Actively inducing disease drives an immune response directed against the CNS, but it also allows for antigen presenting cells (APCs) to naturally process antigen, present peptides, and activate T cells *in vivo*. In contrast, passive EAE involves adoptively transferring activated T cells reactive to myelin antigens into naïve recipient animals (Stromnes and Goverman 2006). Passively transferring EAE provides a useful method for dissecting out the role of immune cells subsets critical for EAE by allowing the cells to be manipulated prior to being

transferred. For instance, cells can be sorted and labeled as a means of tracking migration of these cells *in vivo*. Active and passive EAE have both been fundamental in determining the importance and specific role of individual cell subsets important for the development and progression of EAE and MS.

Immune Responses in EAE and MS

Since 1960, myelin-specific B and T cells have been identified as contributors to the CNS damage observed in EAE (Paterson 1960, Pettinelli and McFarlin 1981, Sriram, Solomon *et al*. 1982, Fillatreau, Sweenie *et al*. 2002). Philip Paterson was the first person to show that the passive transfer of lymphocytes from spinal cord sensitized animals induces EAE in naïve recipients (Paterson 1960). Using this model on T cell-depleted animals clearly established the specific requirement for thymus-derived lymphocytes in the initiation of EAE. Further studies demonstrated that the transfer of myelin-reactive T helper (Th) cells, specifically, induces EAE, whereas exclusion of Th cells can prevent disease initiation (Ben-Nun, Wekerle *et al*. 1981, Ben-Nun, Wekerle *et al*. 1981, Ben-Nun and Cohen 1982, Ben-Nun and Lando 1983, Mokhtarian, McFarlin *et al*. 1984). These and other studies led to the conclusion that EAE is a major histocompatability complex (MHC) class-II restricted T cell-mediated disease (Sriram and Steinman 1983, Steinman, Solomon *et al*. 1983, Waldor, Sriram *et al*. 1983, Zamvil, Mitchell *et al*. 1987). When EAE lesions were examined, both CD8 and CD4 cells were observed in the cellular infiltrate. Interestingly, peripheral myelin-specific T cells were detected in both diseased and naïve animals (Schluesener and Wekerle 1985, Zamvil, Mitchell *et al*. 1987, Genain,

Lee-Parritz *et al*. 1994). These findings among those of others demonstrated a role for T cells as mediators of CNS inflammation and drove researchers to investigate the pathogenic properties of T cell subsets in the context of MS.

Given the localization of the affected organ system and standard challenges with human research, the role of T cells in MS pathogenesis has not easily been elucidated. However, evidence suggests that MS patients share, in part, similar T cell characteristics and roles in driving CNS inflammation. One of the characterizing features of MS is the presence of perivascular cellular infiltrates primarily composed of T cells and macrophages (Frohman, Racke *et al*. 2006). MS patients also have CNS-specific autoreactive T cells in their periphery, but as seen in mice, these cells are also present in healthy individuals. However, characteristics of these autoreactive cells vary greatly, as determined by studies comparing the phenotype, reactivity, and diversity of the myelin-specific T cells from MS patients and healthy individuals (Sun, Olsson *et al*. 1991, Lovett-Racke, Trotter *et al*. 1998, Pelfrey, Rudick *et al*. 2000, Crawford, Yan *et al*. 2004). In terms of T cell receptor (TCR) diversity, the CD4 T cells of MS patients recognize more myelin-specific epitopes than healthy individuals, a potential consequence of epitope spreading (Pelfrey, Rudick *et al*. 2000, Crawford, Yan *et al*. 2004). MS patients have CD4 T cells with predominately activated/memory phenotypes, and display a higher magnitude of Th1 committed cells and IFNɣ production (Lovett-Racke, Trotter *et al*. 1998, Scholz, Patton *et al*. 1998, Pelfrey, Rudick *et al*. 2000). These autoreactive T cells are thought to infiltrate the BBB, gain access to the CNS, and secrete

cytokines and chemokines in order to attract other immune cells and promote myelin damage. These findings demonstrate that CD4 T cells are, at least in part, facilitating CNS inflammation, supporting observations made in EAE. When taken together, evidence from MS patients and animals with EAE indicate an apparent lack in regulation of T cell function and antigen recognition.

Regulatory T Cells (Tregs)

One of the main elements of regulation that is lacking in MS patients is a fully functional regulatory T cell (Treg) population. Under normal conditions, Tregs are essential for negatively regulating the immune system by maintaining immune homeostasis and self-tolerance (Sakaguchi, Sakaguchi *et al*. 1995, Sakaguchi, Sakaguchi *et al*. 2001, Shevach, McHugh *et al*. 2001). Tregs can be separated into two main categories based on their mechanism and location of origin: natural Tregs (nTregs) and inducible Tregs (iTregs). Starting in 1969, predating the naming of nTregs, T cell depletion studies observed multi-organ autoimmune disease in neonatal mice that received thymectomies 2-3 days after birth, demonstrating for the first time the importance of thymic derived T cells in establishing self tolerance (Nishizuka and Sakakura 1969, Penhale, Farmer *et al*. 1973, Kojima and Prehn 1981). Similar observations of autoimmunity were made in rodents following adult thymectomies with subsequent rounds of irradiation, suggesting that thymic derived suppressor cells were also capable of moving into the periphery to maintain tolerance throughout an animal's life, protecting them from autoimmunity. (Penhale, Farmer *et al*. 1973, Penhale, Stumbles

et al. 1990, Fowell and Mason 1993). These thymic derived suppressor T cells were eventually called nTregs because of their ability to confer natural tolerance. The development of these nTregs requires intermediate affinity interactions between their TCR and self-peptide/MHC complexes present on thymic stromal cells, allowing these cells to escape negative selection. The resultant Tregs have a TCR repertoire skewed towards the preferential recognition of self-peptides. Historically, Tregs have been characterized as the CD4+CD25^{hi} population in mice and humans (Sakaguchi, Sakaguchi *et al*. 1995, Itoh, Takahashi *et al*. 1999, Baecher-Allan, Brown *et al*. 2001, Dieckmann, Plottner *et al*. 2001), which also express forkhead box P3 (Foxp3), a transcription factor critical for the function and development of natural and inducible Tregs (Fontenot, Gavin *et al*. 2003, Hori, Nomura *et al*. 2003, Khattri, Cox *et al*. 2003, Fontenot, Rasmussen *et al*. 2005). Identification of these cells provided understanding of one of the immune mechanisms responsible for maintaining immune homeostasis. However attributing lifelong immune tolerance to these thymus derived subset of cells seemed unlikely as it is common knowledge that the thymus involutes with age, yet immune regulation remains intact throughout adulthood.

In the early 2000s, the concept that antigen-specific inducible Tregs (iTregs) can also be generated in the periphery began to emerge. The Horwitz lab was the first to demonstrate that ability to generate CD4+CD25+ T cells from naive CD4 T cells cultured with TGFβ capable of suppressing normal T cell proliferation (Yamagiwa, Gray *et al*. 2001, Zheng, Gray *et al*. 2002). Differentiation of naïve CD4 T cells into fully functional and stable

iTregs requires IL-2, retinoic acid, and TGFβ (Chen, Jin *et al*. 2003, Fantini, Becker *et al*. 2004, Zheng, Wang *et al*. 2004, Mucida, Park *et al*. 2007). TGFβ is a pleiotropic cytokine with broad implications in many cellular processes. TGFβ signaling is initiated by the binding of a TGFβ superfamily protein to (TGFβ1, TGFβ2, or TGFβ) a TGFβR2 homodimer on a cellular surface, resulting in signal transduction (Attisano and Wrana 1996). When bound by its ligand, TGFβR2 acts as a serine/threonine kinase receptor and promotes the recruitment and phosphorylation of TGFβR1 (Wrana and Pawson 1997). Activated TGFβR1 phosphorylates the cytoplasmic proteins SMAD2/SMAD3, which form a complex with SMAD4 (Nakao, Imamura *et al*. 1997). This complex translocates to the nucleus where it can bind SMAD binding domains and promote the transcription of downstream genes such as FOXP3. In 2003, Chen *et al.* established that TGFβ promotes peripheral Treg induction and function through the transduction of Foxp3+ (Chen, Jin *et al*. 2003).

Tregs act in several capacities to prevent immune reactions against self and regulate excessive immune responses to foreign antigens. Some main mechanisms include direct contact inhibition, secretion of anti-inflammatory cytokines, and competition for growth factors (Sojka, Huang *et al*. 2008). CTLA-4 is an inhibitory molecule that binds CD80 and CD86 (B7 molecules) and is constitutively expressed on the surface of Tregs (Takahashi, Tagami *et al*. 2000). CTLA-4 competes with CD28, a co-stimulatory molecule on T cells, and preferentially binds B7 (Krummel and Allison 1995), thus blocking the activation of autoreactive T cells by antigen presenting cells (APCs). Tregs

also secrete the pro-inflammatory cytokines TGFβ and IL-10 (Dieckmann, Plottner *et al*. 2001, Nakamura, Kitani *et al*. 2001, Jonuleit, Schmitt *et al*. 2002, Zheng, Wang *et al*. 2004, Maynard, Harrington *et al*. 2007) both of which can dampen inflammatory processes (Asseman, Mauze *et al*. 1999). Additionally, Tregs can competitively bind IL-2 through their high expression of CD25 leading to the cytokine deprivation of T effector (Teff) cells (Barthlott, Moncrieffe *et al*. 2005, Fontenot, Rasmussen *et al*. 2005). Through these mechanisms, Tregs can modulate the activation, differentiation, and proliferation of other Th cells (Annacker, Pimenta-Araujo *et al*. 2001, Oldenhove, de Heusch *et al*. 2003, Xu, Liu *et al*. 2003, Martin, Banz *et al*. 2004, Stassen, Jonuleit *et al*. 2004, Tadokoro, Shakhar *et al*. 2006, Pandiyan, Zheng *et al*. 2007). Therefore, under normal conditions Tregs have the tools needed to suppress T cell immune responses. However, it is evident that in MS patients Th1 and Th17 responses are unable to be efficiently controlled, facilitating the need to analyze whether Tregs are capable of performing their normal suppressor functions in the context of CNS autoimmunity. Given the importance of TGFβ and Tregs in immunosuppression, research has been done investigating both in an attempt to understand their potential roles in the development of autoimmune diseases.

Function of TGFβ in Immunosuppression

The immunosuppressive effect of TGFβ on immune cells, particularly T lymphocytes, has been well documented. In 1986, Kehrl *et al* were the first to report the ability of TGFβ to inhibit IL-2 dependent proliferation of T cells (Kehrl, Wakefield *et al*. 1986). Additionally, mice with disruptions in the TGFβ1 gene developed multi-organ

autoimmunity (Shull, Ormsby *et al*. 1992, Kulkarni and Karlsson 1993), characterized by the infiltration of lymphocytes, including activated CD4 T cells (Letterio and Roberts 1996). Direct evidence that TGFβ signaling is necessary for the maintenance of T cell homeostasis came from observations of autoimmunity in mice with T cell specific disruptions in TGFβR2 (Gorelik and Flavell 2000). These findings set the stage for dissecting the role of TGFβ in specific T cell subsets.

In adult mice with deficiencies in TGFβ signaling, the number of Tregs appears normal. However, the number of postnatal (3-5 days) Tregs is significantly reduced (Marie, Letterio *et al*. 2005, Marie, Liggitt *et al*. 2006, Liu, Zhang *et al*. 2008). This limited set of Tregs, which are $CD25^{+}$, proliferate in response to increased levels of IL-2, resulting in normal numbers of Tregs (Liu, Zhang *et al*. 2008). However, these Tregs have a reduced capacity to suppress Teff cells as demonstrated by the uncontrolled autoimmunity observed in mice with TGFβ signaling deficiencies (Gorelik and Flavell 2000). The inability of Tregs from TGFβ signaling-impaired mice to suppress autoimmunity has been attributed to decreased Foxp3 expression and a decreased TCR repertoire, indicating that they may have insufficient diversity to respond to self-antigens and prevent autoimmunity. Interestingly, similar observations have been made in MS.

Tregs in EAE and MS

In 2002, the Miller group demonstrated for the first time that nTregs can prevent CNS-specific autoimmunity by transferring purified heterogeneous Tregs from naïve wild-type

mice to mice with MOG-induced EAE (Kohm, Carpentier *et al*. 2002). In another model of EAE, MBP T cell receptor (TCR) transgenic (Tg) mice deficient in recombination-activating gene (rag) 1 spontaneously develop EAE, where as MBP TCR Tg $rag^{+/+}$ mice are protected. This model was used to demonstrate that the passive transfer of antigen-specific Tregs can prevent spontaneous autoimmunity (Hori, Nakano *et al*. 2002). Since 2002, numerous studies have provided evidence that the transfer of antigen-specific or CNS-derived Tregs can allow for disease prevention and recovery in the context of EAE. While these studies demonstrate the ability of transferred Tregs to prevent autoimmunity, they do not address whether endogenous Tregs can be generated to suppress encephalitogenic Teff cells during active disease.

Korn, *et al*. utilized FOXP3/green fluorescent protein knock-in mice along with MOG tetramers to track MOG-specific Treg and Teff cells during active EAE (Korn, Anderson *et al*. 2007). They observed that MOG-specific Tregs were able to expand in the periphery and traffic to the site of damage in the CNS, but when challenged ex vivo, were unable to suppress activated CNS-derived Teff cells (Korn, Anderson *et al*. 2007). These findings indicate that there is an inherent defect in Treg function during CNS autoimmunity, but passive transfer of exogenous Tregs may provide therapeutic benefit.

Similar studies comparing the frequency of Tregs in humans have shown that MS patients and healthy controls (HC) do not have significant differences in their Treg numbers (Putheti, Pettersson *et al*. 2004, Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug

et al. 2005, Haas, Fritzsching *et al*. 2007). However, functional studies indicate that the Tregs of MS patients have diminished suppressive effect on the autoimmune response against myelin-specific T cells (Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Kumar, Putzki *et al*. 2006). Suppressive assays using the noted combinations of Treg and Teff cells (MS Treg with HC Teff cells and HC Treg with MS Teff cells) established that the lack of suppression observed in MS patients is not due to a resistance of Teff cells to suppression, but rather a defect in their Tregs (Baecher-Allan, Brown *et al*. 2001, Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005). Further investigation showed that MS patients have reduced levels of FOXP3 compared to healthy donors (Huan, Culbertson *et al*. 2005, Venken, Hellings *et al*. 2008), which given the importance of FOXP3 in Treg function, could partially explain the lack of Treg function. Interestingly, patient-derived Tregs contained less TCR diversity compared to healthy Tregs, potentially explaining their inability to recognize and suppress myelin-specific autoreactive Teff cells (Haas, Fritzsching *et al*. 2007). Given the inherent lack of Teff cell regulation, numerous therapies attempt to halt disease processes by directly targeting T cells.

Therapies for MS

Disease-modifying therapies (DMTs) are drugs that alter disease course with the potential for slowing down disease progression. Currently, there are 12 DMTs approved by the FDA for the treatment of MS [interferon beta-1a, interferon beta-1b, peginterferon beta-1a, glatiramer acetate (GA), natalizumab, fingolimod, mitoxantrone, alemtuzumab,

dimethyl fumarate (DMF), and teriflunomide] (Wingerchuk and Carter 2014). MS DMTs can be divided into three main groups based on route of administration: self-injectables (IFNβ and GA), oral (DMF, fingolimod, and teriflunomide), and intraveneous (alemtuzumab, mitroxantrone, and natalizumab). Each of these drugs have mechanisms of action (MOA) implicated in the targeting or modulating components of the immune system, including T cells. The two most well characterized drugs are IFNβ derivatives and GA. Both of these drugs are thought to promote their effect by shifting Th cell differentiation from Th1 to Th2, enhancing Treg development, and driving deletion of myelin reactive T cells (Graber, McGraw *et al*. 2010). Similarly, Natalizumab and fingolimod both affect leukocytes, but rather than actively changing the phenotype of T cells, these drugs limit their mobility, preventing their migration into the CNS. For instance, natalizumab is a monoclonal antibody (mAb) that blocks very late antigen 4 (VLA4) and consequently prevents T cells from binding to vascular cellular adhesion molecule 1 (VCAM1) and migrating into the CNS (Ransohoff 2007). Furthermore, fingolimod prevents T cell motility by acting as a sphingosine-1-phosphate (S1P) agonist to block S1P receptor signaling, essentially trapping T cells in the lymph nodes (Cohen and Chun 2011). Whereas alemtuzumab and teriflunomide act through more brute force immunomodulatory mechanisms by depleting circulating T cells and inhibiting T cell proliferation respectively (Claussen and Korn 2012). In addition to having immunosuppressive effects, some of the DMTs are thought to have neuroprotective effects as well. Both DMF and laqunimod are thought to suppress microglia activation and alter their phenotype, limiting CNS inflammation (Wilms, Sievers *et al*. 2010,

Mishra, Wang *et al*. 2014, Peng, Matos *et al*. 2015). Given that current DMTs all have unique immunologic targets and MOA, they are not all equally effective in MS patients and have different risk/benefit profiles.

Currently, interferon beta (IFNβ) and glatiramer acetate (GA) are the most widely accepted first-line therapies for RRMS. While IFNβ and GA are considered only mildly effective compared to other DMTs, they are preferentially prescribed due to their long established use and safety profiles. On the other hand, some of the other drugs such as natalizumab, fingolimod, and alemtuzumab are more effective than traditional therapies, but at a price. These drugs have documented cases of serious and potentially fatal adverse events. For instance, patients on natalizumab who are infected with JC virus are at high risk for developing progressive multifocal leukoencephalopathy, an often fatal disease for which there is no cure. Additionally, patients on alemtuzumab and fingolimod are at risk for developing fatal autoimmune disorders and cardiac problems respectively. While these drugs may be justified due to their increased efficacy, other drugs such as teriflunomid should only be used in the event that a patient is not responding to first or second line therapies, as this treatment is a pregnancy category X drug and no more effective than other safer therapies. Therefore, it is necessary to weigh the potential outcomes and pre-screen patients accordingly when discussing treatment options. Aside from risk/benefit profiles, patient compliance and quality of life must also be considered when choosing a treatment. Therefore, route of administration must be also taken into account. Oral treatments are extremely attractive in that they are convenient and

comfortable to take, however they have not been shown to be superior to other DMTs. Therefore, DMF and fingolimod are really only good options when first line treatments are failing. While all of these drugs have been shown to reduce annual relapse rate and slow down time to progression of disease, it is obvious that current therapy options have many limitations.

Notably, the available treatments are lacking, with only 1/12 DMTs indicated for SPMS and none indicated for PPMS, resulting in minimal chance of recovery especially in patients with progressive forms of the disease. Studies have demonstrated early use of DMTs in patients presenting with a CIS have a delayed conversion to CDMS (Jacobs, Beck *et al*. 2000, Comi, Filippi *et al*. 2001, Kappos, Polman *et al*. 2006, Comi, Martinelli *et al*. 2009, Miller, Wolinsky *et al*. 2014). Therefore, diagnosis of patients early during the disease process while treatments are available is essential for delaying progression of the disease. However, diagnosis is challenging, as there is no single test for MS. Additionally, despite the ability of existing drugs to slow down disease progression, the eventual worsening of symptoms is inevitable. Identification of individuals susceptible to developing MS may allow these patients to be proactive by submitting to routine screening and to prepare themselves for possible diagnosis. Therefore, research aimed at the identification of novel biomarkers and therapeutic targets is imperative for understanding disease activity, measuring treatment efficacy, and stopping disease progression. However, previous attempts at such studies have had limited success in identifying markers with high levels of sensitivity and specificity. This overall lack of

success potentiates the need for innovative strategies for elucidating factors of indicative of disease mechanisms promoting susceptibility and progression of MS. In recent years, miRNAs have emerged as important regulators that affect various biological processes. Even more recently, studies have begun to successfully show the clinical applications of miRNAs in various diseases such as cancer and rheumatoid arthritis (Lu, Getz *et al*. 2005, Calin and Croce 2006, Pers and Jorgensen 2013, Redova, Sana *et al*. 2013). In addition, the accessibility and relatively low number of miRNAs make them ideal potential biomarker candidates.

microRNAs (miRNAs)

miRNA regulation is an active area of investigation for the regulation of cellular pathways and disease processes. The first miRNA was characterized in 1993 by the lab of Victor Ambros in *C. elegans* (Lee, Feinbaum *et al*. 1993), but it was not until the early 2000s that miRNAs were acknowledged as distinct biological regulators (Lagos-Quintana, Rauhut *et al*. 2001, Lau, Lim *et al*. 2001, Lee and Ambros 2001). In the past 22 years, several studies have worked to characterize the properties and functions of miRNAs as epigenetic mediators. miRNAs are small non-coding RNAs approximately 19-24 nucleotides in length. They are transcribed in the nucleus as primary miRNA, which are further processed by the Drosha/Pasha complex, resulting in precursor miRNA (pre-miRNA). Pre-miRNA is subsequently transported from the nucleus to the cytoplasm by the protein Exportin-5. Once in the cytoplasm, pre-miRNA is cleaved by the enzyme Dicer, resulting in the generation of an RNA duplex from which one strand of RNA is

degraded. The remaining single stranded RNA, now called a miRNA, is loaded into the RNA induced silencing complex (RISC) where it promotes its function as a negative regulator of gene expression. miRNAs function at the post-transcriptional level through base-pair binding to complementary sequences of mRNA in the 3' untranslated regions (3' UTRs) of genes (Ambros 2004, Bartel 2004, Bartel 2009).

There are three main mechanisms of miRNA function: 1) the binding and cleaving of mRNA, 2) blocking of machinery crucial for translation, and 3) destabilization of mRNA, resulting in mRNA degradation (Filipowicz, Bhattacharyya *et al*. 2008, Bartel 2009, Chekulaeva and Filipowicz 2009, Fabian, Sonenberg *et al*. 2010). In general terms, miRNAs reduce the expression of specific proteins. Importantly, miRNAs do not bind exclusively to one mRNA target nor are mRNAs only regulated by one miRNA. Rather, miRNAs have the ability to bind to and regulate many different genes, potentially in the same biological pathway. miRNAs are highly conserved among mammals (Lim, Glasner *et al*. 2003) and have conserved binding sequences in approximately 60% of human protein-coding genes (Friedman, Farh *et al*. 2009).

As suggested by their evolutionary conservation, miRNAs are crucial to the maintenance of normal cellular development and function (Ambros 2004, Bartel 2004, Chen, Li *et al*. 2004). Loss of the miRNA processing enzyme dicer in T cells leads to a lack of T cell maturation and differentiation (Cobb, Hertweck *et al*. 2006, Muljo, Kanellopoulou *et al*. 2010). Since these seminal observations, miR-181a (Li,2007), miR-155 (Rodriguez,

Vigorito *et al*. 2007, Thai, Calado *et al*. 2007), and miR-142-3p (Huang, Zhao *et al*. 2009) have been found to influence early T cell development, Th differentiation, and Treg suppressive function, respectively. Interestingly, miRNA levels vary based on cell type and stage of differentiation (Monticelli, Ansel *et al*. 2005, Wu, Neilson *et al*. 2007, Merkerova, Belickova *et al*. 2008). Therefore, comparing miRNA profiles of specific cell types in disease versus healthy state may provide insight into the cellular processes contributing to disease pathogenesis.

As indicated, miRNAs regulate immunoregulatory pathways important for immune development and function. In addition to being biological regulators, miRNAs are easily quantified from cells, tissue, and blood, making them attractive candidates for potential markers for disease. Initial evidence linking human disease to changes in miRNA levels was provided by the Croce lab who demonstrated that the loss of specific miRNAs (mir-15 and miR-16) is associated chronic lymphocytic leukemia (Calin, Dumitru *et al*. 2002) and correlates with increased levels of the antiapoptotic B cell lymphoma 2 (Bcl2) protein (Cimmino, Calin *et al*. 2005). Additionally, the use of miRNA signatures in cancer patients has proven to be effective for identifying tumor origin and predicting outcome and treatment response (Lu, Getz *et al*. 2005, Calin and Croce 2006). Loss of miRNA regulation has also been implicated in autoimmune disease, as shown by casual studies demonstrating that deletion of miRNA processing elements specifically in Tregs leads to uncontrolled autoimmunity in mice (Chong, Rasmussen *et al*. 2008, Liston, Lu *et al*. 2008, Zhou, Jeker *et al*. 2008). In the context of human autoimmune disease, the

deregulation of miRNAs has been correlated with biological process critical for the promotion and progression of several autoimmune diseases. For example, both miR-155 and miR-146a has been found to be differentially expressed in patients with systemic lupus erythematosus and rheumatoid arthritis (Qu, Li *et al*. 2014). These observations promoted further investigation of miRNAs involvement in other human autoimmune diseases.

miRNAs in MS

In recent years, there has been an emerging interest to use miRNA profiling to identify differences between healthy individuals and MS patients. Several studies have evaluated miRNAs levels in cells from the peripheral blood of MS patients (Du, Liu *et al*. 2009, Keller, Leidinger *et al*. 2009, Otaegui, Baranzini *et al*. 2009). In 2009, Otaegui, *et al*. demonstrated for the first time that miRNAs are differentially expressed in the peripheral blood mononuclear cells (PBMCs) of RRMS patients compared to healthy donors. The miRNAs that were significantly altered in relapsing patients (miR-18b and miR-599) were different than those identified in remitting individuals (miR-96), indicating that therapeutically targeting pathways regulated by miR-96 could promote recovery (Otaegui, Baranzini *et al*. 2009). Also in 2009, Keller, *et al*. showed that 165 miRNAs were differentially expressed in the peripheral blood leukocytes of MS patients, 43 of which could be used to detect RRMS patients with an approximately 90% sensitivity, specificity, and accuracy, identifying a novel role for miRNAs in MS as potential biomarkers (Keller, Leidinger *et al*. 2009). Since these initial findings, several miRNAs

have been implicated in specific stages of MS pathogenesis. For example, miR-326, a miRNA that promotes Th17 differentiation, was found to be significantly upregulated in both PBMCs and active lesions of MS patients compared to healthy individuals (Du, Liu *et al*. 2009, Junker, Krumbholz *et al*. 2009). Bitranslational studies showed that silencing of miR-326 results in mice with fewer Th17 cells and less severe EAE (Du, Liu *et al*. 2009). To date, the largest miRNA profiling studying of MS patient PBMCs assessed the levels of 733 miRNAs in 59 treatment naïve patients and 37 healthy controls. Two of the miRNAs (miR-17 and miR-20) that were downregulated in MS patients were found to be important regulators of T cell activation (Cox, Cairns *et al*. 2010).

In addition to miRNA studies on mixed cellular populations, others have been done on defined subsets of cells from the peripheral blood (De Santis, Ferracin *et al*. 2010, Lindberg, Hoffmann *et al*. 2010, Guerau-de-Arellano, Smith *et al*. 2011, Smith, Guerau-de-Arellano *et al*. 2012) and lesions (Junker, Krumbholz *et al*. 2009) of MS patients. In 2010, Lindberg *et al*. and De Santis, *et al*. assessed miRNA levels in CD4+CD25hi Tregs, identifying 23 differentially expressed miRNAs in RRMS patients. Two of the described miRNAs (miR-106b and miR-25) were previously shown to regulate the transforming growth factor beta (TGFβ) pathway, a pathway critical for the development and function of Tregs, indicating a potential defect of TGFβ signaling in these cells (De Santis, Ferracin *et al*. 2010). While the peripheral blood studies can compare miRNA profiles of patients in different disease states, they do not necessarily identify which miRNA are playing a role in active CNS disease. In order to identify miRNA specifically

altered during active CNS damage, Junker *et al* compared the miRNA profiles of active and inactive lesions of MS patients to each other and to normal appearing white matter from healthy subjects and showed a correlation between increased levels of miR-326, miR-155, and miR-34a with decreased levels of CD47 in resident brain cells, a protein that provides inhibitory signals to macrophages (Junker, Krumbholz *et al*. 2009). These studies provide evidence that miRNAs may participate in the many different aspects of MS pathology.

In 2010, Weber *et al* reported the presence of miRNAs in 12 bodily fluids including plasma and CSF, supporting the potential use of miRNAs as easily detectable biomarkers (Weber, Baxter *et al*. 2010). In 2012, Siegel *et al* were the first to demonstrate the differential expression of miRNAs in the plasma of MS patients compared to healthy controls, identifying 7 miRNAs (miR-614, miR-572, miR-648, miR-1826, miR-422a, miR-22, and miR-1979) that could potentially be used as biomarkers for MS (Weber, Baxter *et al*. 2010). A similar study of select miRNAs identified miR-145 as being 3-fold higher in the plasma, PBMCs, and serum of treatment naïve RRMS patients in remission (Søndergaard, Hesse *et al*. 2013). Recently, Gandhi *et al* performed an extensive profiling study of plasmic miRNAs in RRMS and SPMS patients that were treatment naïve, and identified two miRNAs (miR-92 and let-7) that could be used to distinguish RRMS from SPMS (Gandhi, Healy *et al*. 2013). Interestingly, these same miRNAs could be used to differentiate RRMS from amyotrophic lateral sclerosis (ALS), a neurodegenerative

disease, but not SPMS and ALS, suggesting potential overlap in disease processes for SPMS and ALS (Gandhi, Healy *et al*. 2013).

While providing insight into disease mechanisms, the previously mentioned studies do not address whether the observed differences are inherent in the patients or a consequence of disease, which is crucial for identifying specific miRNAs as potential MS susceptibility factors. To this end, our group previously performed a miRNA profiling study on naïve and memory CD4 T cells isolated from the PBMCs of healthy donors and treatment naïve MS patients (Guerau-de-Arellano, Smith *et al*. 2011). This study identified 85 differentially expressed miRNAs in the naïve CD4 T cells of MS patients. In 2011, our group published evidence that two miRNAs (miR-128 and miR-27b) which are significantly upregulated in the naïve CD4 T cells of MS patients influence the development of MS through the inhibition of Th2 differentiation and promotion of Th1 responses (Guerau-de-Arellano, Smith *et al*. 2011). Evidence from Smith *et al* demonstrating a decrease in miR-29b levels also supports that the naïve CD4 T cell miRNA profile of MS patients is preferential to Th1 polarization (Smith, Guerau-de-Arellano *et al*. 2012). Additional bioinformatics analyses of data from our group predicted that 25 of the 85 differential expressed miRNAs target four genes of the TGFβ signaling pathway, TGFβ receptor 1 (TGFβR1), TGFβ receptor 2 (TGFβR2), SMAD2, and SMAD4.

Rationale

MS is an inflammatory demyelinating disease of the CNS, which devastatingly affects 450,000 people in the United States alone (Hersh and Fox 2010). The cause of MS remains unknown, and the mechanisms that propagate the pathology of the disease are poorly understood. This lack of knowledge has made preventing MS impossible and effectively halting disease progression challenging. The ***long-term goals*** of the Lovett-Racke lab are to determine mechanisms that underlie the pathophysiology of MS and identify candidate regulators of disease susceptibility and novel therapeutic targets. The CNS damage observed in MS patients is thought to be immune-mediated and promoted by myelin-specific pro-inflammatory T cells. The lack of immune regulation in MS patients has clearly been established and partially attributed to an inherent defect in Tregs. While being normal in number, Tregs of MS patients demonstrate diminished suppressive effect, reduced Foxp3 expression, and a less diverse TCR repertoire (Putheti, Pettersson *et al*. 2004, Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Haas, Fritzsching *et al*. 2007, Venken, Hellings *et al*. 2008). However, the mechanisms for the Treg defects in MS patients are unknown. Understanding what causes this pro-inflammatory shift in T cell differentiation and lack of regulation in MS patients is critical for identifying potential MS susceptibility factors and therapeutic targets.

The ***main objective*** of my graduate work was to determine the extent to which miRNAs identified as being differentially expressed in MS patients regulate genes critical for the development of functional Tregs. To this end, our lab previously performed miRNA

profiling studies on the naïve CD4+ T cells of MS patients and healthy donors and identified 85 miRNAs to be differentially expressed in MS patients. In a published report, the lab demonstrated that dysregulation of a select group of these miRNA in the naïve CD4 T cells of MS patients promotes Th1 polarization. Further pathway analysis of the miRNA data, led to the discovery of 19 differentially expressed miRNAs in the naïve CD4 T cells of MS patients that are predicted to target and inhibit genes of the TGFβ signaling pathway. TGFβ is a pleiotropic cytokine that is critical for the development and suppressor function of Tregs (Yamagiwa, Gray *et al*. 2001, Zheng, Gray *et al*. 2002, Chen, Jin *et al*. 2003). Interestingly, it has been observed that adult mice deficient in TGFβ signaling develop multi-organ autoimmunity (Shull, Ormsby *et al*. 1992, Kulkarni and Karlsson 1993, Gorelik and Flavell 2000). This has been attributed to a defect in Tregs. These mice exhibit a Treg phenotype that mirrors observations made in MS. Taken together, these findings suggested a potential link between the Treg defect observed in MS patients and TGFB signaling deficiencies. Therefore, we ***hypothesized*** *that dysregulated miRNAs in the naïve CD4+ T cells of MS patients target the TGFβ-signaling pathway, resulting in defective Tregs and enhanced susceptibility to developing MS*. The ***rationale*** for the proposed project is that investigating the role of miRNAs in the development of Tregs, will enable the: (1) identification of miRNAs responsible for the regulation of the TGFβ signaling pathway, (2) correlation of miRNA expression levels with Tregs deficits and (3) identification of miRNAs that potentially regulate MS pathogenesis through their binding of TGFβ-associated genes.

Specific Aims

To accomplish my overall objective, the following aims were proposed:

Aim 1: Determine the extent to which the TGFβ signaling pathway is inhibited by the miRNAs identified as dysregulated in the naïve CD4+ T cells of MS patients. The *working hypothesis* was that differentially expressed miRNAs bind to specific genes of the TGFβ-signaling pathway in naive CD4 T cells and inhibit the translation of their protein products.

Aim 2: Identify which TGFβ-targeting miRNAs alter the development of inducible Tregs (iTregs) *in vitro*. The *working hypothesis* was that over expression of miRNAs that target specific genes known to promote TGFβ-pathway signaling would cause a decrease in signaling and attenuates the development of iTregs.

Aim 3: Elucidate the effect of miRNA dysregulation on disease susceptability *in vivo* and iTreg generation directly *ex vivo*. The *working hypothesis* was that the overexpression of TGFβ-targeting miRNAs *in vivo* would increase disease severity and reduce capacity to generate iTregs *ex vivo*.

The work proposed in aim 1 was expected to yield the following *outcome*: specific miRNAs would be identified as key negative regulators of the TGFβ-signaling pathway. Importantly, aim 2 would allow the identification of specific TGFβ-targeting miRNAs that have a diminishing effect on Treg development. The work proposed in aim 3 was

predicted to identify specific combinations of miRNAs that enhance onset and severity of CNS autoimmunity. The overall expected outcomes of this project proposed to ***positively impact*** the field by contributing to the understanding of the underlying pathophysiology of Treg dysfunction in MS patients and by identifying candidate regulators of disease susceptibility, as well as, potential therapeutic targets.

Chapter 2: General Methods

Human Subjects (Chapters 3-5)

The PBMCs used in Chapter 3 were obtained by leukopharesis at the University of Texas Southwestern Medical Center and the Washington University in Saint Louis. All PBMCs used in Chapter 3 were frozen and stored in liquid nitrogen. The MS PBMCs used in Chapters 4 and 5 were obtained by blood draw at The Ohio State University (OSU) MS Clinic. The healthy control PBMCs used in Chapters 4 and 5 were obtained via blood draw from donors at the American Red Cross. The donor blood was screened by Red Cross and found negative for specific pathogens. Both OSU MS Clinic and Red Cross samples were used directly *ex vivo* within 24 hr of being obtained.

PBMC Isolation (Chapters 3-5)

PBMCs were isolated from peripheral blood with Ficoll-Paque Plus (GE Healthcare). The blood was layered over Ficoll and lymphocytes were isolated via density centrifugation according to the manufacturer's protocol. Cells were immediately used and not frozen.

PBMC Transfection (Chapters 3-5)

PBMCs were plated on a 6-well plate in 2.5 ml of complete growth medium at a density of 8×10^6 cells per well. Transfection complexes were made by mixing 8 µl of TransIT-

TKO Transfection Reagent (Mirus) with 0.05 µM of single miRNA mimics (Dharmacon; miR-NS, miR-27b, miR-103a, miR-128, miR-141, miR-212, miR-500a, miR-628-3p, miR-708, let-7a, and let-7b) or 0.05 µM of miRNAs mimics in combination (miR-103/212/708; miR-141/500a/let-7b; miR-128/628-3p/let-7ab) in 250 µl of serum free medium and incubated for 30 min at room temperature. A nonsense miRNA (miR-NS) was used as a negative control for miRNA transfection. Transit TKO:miRNA complexes (250 µl) were added in a drop-wise fashion to the cells. Cells were incubated for 48 hr at 37 °C.

In Vitro Human Treg Induction (Chapters 4 and 5)

In general, PBMCs were rested for 2 hr, in order to get rid of adherent cells. The cells remaining in suspension were counted and plated at concentration of 1.5 x 10^6 cells/ml on a 48-well antibody-coated plate. Plates were coated with 500 µl of anti-human CD3 (1 µg/ml) and anti-human CD28 (1 µg/ml) diluted in PBS and incubated overnight at 4 °C or 2 hr at 37 °C. In order to generate iTregs, cells were centrifuged and resuspended in complete human growth media [1650 RPMI, 1% L-glutamine, 1% HEPES Buffer, 1% penicillin/streptomycin, and 5% human serum (AB)] supplemented with of 1 U/ml IL-2, 0.5 ng/ml TGFβ1, and 2.5 nM all-trans retinoic acid. Cells were subsequently incubated for 72 hr at 37 °C.

Flow Cytometry (Chapters 3-5)

Flow cytometry was used to look at cell surface markers CD4 (BD Biosciences), CD45RA (BioLegend), and CD25 (BD Biosciences) and intracellular components of FOXP3 (eBioscience), SMAD4 (R&D Systems), and TGFBR1 V 1-22 (Santa Cruz). Cells were collected, centrifuged, and resuspended in Fc Block (Miltenyi Biotec) diluted with staining buffer (PBS with 1% bovine serum albumin). Cells were incubated in diluted Fc Block for 10 min at room temperature. After blocking, the anti-human surface antibodies were diluted with staining buffer, added to cells, and incubated for 30 min at 4 °C. The cells were subsequently washed in staining buffer, resuspended in 1X fixation/permeabilization concentrate (eBioscience), and incubated for 30 min at 4 °C. After washing with 1X permeabilization buffer, cells were resuspended in Fc Block diluted in permeabilization buffer and incubated for 10 min at room temperature. After blocking, anti-human intracellular antibodies were diluted with 1X permeabilization buffer, added to cells, and incubated for 30 min at 4 °C (SMAD4 and TGFBR1) or 45 min at room temperature (FOXP3). The TGFβR1 detection required an additional staining with FITC conjugated goat anti-rabbit secondary antibody (Abcam) for 30 min at 4 °C. Cellular markers were measured with a FACSCanto II (BD Biosciences), and all data was analyzed using FlowJo software (Tree Star).

Mice (Chapter 5)

All mice used were B10.Pl wild-type mice. Breeder mice were initially purchased through Jackson Laboratory. All mice were bred, housed, and maintained in a specific-

pathogen free facility at OSU. All animal procedures performed were outlined in protocols approved by the OSU Institutional Animal Care and Use Committee (IACUC).

Chapter 3: Differentially Expressed miRNAs in the Naïve CD4 T cells of MS Patients Target the TGFß Signaling Pathway

Introduction

While epidemiological studies have identified both environmental and genetic components as potential risk factors for MS, the true cause of the disease is unknown. The CNS demyelination observed in MS patients is hypothesized to be an immune-mediated response driven in part by pro-inflammatory T cells activated against myelin antigens. Genetics studies have identified variants of immune-related genes as potential contributors to disease susceptibility (Mackay and Myrianthopoulos 1966, Ebers, Bulman *et al*. 1986, Willer, Dyment *et al*. 2003), indicating the importance of investigating differences in immune components between MS patients and healthy individuals. However, these studies have had limited success, necessitating the need to uncover additional genetic contributors.

The recent discovery of miRNAs as novel biological regulators, and the evidence supporting the role of miRNA dysregulation in mediating immune-related disease processes drove researchers to investigate miRNAs in the context of MS. Several studies have identified dysregulated miRNAs in both bodily fluids and isolated cellular subsets from the lesions and peripheral blood of MS patients (Junker, Krumbholz *et al*. 2009, De

Santis, Ferracin *et al*. 2010, Lindberg, Hoffmann *et al*. 2010, Guerau-de-Arellano, Smith *et al*. 2011, Smith, Guerau-de-Arellano *et al*. 2012). However, these studies do not address whether the observed miRNA dysregulation is an inherent defect in MS patients that increases their disease susceptibility or a mere consequence of disease.

To this end, our lab performed a miRNA profiling study of the naïve and memory CD4 T cells of MS patients and healthy individuals. Naïve CD4 T cells have not encountered antigen or undergone activation, suggesting that any observed differences in MS patients are indicative of inherent defects and not a repercussion of MS. In the naïve CD4 T cells, 85 miRNAs were found to be significantly up or down regulated in MS patients compared to healthy controls. In a published report, our lab demonstrated that dysregulation of two of the 85 identified miRNAs (miR-27b and miR-128) promotes decreased Th2 differentiation and enhances Th1 polarization (Guerau-de-Arellano, Smith *et al*. 2011). These findings suggested that miRNAs have biological significance and therapeutic potential through their regulation of T cell phenotypes in MS.

Further pathway analysis of the miRNA profiling data identified 19 differentially expressed miRNAs predicted to target and inhibit the TGFß-signaling pathway. TGFβ is a cytokine critical for immune suppression, as made evident by the uncontrolled multi-organ autoimmunity observed in mice with deficiencies in TGFß signaling (Shull, Ormsby *et al*. 1992, Kulkarni and Karlsson 1993, Gorelik and Flavell 2000). Interestingly, these mice have similar immune characteristics as MS patients. The initial

aim of my dissertation was to determine the extent to which the TGFß signaling pathway is inhibited by the dysregulated miRNAs in the naïve CD4+ T cells of MS patients. *The working hypothesis is that a portion of the 19 differentially expressed miRNAs directly bind to genes of the TGFß-signaling pathway and disrupt the translation of their protein products, thereby diminishing signaling of the TGFß-pathway in the naïve CD4 T cells of MS patients*. The *rationale* for the work described in Chapter 3 is that identifying specific TGFß-associated genes that are decreased in MS patients would allow us to validate that the TGFß pathway is suppressed in MS patients and assist us in narrowing down the scopes of our study.

Materials and Methods

Human Naïve CD4 T Cell Isolation

Naïve CD4+CD45RA+ T cells were isolated from the frozen MS patient and healthy control PBMCs that were obtained as described in Chapter 2. First, frozen cells were thawed, washed with complete human medium, and centrifuged. Dead cells were eliminated with the Dead Cell Removal Kit (Miltenyi Biotec). The naïve CD4 T cells were subsequently isolated with the Human Naïve CD4+ T Cell Isolation Kit II (Miltenyi Biotec). The remaining live cells were incubated with the a cocktail of biotinylated anti-human CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCRα/β, anti-HLA-DR, and CD235a antibodies, magnetically labeling Memory CD4 T cells and non-CD4 T cells. The labeled cells were subsequently magnetically depleted with the autoMACS® Pro Separator. The "depletes" program automatically

retains the labeled cells on the column as the positive fraction and dispels the untouched naïve CD4 T cells in the negative fraction. The Dead Cell Removal Kit and the Human Naïve CD4+ T Cell Isolation Kit II were used according to the manufacturer's manual magnetic labeling and autoMACS® Pro Separator protocols.

Flow Cytometry: Naïve CD4 T Cell Purity Check

A small sample of the negative fraction was saved to determine the purity of the naïve CD4 T cells recovered. The positive fraction was used for compensation set up. Flow cytometry was performed as described in Chapter 2. Cells were labeled with the surface antibodies anti-human CD4-Pacific Blue™ (PB; BD Biosciences), anti-human CD45RA-allophycocyanin (APC; BioLegend), and anti-human CD45RO-phycoerythrin (PE; BioLegend). Naïve cells were defined as cells that were CD4+CD45RA+CD45RO-.

RNA Isolation

RNA was only extracted from MS patient and healthy donor sample Naïve CD4 T cell isolates with CD4+CD45RA+ purity of 95% or higher. RNA was isolated from the naïve CD4 T cells with the *mir*Vana™ miRNA Isolation Kit (Ambion™). Total RNA including miRNAs was isolated according to the manufacturer's protocol and stored at -80 °C.

Real-Time Polymerase Chain Reaction (PCR): TGFβ Signaling Genes

The expression levels of TGFβR1, TGFβR2, SMAD2, and SMAD4 were measured with quantitative real-time PCR. The RNA samples used were the same samples from the miRNA profiling array. cDNA was reverse transcribed from the RNA of these samples using random primers. TaqMan® real-time polymerase chain reactions were performed using human TGFβR1, TGFβR2, SMAD2, SMAD4, and HPRT Applied Biosystems® (ABI) primer/probe sets. TaqMan® real-time PCR was performed with a 20 μl reaction volume containing 10 μl TaqMan® Universal PCR Master Mix (2X; ABI), 1 μl primer/probe assay mix (20X; ABI), 1 μl cDNA, and 8 μl RNAse free water. Human HPRT was measured and used as the endogenous control. Results were analyzed using the comparative Ct method. Relative fold change expression was calculated relative to the median Ct of the healthy controls group.

Pathway Analysis

The online software TargetScanHuman (Whitehead Institute for Biomedical Research) was used to identify predicted target genes of the miRNAs that were significantly different between the MS patients and healthy controls groups. The software determined target genes based on 3'UTR base-pairing. The resulting list of predicted target genes was loaded into Ingenuity® Pathway Analysis (QIAGEN) to identify if any of the target genes belonged to the same signaling pathway.

Flow Cytometry: SMAD4 and TGFβR1

The naïve CD4 T cells of MS patients and healthy individuals in Figure 3.3 were analyzed for protein levels of SMAD4 and TGFβR1 via flow cytometry as described in Chapter 2. Vials of frozen PBMCs from the samples used in the miRNA profiling and real-time PCR assays described above were thawed and used for flow cytometric analysis. The cells were stained with the surface antibodies anti-human CD4-PB (BD Biosciences) and human anti-CD45RA-APC (BioLegend). The cells were also stained with the intracellular antibody anti-human SMAD4-PE (R&D systems) and the primary antibody anti-human TGFβR1 V 1-22 (Santa Cruz). A second intracellular staining step was performed with the secondary antibody goat anti-rabbit IgG-fluorescein isothiocyanate (FITC; abcam®). The cells were first sub-gated on naïve CD4 T cells (CD4+CD45RA+). The geometric mean fluorescence intensity (MFI) of SMAD4 and TGFβR1 was determined for the CD4+CD45RA+ cells. The levels of SMAD4 and TGFβR1in the naïve CD4 T cells of the miRNA transfected healthy PBMCs shown in Figure 3.7 and Figure 3.8 were analyzed via flow cytometry as described above.

Vector Preparation

In order to prepare the vector for the luciferase assays, plasmids were replicated and digested with restriction enzymes. One Shot® TOP10 chemically competent *E.coli* cells (Invitrogen™) were transformed with the pGL3 Luciferase Reporter Vector (Promega). The resulting transformants were grown on liquid broth (LB) agar plates containing

ampicillin. Ampicillin was chosen since pGL3 plasmids are resistant to that specific antibiotic. Single bacteria colonies were picked and grown in small volume cultures (2 ml LB and 2 μl ampicillin (100 mg/ml)) at 37 °C overnight. The small volume cultures were expanded to large volume cultures (2 ml small volume culture and 48 ml LB) at 37 °C for 12 hr. Resultant bacteria were pelleted and stored at -20 °C until further use. Bacterial pellets were thawed, and pGL3 plasmids were isolated with the Plasmid Midi Kit (QIAGEN) according to the manufacturer's protocol. The purified plasmids were subsequently digested with the restriction enzyme XbaI in order to prep the plasmid for ligation of a 3'UTR insert.

3'UTR Insert Preparation

Segments of the 3'UTRs for TGFβR1 and SMAD4 were PCR-amplified and prepared for insertion into the pGL3 luciferase vector. The binding sites for the miRNAs predicted to target the TGFβ signaling pathway were mapped throughout the 3'UTR of TGFβR1 (NM_004612) and SMAD4 (NM_00539) using TargetScan Human 6.2 (Whitehead Institute for Biomedical Research). RNA was extracted from the PBMCs of healthy donors according to the manufacturer's protocol for TRIzol® RNA isolation (Life Technologies). Resulting RNA was checked for RNA degradation using gel electrophoresis. cDNA was obtained by reverse transcribing 2 μg of the isolated RNA with random primers (Applied Biosystems, ABI) using the High Capacity cDNA Reverse Transcription (RT) Kit Protocol (ABI). Custom primers (Invitrogen™) were designed against segments of the TGFβR1 and SMAD4 3'UTRs that contain multiple miRNA

binding sites and that were approximately ≤ 1 kilo base pairs (bp) long. It is important to note that these primers added XbaI sites onto the ends of each PCR product.

Subsequently, a standard polymerase chain reaction (PCR) was performed for each primer set in order to amplify the desired 3'UTR segments. The Finnzyme Fusion Protocol was performed according to manufacturer specifications. The final reaction volume was 50 µl consisting of 10 µl Phusion Buffer (5X), 1 µl dNTPs (10 mM), 2 µl forward primer, 2 µl reverse primer, 1 µl cDNA (RT reaction product), 0.5 µl DNA polymerase, and DNAse free water. The PCR program was as follows: 94 °C for 30 sec, 35 amplification cycles (94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min), and 72 °C for 10 min. To verify that the PCR products were the correct size they were analyzed using gel electrophoresis. To prepare the PCR product for insert into the PGL3 vector, the PCR products were purified using the PCR Purification Kit (QIAGEN) and digested with Xbal.

Ligation and Transformation of Digested Plasmids and 3'UTR Segments

In order to make complete constructs for the luciferase assays, individual digested 3'UTR inserts were ligated into digested pGL3 vectors. Ligation reactions consisting of 1 µl buffer, 0.5 µl vector (XbaI-digested), 1 µl insert (XbaI-digested), and 0.5 µl ligase enzyme were incubated at room temperature for 2 hr. Subsequently, One Shot® TOP10 chemically competent *E. coli* cells were transformed with 4 µl of the ligation reaction. The resulting transformants were grown on LB agar plates, selected, and grown in LB

cultures as described above. From the resultant bacteria, plasmids were isolated and purified also as previously described. To verify that the plasmids contained the correct insert, enzyme digestion of the constructs was performed and the resulting fragment sizes were determined via gel electrophoresis. Additionally, the plasmid sequences were verified through DNA sequencing at the OSU Nucleic Acid Shared Resource facility.

Luciferase Assay

The ability of miRNAs to bind and inhibit their predicted target genes was analyzed via luciferase assays. For human TGFβR1 (NM_004612), two segments of the 3'UTR were individually cloned into pGL3 vectors (Promega). TGFβR1 3'UTR base pairs 1563-2752 contain binding sites for miR-27b and miR-128. TGFβR1 3'UTR base pairs 3275-4038 contain binding sites for miR-141, miR-500a, and let-7. For human SMAD4 (NM_00539), four segments of the 3'UTR were made and cloned into pGL3 vectors. SMAD4 3'UTR base pairs 1-787 contain binding sites for miR-708 and miR-212. SMAD4 3'UTR base pairs 1525-2275 contain binding sites for miR-500a, miR-27b, and miR-128. SMAD4 3'UTR base pairs 3927-4582 contain binding sites for miR-128, miR-628-3p, miR-141, and miR-27b. SMAD4 3'UTR base pairs 4570-5563 contain binding sites for miR-103a, miR-141, and miR-18a.

Cos-7 cells were grown in complete growth medium (Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin) at 37 °C until cells were 60-80% confluent. Cells were subsequently transfected with a complete

construct (pGL3 with 3'UTR segment) and a single miRNA. Transfection complexes were generated by incubating plasmid DNA (150 ng), miRNA mimic (50 pmol), and 250 µl transfection media [6 µl Lipofectamine 2000 (Life Technologies) and 250 µl DMEM] together for 20 min at room temperature. Nonsense scrambled miRNA (miR-NS) was used as the negative control. miRNA:plasmid complexes (250 µl) were added in a drop-wise fashion to the cells. Cells were incubated at 37 °C overnight. After incubation, media was removed, and the transfected cells were lysed in 250 µl Luciferase Lysis Buffer (1X; Promega). Lysates were stored at -80 °C until further use. The cell lysates were analyzed with the Luciferase Assay System (Promega). 100 µl of Luciferase Assay Reagent (Promega) was added to 20 µl of lysate in opaque 96-well microplates (BD Falcon). A luminometer measured the light produced in relative light units (RLU). It is important to note that luciferase assays were performed in biological triplicates for all the miRNA and construct combinations. For the RLU readings, experimental triplicates were used.

miRNA Transfection

PBMCs from healthy individuals were transfected with individual miRNA mimics (miR-NS, miR-27b, miR-103a, miR-128, miR-141, miR-212, miR-500a, miR-628-3p, miR-708, let-7a, and let-7b; GE Healthcare Dharmacon) or combinations of miRNA mimics (miR-103/212/708; miR-141/500a/let-7b; miR-128/628-3p/let-7ab). Source leukocytes were obtained and kept at room temperature. PBMCs were isolated from the source

leukocytes within 24 hr of donation. Cells were plated, transfected, and cultured as described in Chapter 2.

Statistical Analysis

Statistical analysis for the miRNA profiling array data shown in Figure 3.4 and Table 1 was performed using parametric Limma test as previously described by our lab (Guerau-de-Arellano, Smith *et al*. 2011). The fold changes for Figure 3.2 were calculated using the comparative Ct method. Fold change calculations were normalized to the median Ct value of the healthy controls group. Statistical significance for Figure 3.2 and Figure 3.3 were calculated using a Mann-Whitney (nonparametric) t-test. Statistical significance ($p < 0.05$) for the luciferase assay data shown in Figure 3.5 and Figure 3.6 was performed using a two-tailed paired t-test comparing percent RLU of the miR-NS control and each miRNA group. RLU values were normalized to the protein concentration of the lysates and percent RLU was calculated. Percent RLU was calculated as follows: (average RLU_{miRNA}/ average $RLU_{control}$) x 100. Statistical significance ($p < 0.05$) for Figure 3.7 and Figure 3.8 was calculated with a Wilcoxon (nonparametric) matched pairs test. All statistical analyses were calculated using GraphPad Prism 4 Software (GraphPad Software).

Results

Differentially Expressed miRNAs in the Naïve CD4 T cells of MS Patients are Predicted to Target and Inhibit the TGFβ Signaling Pathway

In order to investigate differences in the CD4 T cells of MS patients and healthy individuals, we previously performed a miRNA profiling study on the naïve and memory CD4 T cells of individuals with MS and healthy donors (Guerau-de-Arellano, Smith *et al*. 2011). We identified 85 differentially expressed miRNAs in the naïve CD4+CD45RA+ T cells of MS patients compared to healthy controls. Pathway analysis identified that 19 of the 85 dysregulated miRNAs were predicted to target and inhibit the TGFβ signaling pathway (Figure 3.1). However, in order to narrow the scope of this study, my initial experiments were limited to investigating the upregulated miRNAs predicted to target TGFβR1, TGFβR2, SMAD2, and SMAD4. Additionally, we required that the miRNA meet an inclusion criterion of a fold-change (FC) greater than 5. The fold change values for the upregulated miRNAs that met the inclusion criteria are shown in Table 1. As a result of these findings, we hypothesized that the miRNAs differentially expressed in the naïve CD4 T cells of MS patients inhibit the TGFβ signaling pathway, resulting in decreased levels of their TGFβ-associated target genes.

TGFβR1 and SMAD4 Expression Levels are Reduced in the Naïve CD4 T cells of MS Patients

Given that miRNAs can cause decreased in mRNA and protein levels, I performed two separate experiments to investigate if TGFβ signaling is decreased in the naïve CD4 T

cells of MS patients. To determine if mRNA levels of the TGFβ-associated genes were reduced in MS patients, real-time PCR was performed on the RNA from the naïve $CD4^{+}CD45RA^{+}$ T cells of treatment naïve MS patients (n = 22) and healthy controls (HC; n = 7). The RNA utilized for this experiment was from the same samples used in the miRNA profiling study. This allowed us to analyze whether the set of MS patients identified to have dysregulated miRNAs that were predicted to target the TGFβ signaling pathway also had lower expression of TGFβ-associated genes (TGFβR1, TGFβR2, SMAD2, and SMAD4). Of the four predicted genes evaluated, TGFβR1 and SMAD4 were significantly decreased in MS patients compared to HC (Figure 3.2). TGFβR2 trended towards a decrease in MS patients, but was not statistically significant (Figure 3.2). Thus, TGFβR1 and SMAD4 were the focus of my study.

In order to determine if protein levels of TGFβR1 and SMAD4 were also decreased, we performed flow cytometry on the Naïve CD4+CD45RA+ T cells from a selection of the previously analyzed MS (n = 14) and HC (n = 4) samples. Given that almost 100% of naïve CD4 T cells endogenously express TGFβR1 and SMAD4, I analyzed the geometric MFI of each protein. This strategy allowed us to evaluate the amount of TGFβR1 and SMAD4 on a per cell level basis and thus, determine if TGFβ signaling was reduced in MS patients. TGFβR1 was not significantly decreased when comparing total MS patients compared to HC (Figure 3.3). However, when patients were grouped by their subtype of MS and compared to HC, TGFβR1 protein levels were significantly decreased in the

SPMS patient (Figure 3.3). The protein levels of SMAD4 were significantly decreased in total MS patients compared to HC (Figure 3.3).

Therefore, my graduate studies focused on the miRNAs predicted to target TGFβR1 and SMAD4. The expression levels, represented as fold change, for the dysregulated miRNAs predicted to target TGFβR1 and SMAD4 are shown in Figure 3.4. These data demonstrate that TGFβR1 and SMAD4 levels are significantly decreased in the same MS patients who have differentially expressed miRNAs predicted to target the TGFβ signaling pathway.

miRNAs Dysregulated in the Naïve CD4 T cells of MS Patients Directly Bind and Regulate TGFβR1 and SMAD4

In order to identify which miRNAs directly bind and potentially regulate their predicted target genes, TGFβR1 and SMAD4, luciferase assays were performed. Both of these genes contain very large 3'UTRs and therefore, it was necessary to generate multiple fragments for the assay. These segments of the 3'UTRs of TGFβR1 (Figure 3.5A) and SMAD4 (Figure 3.6A), which contain miRNA binding sites, were inserted into luciferase vectors and co-transfected into cos-7 cells with the appropriate individual miRNA. A reduction in luciferase activity would be indicative of miRNA regulation. miR-NS was used as a control to indicate the normal levels of luciferase activity. All of the miRNAs predicted to target TGFβR1 were shown to cause a significant reduction in the relative light units (RLU) when co-transfected with the appropriate TGFβR1 construct:miRNA

pairs (Figure 3.5B). All the miRNAs predicted to target SMAD4, except miR-18a, had a significant reduction in RLU (Figure 3.6B). These data indicate that the identified miRNAs, with the exception of miR-18a, can in fact bind their predicted target genes, suggesting that these miRNAs have the potential to modulate TGFβ signaling through their regulation of TGFβR1 and SMAD4. As a result of these findings, we hypothesized that overexpression of miR-27b, miR-103, miR-128, miR-141, miR-212, miR-500, miR-628-3p, miR-708, let-7a, let-7b, and let-7f in naïve CD4 T cells would result in decreased levels of TGFβR1 and SMAD4.

Overexpression of MS-Associated miRNAs Decrease TGFβR1 and SMAD4 Levels

Given that MS-associated miRNAs can directly bind and inhibit TGFβR1 and SMAD4, I wanted to further verify that these miRNAs could directly regulate translation of these genes in naïve CD4 T cells. To determine if the TGFβ-targeting miRNAs could decrease these genes in naïve CD4 T cells, miRNAs were overexpressed in healthy donor PBMCs. PBMCs from healthy individuals, not MS patients since they already have altered miRNA levels, were transfected with single miRNAs or miRNAs in combination. Subsequently, flow cytometry was used to analyze the TGFβR1 and SMAD4 levels in the naïve $CD4^{+}CD45RA^{+}$ T cells. TGFβR1 and SMAD4 are constitutively expressed at high levels in approximately 100% of T cells. Therefore, to detect changes in protein levels on a per cell basis, I measured the mean fluorescence intensity (MFI) of these proteins.

Figure 3.7A illustrates the changes in TGFβR1 levels following miRNA transfection for one individual PBMC sample. The quantification for this representative sample is shown in red in Figure 3.7B, as well as the changes for each miRNA in 15 PBMC samples. For TGFβR1, 6 of the 7 miRNAs (miR-27b, miR-141, miR-500a, let-7a, let-7b, and let-7f) significantly decreased the MFI (Figure 3.7B). Since we typically observed multiple dysregulated miRNAs in MS patients, I wanted to determine if having multiple miRNAs overexpressed augments changes in TGFβR1 levels. In the last panel of Figure 3.7B, I demonstrate that a combination of miR-141, miR-500a and let-7b results in a more significant decrease in TGFβR1 levels.

Similarly, Figure 3.8A shows the effect of miRNA overexpression on SMAD4 levels for a single PBMC sample. This sample is represented in red in Figure 3.8B, as well as data for 14 additional samples. For SMAD4, 5 of the 8 miRNAs (miR-141, miR-212, miR-500a, miR-628-3p, and miR-708) significantly decreased the MFI of SMAD4 (Figure 3.8B). In the last panel of Figure 3.8B, I show that the overexpression of miR-103, miR-212, and miR-708 in combination can have a synergistic effect, resulting in enhanced suppression of SMAD4. Overall, the changes in MFI demonstrated in Figures 3.7 and 3.8 indicate that elevated levels of TGFβ-targeting miRNAs, especially in combination, can reduce the TGFβ signaling capacity of naïve CD4 T cells. Given that TGFβ signaling is required for the development of Tregs, the elevated expression of these miRNAs in MS patients' naïve CD4 T cells suggests that these miRNAs play a role in the Treg defect

observed in MS patients. Therefore, we hypothesized that overexpression of these miRNAs in naïve CD4 T cells would suppress their capacity to differentiate into iTregs.

Discussion

When taken together, our findings demonstrate that TGFβ signaling genes are diminished in MS patients and suggest that the identified differentially expressed miRNAs may be responsible for the observed inhibition of TGFβ signaling genes. It is important to note that while we investigated 19 individual miRNAs predicted to target 4 genes of the TGFβ signaling pathway, there were other miRNAs among the 85 identified that were predicted to target this pathway. However, given the extensive list of differentially expressed miRNAs and target genes, we had to determine exclusion criteria throughout my work. In order to limit our initial miRNA list, we chose to only include statistically significant miRNAs that had a fold change greater than 5 when comparing MS patients and healthy controls. The miRNAs also had to be differentially expressed specifically in RRMS, since RRMS is the most common form of MS. Therefore, there are other miRNAs predicted to target the TGFβ signaling pathway that may contribute to the reduced levels of TGFβR1 and SMAD4.

Additionally, other than the investigated target genes (TGFβR1, TGFβR2, SMAD2, and SMAD4), SMAD7 was also predicted to be targeted. For my graduate project, we decided to focus on the miRNAs that were upregulated, allowing for a more simplified and cohesive working hypothesis. If the miRNAs were upregulated, their targets were

predicted to be downregulated, thus resulting in diminished TGFβ signaling. Thus, we decided to not investigate the downregulated miRNAs predicted to target SMAD7. However, this does not mean that effect of miRNAs on SMAD7 expression should not be addressed, but this will be the focus of future studies. TGFβR2 may also be of interest in future studies. While the reduction in this gene was not statistically significant, its levels trended towards a decrease in MS patients. When looking at the data, it is evident that the range of TGFβR2 levels is very diverse within the sampled MS population. The lack of significance could merely be a result of insufficient sample numbers to account for this dispersal of TGFβR2 levels in the tested individuals.

The most significant decrease of TGFβR1 and SMAD4 in the naïve CD4 T cells of MS patients was observed at the mRNA level. Several factors may contribute to the statistical differences in mRNA and protein levels. Due to the limited quantities of cells available from the individuals used in the initial miRNA profiling study, I was only able to include 4 HC samples. Increasing HC numbers would allow for a more accurate representation of the healthy population and could potentially magnify the difference observed. Additionally, lack of flow antibodies able to detect TGFβR1 on the surface of cells confined me to using a suboptimal alternative. The TGFβR1 antibody used was an unconjugated polyclonal antibody against an epitope within the C-terminal cytoplasmic domain of TGFβR1, which poses a couple concerns. Given that the antibody is polyclonal, it has disadvantages when applied to flow cytometry, such as excessive background fluorescence due to non-specific binding and inaccurate quantification due to

the binding of multiple epitopes on the same protein. Additionally, levels of TGFβR1 measured with this intracellular antibody may not be representative of the amount of active TGFβR1 available on the surface of the cells to bind TGFβ and promote signaling. Alternative methods for future studies could include isolation of protein from the naïve CD4 T cells of MS patients for the detection of extracellular TGFβR1 epitopes via western blotting. However, similar issues of non-specific binding and misrepresentation of active TGFβR1 levels may occur my primary method for determining if TGFβ signaling genes are decreased in MS patients, real-time PCR, is highly sensitive and indicate that both TGFβR1 and SMAD4 are reduced in MS patients compared to healthy individuals. Despite possible protein detection sensitivity issues, the protein levels do appear to support my conclusions drawn from the mRNA data. These findings provided justification for focusing my studies on miRNAs specifically predicted to target TGFβR1 and SMAD4.

My further studies with these genes shown in Figures 3.5 -3.8 demonstrate that most of the miRNAs predicted to target the TGFβ signaling pathway are capable of directly binding and inhibiting TGFβR1 and SMAD4 expression. This was first demonstrated in the luciferase assays. The extensive size of the 3'UTRs of these genes made generating the luciferase constructs very complex. The initial challenge was dividing the 3'UTR in way that retained all the necessary miRNA binding sites, while keeping the number of segments minimal and their length optimal. It took many attempts to design primers that were efficient in exclusively amplifying the desired gene segments. Additionally, having

used a single enzyme for the digestions, the inserts could either bind to the plasmid in the 3' to 5' or 5' to 3' direction. Gel electrophoresis only allowed us to identify whether the insert was the correct size, not if the insert was in the correct orientation. Therefore, I had to perform secondary verifications using DNA sequencing.

Additionally, as a result of complications with my cos-7 cell cultures, I switched to an alternative cell line. I chose RAW 264.7 macrophages because they are hearty adherent cells, which were previously transfected in the lab with success. Additionally, there were reports documenting their successful application in Promega luciferase assays. However, I was continually observing very low, almost undetectable, RLU values. Despite several attempts to optimize culture conditions according to published protocols, the assay failed to work. Upon additional thought and research, I discovered the key detail that I was missing. The pGL3 vector contains Simian Virus 40 (SV40) promoter and enhancer regions. Engagement of these regions with SV40 T antigen promotes vector replication, which can result in enhanced light detection. Given that RAW cells are not infected with SV40 there is not SV40 T antigen available in these cells. Interestingly, SV40 is commonly found in monkeys, the origin of cos-7 cells. Therefore, repeating the luciferase assays in cos-7 cells containing SV40 T antigen resulting in the production of detectable RLU levels, and useable data. Successful competition of these assays allowed me to determine which miRNAs did not regulate their predicted target genes, and thus allowed me to exclude miR-18a from future experiments, further focusing my work.

Overall the luciferase assays were essential for confirming the miRNA and target gene interactions and promoting further investigation. However, the assay is very artificial in terms of having to use cell lines versus primary cells. Additionally, the effect of miRNAs on protein levels of TGFβR1 and SMAD4 is not directly established. Therefore, a more biologically relevant experiment was necessary, thus driving the miRNA transfection experiments in Figures 3.7 and 3.8. Given the challenges caused by human variation and the control provided in murine models, I initially performed the same miRNA transfection experiments in wild-type B10.Pl mice. However, unlike later studies with human PBMCs, I did not consistently observe changes in the TGFβ signaling genes when the various miRNAs were overexpressed. While doing these experiments, I was subsequently doing the miRNA mapping for the luciferase assays. I discovered that while the miRNAs themselves are highly conserved among mice and humans, their binding sites are not. This was an extremely important fact to take into account when planning several of my other experiments, and drove me to perform most of my experiments in human PBMCs.

Having to use PBMCs proved beneficial in that it was more biologically relevant, given that initial observations were made in the PBMCs of MS patients and healthy donors. For the miRNA transfection studies, we elected to use healthy donor PBMCs rather than MS patient PBMCs. Our rationale was that MS patients typically already have elevated levels of multiple miRNAs, making dissecting the effect of an individual miRNA difficult. Therefore, by overexpressing miRNAs in healthy PBMCs, we were able elucidate the

effect of individual miRNAs and mirror specific patterns that we observe in MS patients in a controlled setting.

Additionally, there were several technical benefits to my PBMC transfection experiments. By using flow cytometry, I was able to look at the effect of overexpressing individual miRNAs directly on TGFβR1 and SMAD4 expression levels. Importantly, I was able to assess the levels of these proteins specifically in naïve CD4 T cells on a per cell level basis, allowing me to observe changes in our primary cell of interest. Additionally, we chose miRNAs that are double-stranded RNAs designed to mimic endogenous, mature miRNAs. By being double-stranded, they require the cells own machinery to process it and load it into the RISC complex. In order to allow time for the miRNA machinery to work, I waited 48 hr after transfection before analyzing the cells. I also chose a 48 hr time point because SMAD4 is a very long-lived endogenous proteins, with a half-life over 12 hr. All of the described advantages to this method provided us with a biologically relevant model to use in future studies. Therefore, our next objective was to use this model to address our hypothesis of whether the overexpression of the TGFβ targeting miRNA has an effect on the development of iTregs.

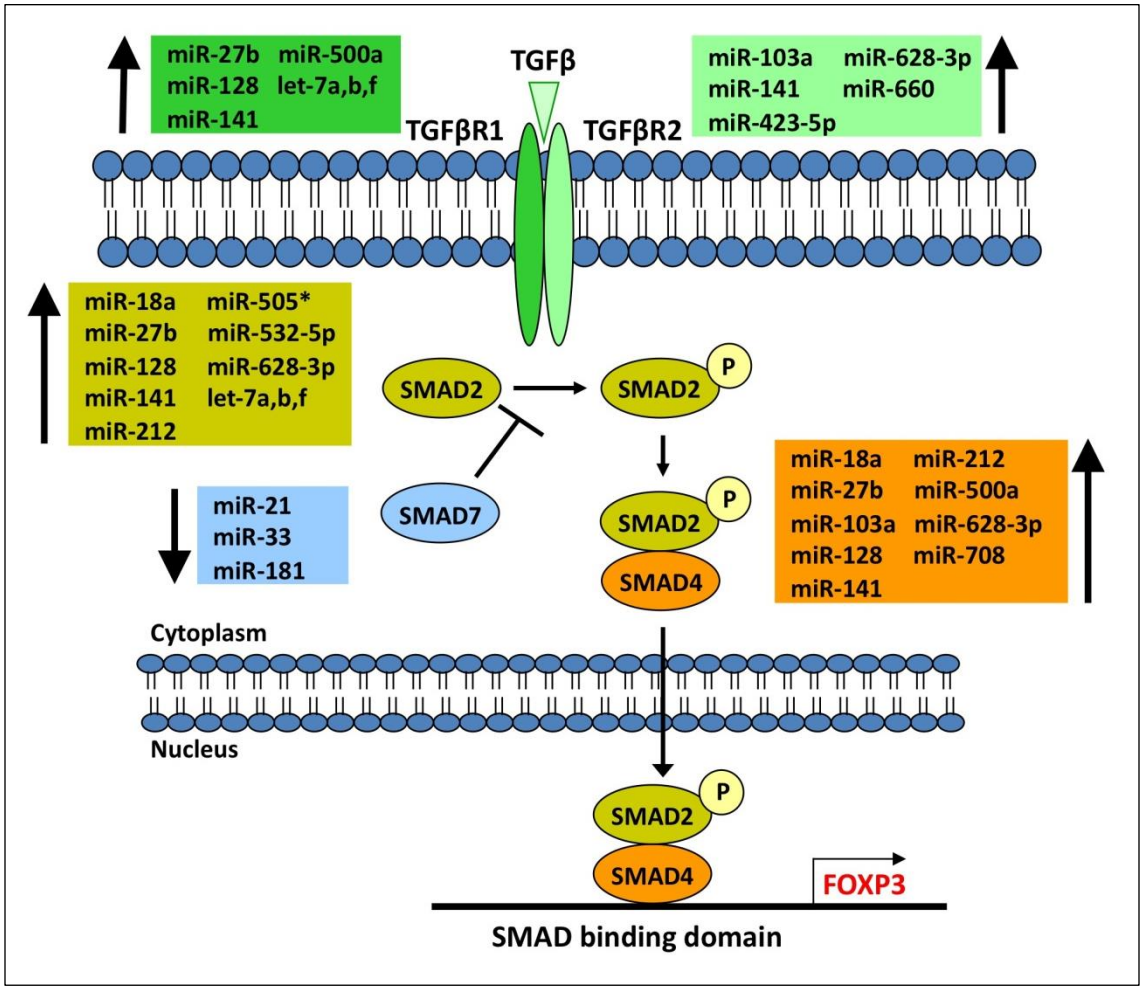


Figure 3.1 Differentially expressed miRNAs in the naïve CD4 T cells of MS patients are predicted to target the TGFβ signaling pathway.

A TaqMan® miRNA profiling array was performed on the naïve CD4+CD45RA+ T cells of healthy controls (HC; n = 16) and MS patients (MS; n = 22). Of the 85 differentially expressed miRNAs, 19 miRNA with a fold change >5 were predicted to target the TGFβ signaling pathway. The miRNAs are color matched with their predicted targets (TGFβR1, green; TGFβR2, light green; SMAD2, yellow; SMAD4, orange; and SMAD7, light blue).

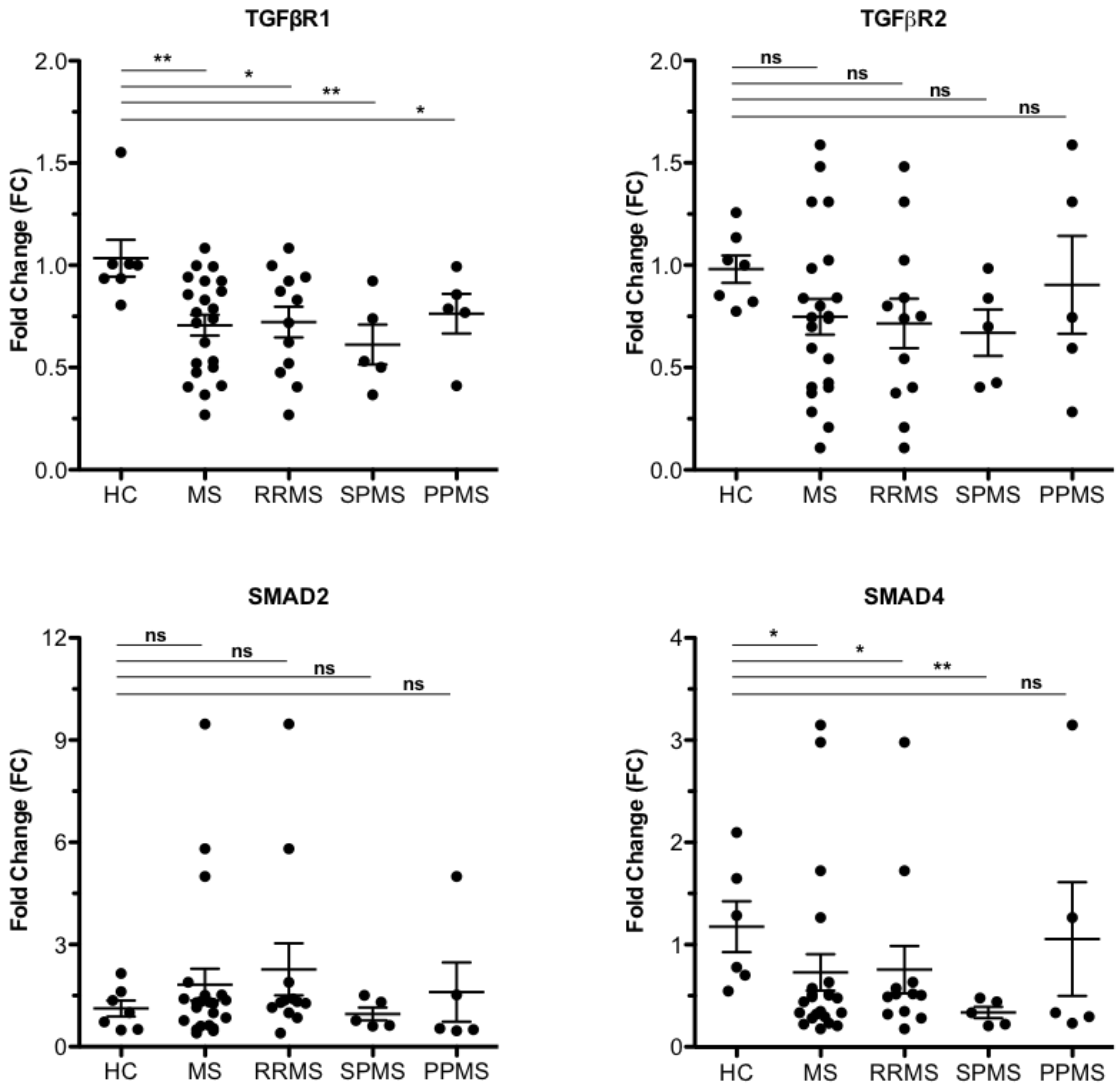


Figure 3.2 TGFβR1 and SMAD4 mRNA is significantly decreased in the naïve CD4 T cells of MS patients.

TaqMan® real-time PCR performed on the naïve CD4+CD45RA+ T cells of MS patients (RRMS, n = 12; SPMS, n = 5; and PPMS, n = 5) and healthy controls (HC; n = 7). Fold Change (FC) was calculated and normalized relative to median Ct of the HC group. Statistical significance was calculated with a Mann-Whitney (nonparametric) t-test comparing HC to each MS group (*, $p \leq 0.05$; **, $p \leq 0.01$; and ns, $p > 0.05$). Mean FC for each sample group is represented by a line in each column. TGFβR1 and SMAD4 were found to be significantly decreased ($p < 0.05$) in MS patients.

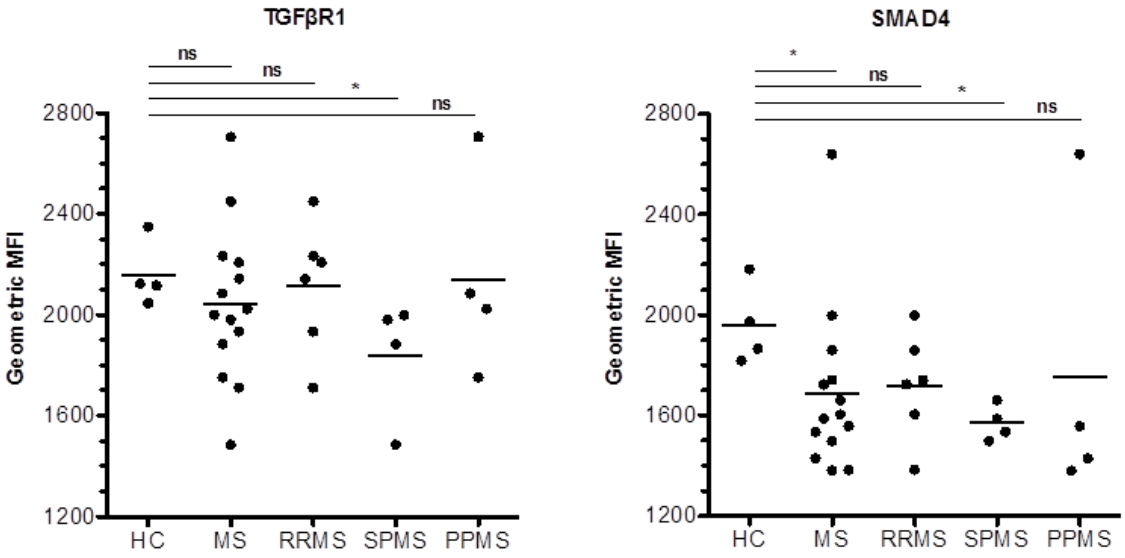


Figure 3.3 TGFβR1 and SMAD4 proteins are decreased in the naïve CD4 T cells from select MS patient groups.

Flow cytometry was performed on the naïve CD4+CD45RA+ T cells of MS patients (RRMS, n = 6; SPMS, n = 4; and PPMS, n = 4) and healthy controls (HC; n = 4). Geometric MFI was measured and statistical significance was calculated with a Mann-Whitney (nonparametric) t-test comparing HC to each MS group (*, $p \leq 0.05$ and ns, $p > 0.05$). Mean geometric MFI for each sample group is represented by a line in each column. TGFβR1 and SMAD4 were found to be significantly decreased ($p < 0.05$) in SPMS patients.

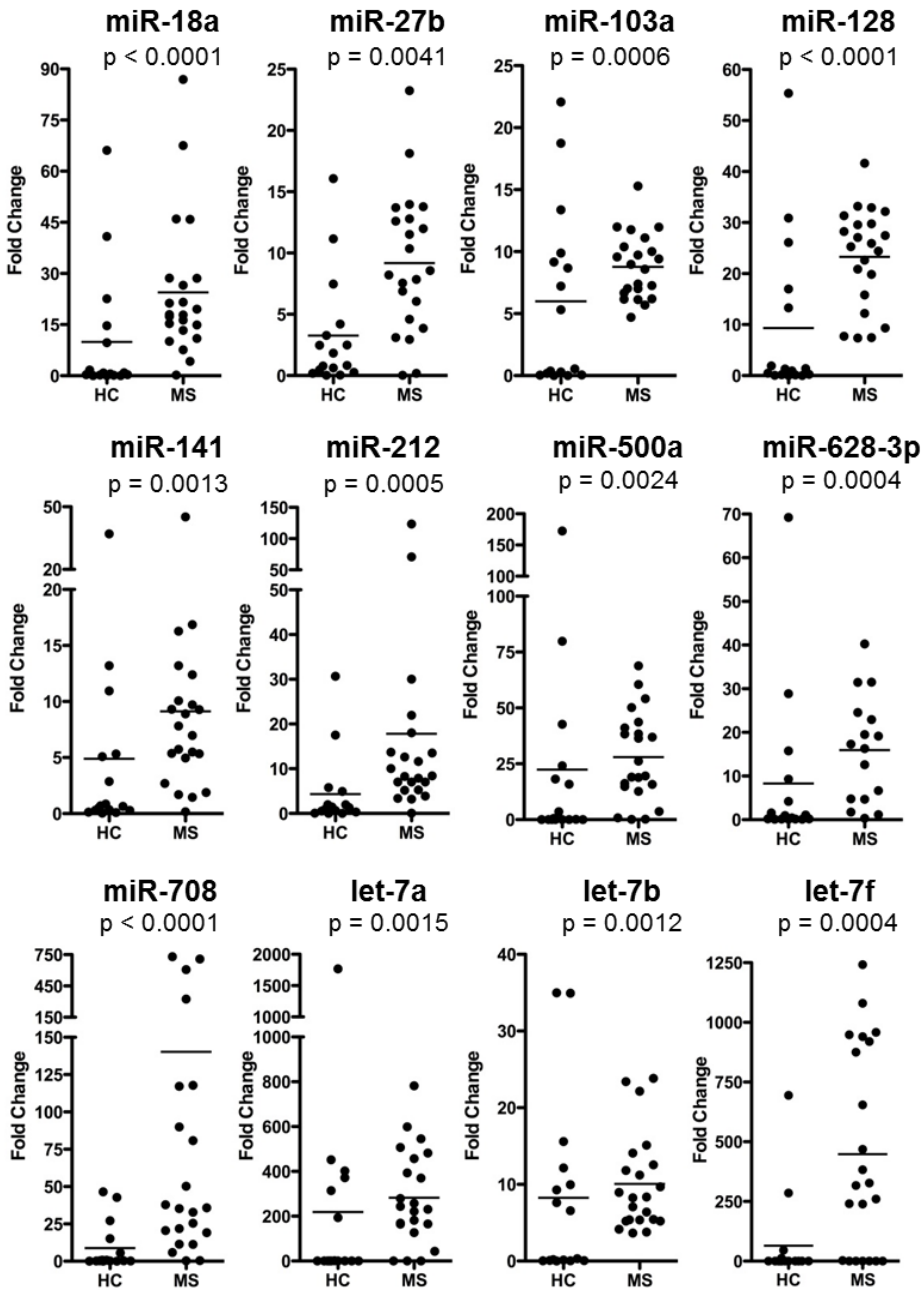


Figure 3.4 TGFβR1 and SMAD4 targeting miRNAs are upregulated in the naïve CD4 T cells of MS patients.

For the miRNAs predicted to target TGFβR1 and SMAD4, fold change (FC) was calculated and normalized relative to the geometric mean Ct of the HC group. Mean FC for each sample group is represented by a line. Statistical significance ($p < 0.05$) was calculated with a parametric Limma test. P values for the comparisons of the HC (n = 16) and MS (n = 22) groups are shown.

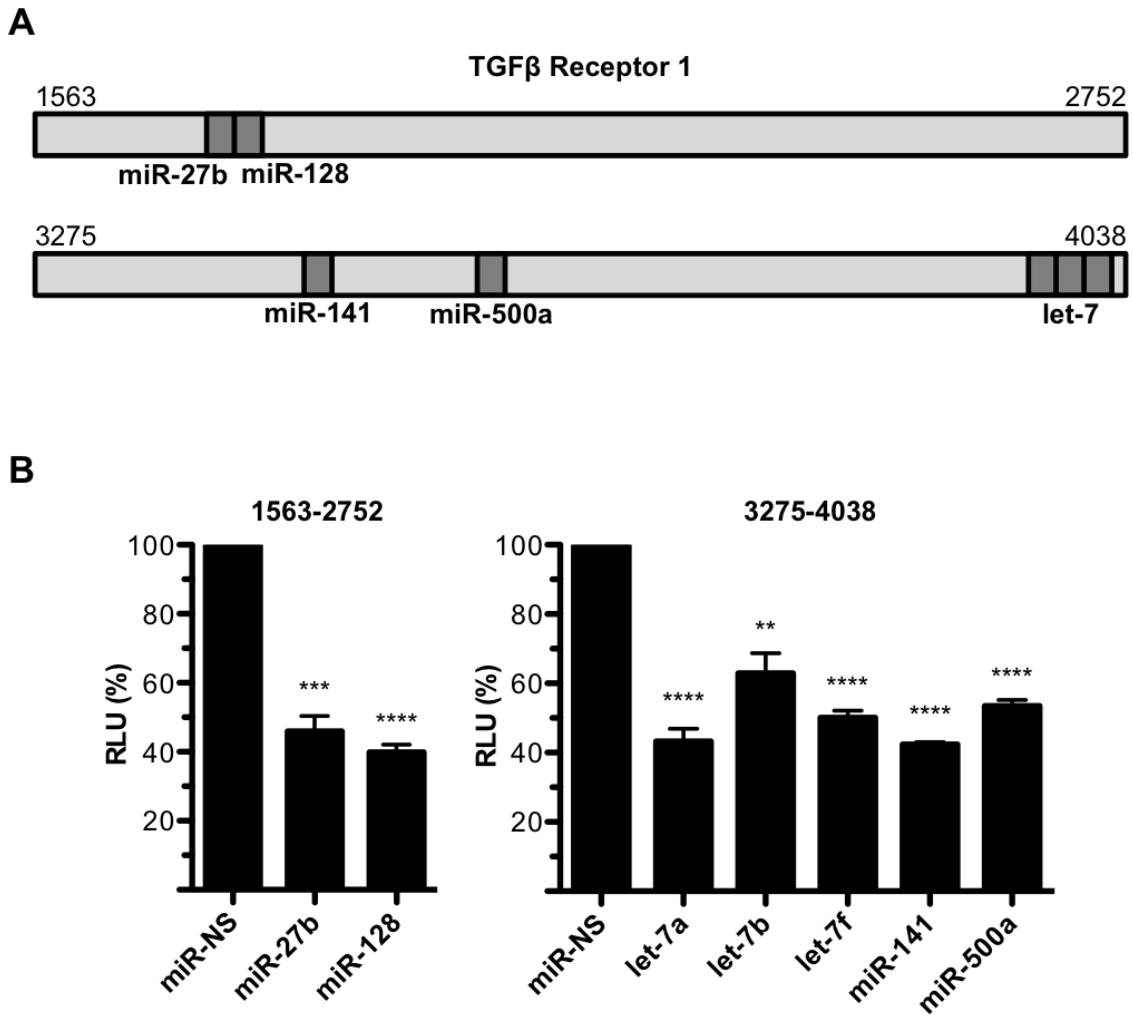


Figure 3.5 Differentially expressed miRNAs directly bind and regulate TGFβR1.

Luciferase assays were conducted using pGL3 vector constructs containing segments of the 3'UTR of TGFβR1. (A) The 3'UTR of TGFβR1 was amplified as two separate segments. The miRNA binding sites within each segment are noted. (B) Significance was calculated using an unpaired t-test comparing the relative luciferase units (RLU) of the miR-NS control and individual miRNA groups (**, $p \leq 0.01$; ***, $p \leq 0.001$; and **** $p \leq 0.0001$). All of the miRNAs predicted to target TGFβR1 caused a reduction in RLU.

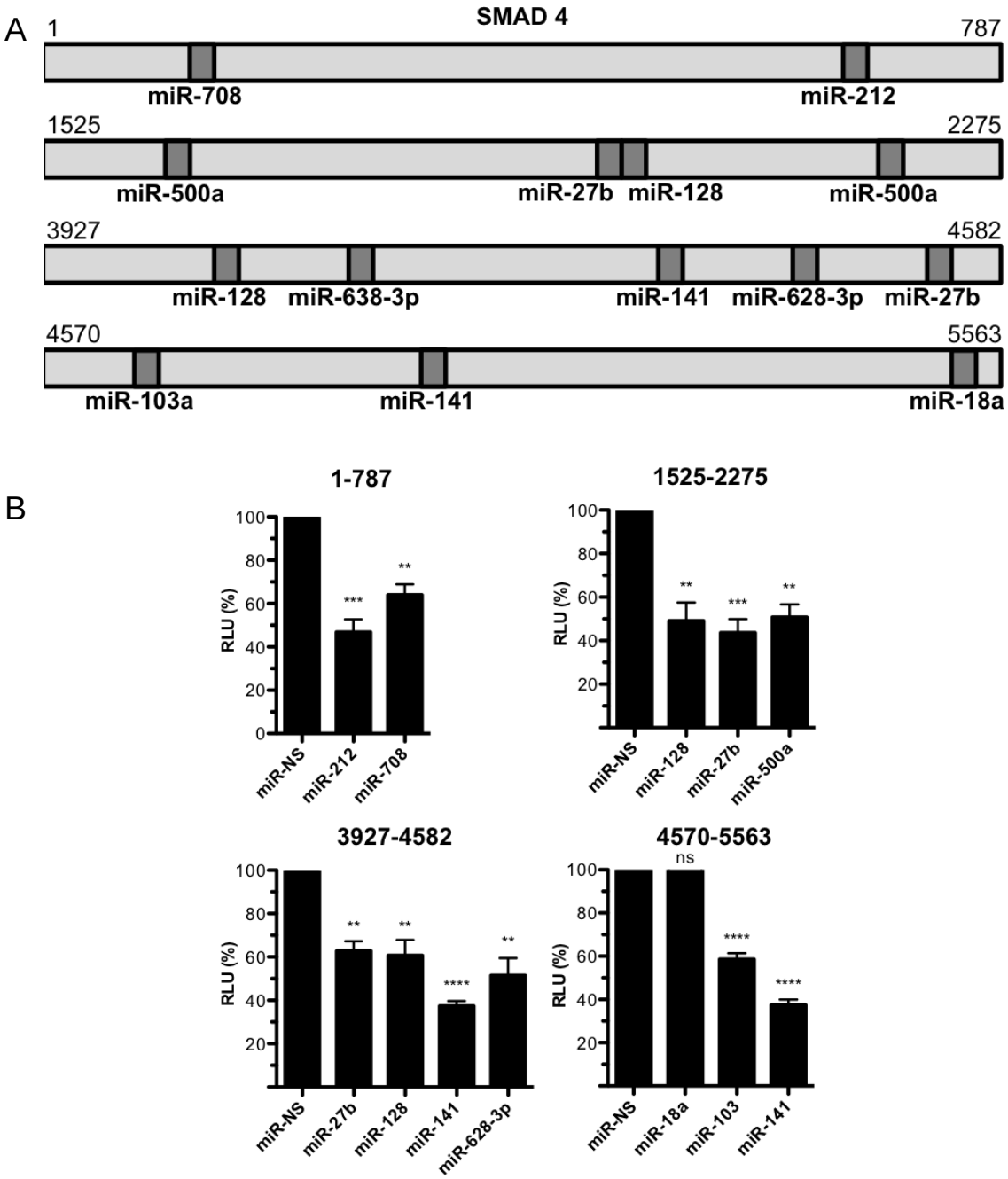


Figure 3.6 Dysregulated miRNAs directly bind and regulate SMAD4.

Luciferase assays were conducted using vector constructs containing 3'UTR segments of SMAD4. (A) The 3'UTR of SMAD4 was divided into four separate segments. The miRNA binding sites within each segment are noted. (B) Significance was calculated using an unpaired t-test comparing the RLU of the miR-NS control and each miRNA groups (**, $p \leq 0.01$; ***, $p \leq 0.001$; and **** $p \leq 0.0001$). All of the miRNAs tested, except miR-18, SMAD4 reduce RLU.

Figure 3.7 Overexpression of TGFβ-targeting miRNAs reduces TGFβR1 expression.

Individual or combinations of miRNAs predicted to target TGFβR1 were transfected into PMBCs from healthy controls. Flow cytometry was used to analyze changes in TGFβR1 levels. (A) Flow plots representative of data from a single individual. (B) Each line represents a unique PBMC sample (n = 15) with the red line representing the data in panel A. The geometric mean florescence intensity (MFI) was measured and compared between miR-NS and individual or combined miRNA groups. A Wilcoxon matched pairs test calculated significance ($p < 0.05$).

Figure 3.7

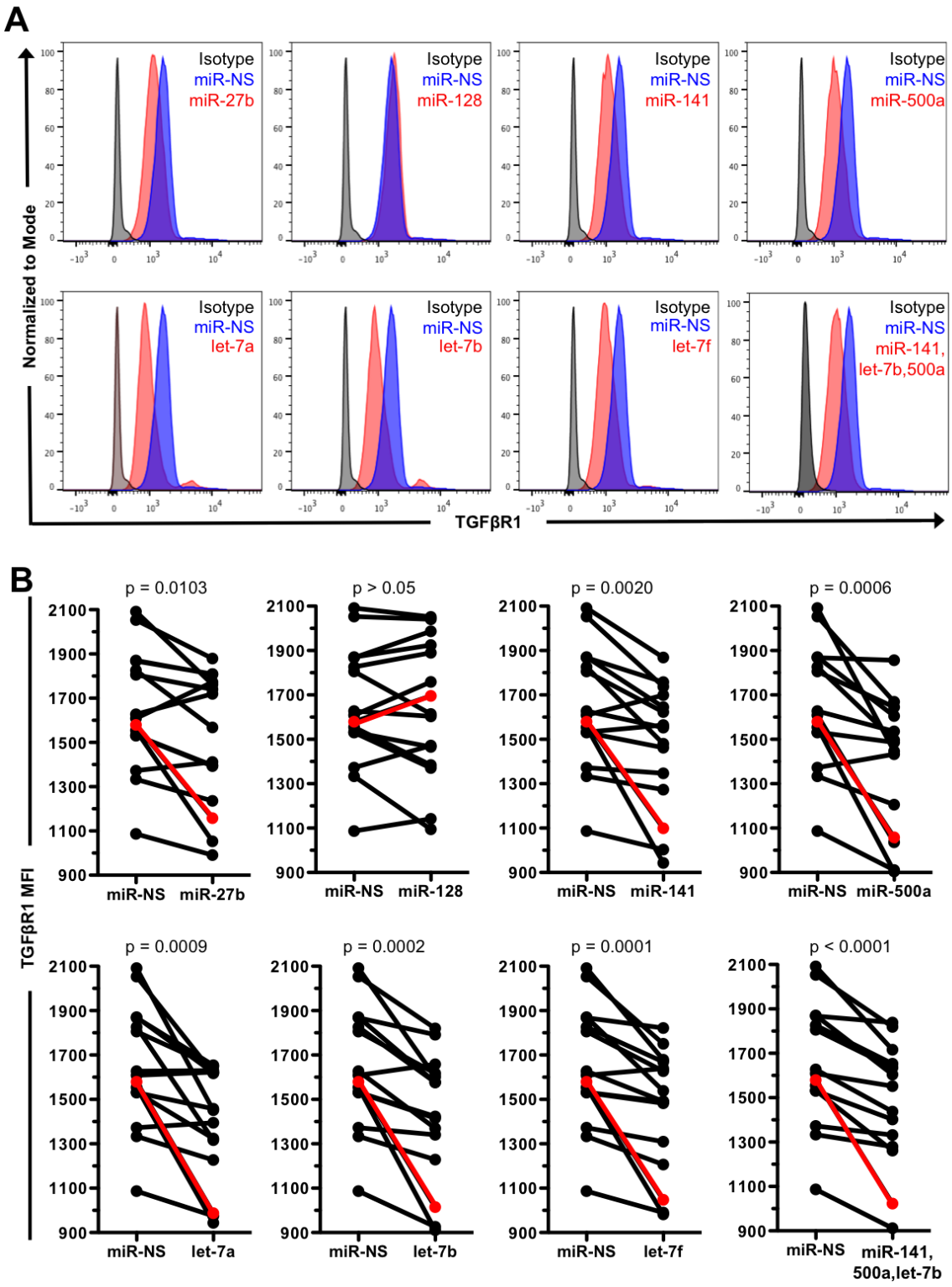

A
Isotype
miR-NS
miR-27b
miR-128
miR-141
miR-500a
let-7a
let-7b
let-7f
miR-141,
let-7b,500a
Normalized to Mode
TGFβR1
B
TGFβR1 MFI
p = 0.0103
p > 0.05
p = 0.0020
p = 0.0006
p = 0.0009
p = 0.0002
p = 0.0001
p < 0.0001
miR-NS
miR-27b
miR-128
miR-141
miR-500a
let-7a
let-7b
let-7f
miR-141,
500a,let-7b

Figure 3.8 Overexpression of TGFβ-targeting miRNAs reduces SMAD4 expression.

Individual or combinations of miRNAs predicted to target SMAD4 were transfected into PMBCs from healthy controls. Flow cytometry was used to analyze changes in SMAD4 levels. (A) Flow plots representative of data from a single individual. (B) Each line represents a unique PBMC sample (n = 15) with the red line representing the data in panel A. The geometric mean florescence intensity (MFI) was measured and compared between miR-NS and individual or combined miRNA groups. A Wilcoxon matched pairs test calculated significance ($p < 0.05$).

Figure 3.8

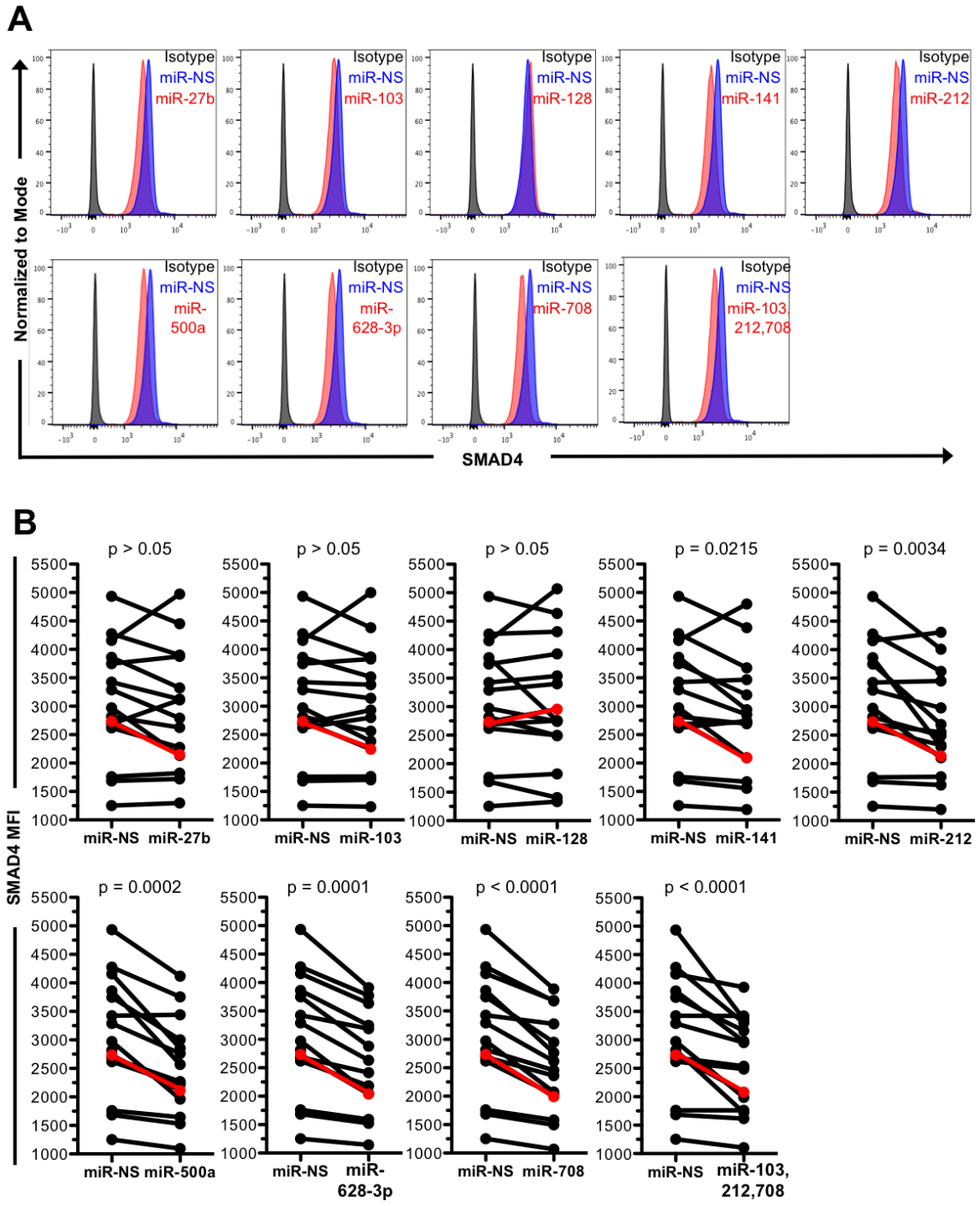

A
Isotype
miR-NS
miR-27b
miR-103
miR-128
miR-141
miR-212
miR-500a
miR-628-3p
miR-708
miR-103, 212,708
Normalized to Mode
SMAD4
B
SMAD4 MFI
p > 0.05
p = 0.0215
p = 0.0034
p = 0.0002
p = 0.0001
p < 0.0001
miR-NS
miR-27b
miR-103
miR-128
miR-141
miR-212
miR-500a
miR-628-3p
miR-708
miR-103, 212,708

Table 1. Select miRNAs upregulated in the naïve CD4 T cells of MS patients.

miRNA	Fold Change	P value
hsa-miR-18a	16.35	5.45E-05
hsa-miR-27b	5.66	4.09E-03
hsa-miR-103a	8.41	6.24E-04
hsa-mIR-128	20.76	8.07E-06
hsa-miR-141	5.86	1.27E-03
hsa-miR-212	8.52	5.20E-04
hsa-miR-423-5p	15.96	3.19E-03
hsa-miR-500	14.26	2.40E-03
hsa-miR-505*	10.58	2.13E-03
hsa-miR-532-5p	14.75	2.64E-04
hsa-miR-660	191.66	3.41E-06
hsa-miR-let-7a	88.38	1.45E-03
hsa-miR-let-7b	8.52	1.23E-03
hsa-miR-let-7f	60.78	4.11E-04

Chapter 4: Overexpression of TGFβ-Targeting miRNAs Decrease Treg Development

Introduction

One of the mechanisms hypothesized to propagate MS pathology is an inflammatory attack driven by activated myelin-specific T cells that are targeting the CNS. Interestingly, these autoreactive T cells are found in healthy individuals as well (Sun, Olsson *et al*. 1991, Lovett-Racke, Trotter *et al*. 1998, Pelfrey, Rudick *et al*. 2000, Crawford, Yan *et al*. 2004), suggesting that MS patients lack the normal regulatory mechanisms needed to suppress such immune responses. One of the main elements of regulation that is absent in MS patients is a fully functional Treg population. The Tregs of MS patients have diminished suppressive effect on the autoimmune response against myelin-specific T cells (Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Kumar, Putzki *et al*. 2006) and have low expression of the transcription factor FoxP3 (Huan, Culbertson *et al*. 2005, Venken, Hellings *et al*. 2008), which is required for the development and function of Tregs (Fontenot, Gavin *et al*. 2003, Hori, Nomura *et al*. 2003, Khattri, Cox *et al*. 2003, Fontenot, Rasmussen *et al*. 2005).

TGFβ has also been shown to be critical for maintaining the differentiation and suppressive capacity of Tregs, through its role in promoting FoxP3 transcription (Chen, Jin *et al*. 2003). Interestingly, TGFβ1 and TGFβ receptor deficient mice develop a multi-

organ autoimmune disease similar to humans with mutated FoxP3 genes (Shull, Ormsby *et al*. 1992, Kulkarni and Karlsson 1993, Gorelik and Flavell 2000, Bennett, Christie *et al*. 2001, Brunkow, Jeffery *et al*. 2001, Wildin, Ramsdell *et al*. 2001). These findings not only suggest a relationship between TGFβ and FoxP3, but also provide a link between FoxP3/TGFβ deficiencies and autoimmunity. The uncontrolled autoimmunity in these mice has been attributed to a defect in their Tregs. These mice exhibit a Treg phenotype with normal numbers, diminished suppressive effect, a limited TCR repertoire, and low FoxP3 expression, mirroring observations in MS patients. While these observations suggest a connection between inhibition of TGFβ signaling and Treg defects, the mechanisms by which Tregs become defective in MS have not yet been determined. Understanding the relationship between T cell driven autoimmune responses and the dysregulation of Tregs in MS patients can provide valuable understanding of the cause and course of this disease.

As demonstrated in Chapter 3, several of the miRNAs identified as dysregulated in the naïve CD4 T cells of MS patients are capable of suppressing TGFβ signaling. Given the importance of TGFβ signaling in Treg development and the Treg defect observed in MS patients, the logical next step was to investigate the ability of these TGFβ-targeting miRNAs to impede Treg development. Therefore, the *objective* of aim 2 was to identify which TGFβ-targeting miRNAs alter the development of iTregs *in vivo*. *The working hypothesis was that over expression of miRNAs that target specific genes known to promote TGFβ-pathway signaling would cause a decrease in signaling and attenuate the*

development of iTregs. The *rationale* for the work described in Chapter 4 was that the identification of specific miRNAs capable of inhibiting Treg induction would potentially enhance understanding of the mechanisms underlying the Treg defect seen in MS patients.

Materials and Methods

miRNA Transfection

PBMCs were obtained from the American Red Cross and isolated as described in Chapter 2. PBMCs from healthy individuals, as defined by the Red Cross, were transfected with individual miRNA mimics (miR-NS, miR-27b, miR-103a, miR-128, miR-141, miR-212, miR-500a, miR-628-3p, miR-708, let-7a, and let-7b; GE Healthcare Dharmacon) or miRNA mimics in combination (miR-103/212/708; miR-141/500a/let-7b; miR-128/628-3p/let-7ab). miR-NS was included as a negative control for each individual PBMC sample transfected. Source leukocytes were obtained and kept at room temperature. PBMCs were isolated from the source leukocytes within 24 hr of donation. Cells were plated, transfected, and cultured as described in Chapter 2.

Treg Induction

After the 48 hr transfection, cells were washed, counted, and cultured according to the *In Vitro Treg Induction* protocol in Chapter 2. Suboptimal iTreg culture conditions were used to avoid saturating the naïve CD4 T cells and masking differences in Treg induction.

Flow Cytometry

After the 48 hr incubation, the miRNA transfected PBMCs were analyzed for Treg induction via flow cytometry as described in Chapter 2. In order to look at differentiation of Tregs from naïve CD4 T cells, the PBMCs were stained with the surface antibodies human anti-CD4-PB (BD Biosciences), human anti-CD45RA-APC (BioLegend), and human anti-CD25-FITC (BD Biosciences). The cells were also stained with the intracellular antibody human anti-FoxP3-PE (eBioscience) and the primary antibody anti-human TGFβR1 V 1-22 (Santa Cruz). When analyzed, the cells were first subgated on naïve CD4 T cells (CD4+CD45RA+). The Tregs were defined as the CD4+CD45RA+CD25^{hi}FoxP3+ cells.

IL-10 Enzyme-Linked Immunosorbent Assay (ELISA)

Supernatants were collected from the iTreg cultures and analyzed for IL-10. ELISA was performed as follows: purified rat anti-human IL-10 capture antibody (BD Biosciences) was diluted to 2 µg/ml in $NaHCO_3$ (0.1M; pH 8.2); Immulon II 96-well plates were coated with 50 µl of diluted antibody and incubated at 4 °C overnight; plates were washed twice with 200 µl PBS/Tween 20; 200 µl 1% BSA/PBS was added to each well and incubated at room temperature for 2 hr; plates were washed twice with 200 µl PBS/Tween 20; 100 µl diluted recombinant human IL-10 standards (2 ng/ml to 0 ng/ml) was added to triplicate wells; 100 µl supernatant was added to duplicate wells; plates were incubated at 4 °C overnight; plates were washed four times with PBS/Tween 20; biotinylated anti-IL-10 antibody (BD Biosciences) was diluted to 1 µg/ml in 1% BSA/PBS; 100 ul of diluted detection antibody was added to each well; plate was

incubated for 60 min at room temperature; plate was washed six times with PBS/Tween 20; avidin-peroxidase was diluted to 2.5 µg/ml in 1% BSA/PBS; 100 µl diluted avidin-peroxidase was added to each well; plate was incubated for 30 min at room temperature; plate was washed eight times with PBS/Tween 20; 100 µl of H_2O_2/ABTS substrate was added to each well; plate developed for approximately 10 min at room temperature in the dark; plates were read at OD 405 nm on a microplate reader. IL-10 concentrations were calculated from known standards of recombinant IL-10 protein (R&D Systems) and analyzed via SoftMax Pro Software (Molecular Devices).

Statistical Analysis

Statistical significance (p value < 0.05) for Figures 4.1 and 4.2 was calculated with a Wilcoxon (nonparametric) matched pairs test. All statistical analyses were calculated using GraphPad Prism 4 Software (GraphPad Software). For Figure 4.3, a non-parametric Pearson correlation test analyzed the degree of relatedness between the levels of IL-10 and percent of iTregs generated.

Results

Overexpression of Individual TGFβ-Associated miRNAs Decrease Treg Development

Given the dysregulation of TGFβ-targeting miRNAs in MS patients and the importance of TGFβ in Treg development, we wanted to determine if overexpression of these TGFβ-associated miRNAs could decrease the differentiation of naïve $CD4^+CD45RA^+$ T cells into iTregs. PBMCs from healthy individuals were transfected with individual miRNAs

(miR-NS, miR-27b, miR-103, miR-128, miR-141, miR-500a, miR-628-3p, miR-708, let-7a, let-7b, and let-7f). Following a 48 hr incubation, flow cytometry was used to analyze the percentage of naïve CD4 T cells (CD4+CD45RA+) that were able to differentiate into iTregs ($CD25^{hi}$FoxP3+). Figure 4.1A illustrates the percentage of iTregs generated from one PBMC sample when the miRNAs are overexpressed individually. Analysis of a panel of PBMCs (n = 14-19) found that miR-27b, miR-103, miR-128, miR-628-3p, and miR-708 significantly decreased iTreg development (Figure 4.1B, the sample shown in Figure 4.1A is indicated by red line).

Overexpression of TGFβ-Associated miRNAs in Combination Enhance Suppression of Treg Development

Similar to MS patients, overexpression of a single TGFβ-targeting miRNA was not unusual in the healthy control population, as seen in Figure 3.4. However, unlike healthy individuals, we observed that MS patients typically had multiple TGFβ-targeting miRNAs overexpressed. Therefore, I tested combinations of miRNAs found to be overexpressed in the MS patients' naïve CD4 T cells to determine if there may be a synergistic effect of the TGFβ-targeting miRNAs. I transfected three different miRNA combinations (miR-141/500/let-7b; miR-103/212/708; or miR-128/628-3p/let-7ab) observed in MS patients into PBMCs of healthy controls (HC) and evaluated for iTreg development. Figure 4.2A shows representative flow plots from a single PBMC sample that demonstrates an approximately 50% decrease in the percent of Tregs induced when miRNAs are overexpressed in combination. Analysis of a panel of PBMCs (n = 14-19)

found that all three combinations demonstrated highly significant reductions in iTregs (Figure 4.2B, the sample shown in Figure 4.2A is indicated by red line). In fact, miR-141/500/let-7b, which had no significant effect individually on iTreg development, showed a significant decrease in combination, indicative of the synergistic effect. Similarly, the percent decrease in the other two miRNA combinations were more significant than the individual miRNAs, suggesting that the elevated expression of multiple TGFβ-targeting miRNAs in naïve CD4 T cells of MS patients diminishes their capacity to generate iTregs.

IL-10 Production is not Inhibited in iTregs Generated from miRNA Transfected Naïve CD4 T Cells

One of the ways in which Tregs function is by secreting anti-inflammatory cytokines which can act on other immune cells to dampen pro-inflammatory responses. To begin to address the functional status of the Tregs generated from naïve T cells overexpressing these miRNAs, IL-10 levels in the supernatants of the miRNA transfected iTregs were analyzed. Figures 4.3A-B demonstrates data from a single PBMC sample transfected with TGFβ-targeting miRNAs. When PBMCs were transfected with TGFβ-targeting miRNAs and cultured in iTreg conditions, overall Treg numbers were decreased (Figure 4.3A), as were levels of secreted IL-10 (Figure 4.3B). However, I saw a positive correlation between the numbers of iTregs and levels of IL-10, indicating that the Tregs that did develop were able to produce normal levels of IL-10 (Figure 4.3C). These data

suggest that these miRNAs limit Treg development, but not their ability to produce the anti-inflammatory cytokine IL-10.

Discussion

When taken together, my findings demonstrate that the overexpression of TGFβ-targeting miRNAs can directly decrease the number of iTregs (CD4+CD25^{hi}Foxp3+ cells) generated. While I have shown that IL-10 production does not appear to be altered in these cells, I think further investigation into the ability of these cells to secrete other anti-inflammatory cytokines and suppress effector T cell proliferation is necessary. Importantly, my work is the first to link the enhanced negative regulation of the TGFβ-signaling pathway by specific miRNAs to a reduction in Treg development, potentially explaining the Treg defect observed in MS patients.

While straightforward in theory, the transfection experiments I performed proved to be challenging for several reasons. When I was initially designing these experiments, I chose Treg inducing conditions that were commonly described in the literature and proven to successfully generate iTregs. This protocol consisted of culturing PBMCs on anti-CD3/CD28 coated plates in the presence of IL-2 (100 U/ml), tRA (2.5 nM), and TGFβ1 (5 ng/ml). With these conditions, I was able to generate high percentages of iTregs. However, the differences in Treg induction between the control and miRNA groups were very modest. Typically, we would take this to mean that miRNAs were not as effective as hypothesized in inhibiting Treg development. However, I occasionally had PBMC

samples that demonstrated a significant decrease in the ability to generate iTregs when TGFβ-targeting miRNAs were overexpressed. Around the time that I was finishing up my twentieth PBMC sample with only marginal success, I attended a talk that demonstrated that high levels of anti-CD3/CD28 can mask differences in Treg development. After this talk, I thought about the possibility that I could be overstimulating my cells, allowing the cells to overcome any defects caused by the miRNAs. However, no difference was observed when I reduced the concentrations of anti-CD3/CD28 used to coat the plates. Fortunately, I attended another seminar by a guest lecturer who discussed the extreme sensitivity of Tregs to IL-2, demonstrating that Tregs can respond to levels of IL-2 100-fold lower than I was using. Interestingly, previous reports have demonstrated that reductions in Treg numbers are observed in TGFβ deficient mice post-natal, but that defect is eventually masked by Treg expansion via peripheral IL-2. I postulated that a similar effect was happening to my Tregs *in vivo* due to excessive amounts of IL-2 present in the culture. With these findings in mind, I performed several titration experiments with both IL-2 and TGFβ and eventually developed my current Treg induction protocol using only 1 U/ml IL-2 and 0.5 ng/ml TGFβ. Using these suboptimal conditions allowed me to observe differences between my control and miRNA conditions.

In addition to the culture conditions, there was another flaw in my initial set of experiments. When I was calculating the percent of iTregs generated, I was gating on CD4+CD25^{hi}FoxP3+ cells. This was problematic for a couple of reasons. In humans,

CD25 and FoxP3 are commonly accepted as Treg markers, yet they are not exclusive to Tregs. Both markers are transiently expressed during normal T cell activation. Therefore, it was possible that I was detecting T cells in this transient stage of activation that were not Tregs. Additionally, by gating on total CD4+ cells, I was not solely addressing whether overexpression of TGFβ-targeting miRNAs decreased differentiation of Tregs from naïve CD4 T cells. However, inadvertantly I noticed that CD45RA, an established marker for naïve/resting T lymphocytes, is not immediately down regulated when cells are stimulated. Rather, I was able to detect CD45RA on naïve CD4 T cells 72 hr after activation via flow cytometry, thus allowing me to detect the percentage of naïve CD4+CD45RA+ T cells that differentiated into iTregs. By switching to the suboptimal Treg protocol and the naïve T cell method of gating, I was able to observe significant differences in Treg development when select miRNAs were overexpressed.

Additionally, as with any experiment using human samples, there was a lot of sample and experimental variation. This made pooling data extremely difficult. In order to ensure that the differences observed were not a consequence of this variation, I included a miR-NS control for every PBMC sample transfected, and used a matched pairs test to analyze the data. Additionally, I had to perform this experiment on multiple different PBMC samples to ensure accurate representation of the effect of miRNAs on Treg development in the population as a whole. High sample number was also necessary to observe differences between groups, since some individuals were non-responsive to increased miRNA levels. Some individuals responded opposite to what we would have expected. As evident in

Figure 4.1B, some samples made more iTregs when transfected with the various miRNAs. It is important to note that it was the same two individuals that demonstrated the strongest reverse responses to each miRNA. It is possible that there is an off target effect of the miRNAs that enhance Treg development in those individuals.

Additionally, it is important to highlight the advantages our model has in terms of its use in mimicking miRNA profiles observed in MS patients. As mentioned in Chapter 3, by transfecting healthy PBMCs rather than MS patient PBMCs, we were able to look at the effects of individual miRNAs in a controlled setting. When I transfected the healthy PBMCs with specific TGFβ-targeting miRNAs, I observed a modest decrease in Treg induction. Interestingly, upregulation of a single miRNA is not exclusively observed in MS patients. As shown in Chapter 3, occasionally we would observe an increased level of TGFβ-targeting miRNAs in healthy individuals as well. While, outside the scope of our study, I think it would be interesting if a follow-up could have been done to investigate if any of the healthy donors from the initial profiling study demonstrating elevated miRNA levels eventually developed MS.

While my data indicates that it is possible for an individual miRNA to have an effect on Treg development, the effect of miRNA dysregulation is much more robust when they are expressed in combination. This is important since most MS patients, unlike healthy individuals, demonstrate increased levels of multiple TGFβ-targeting miRNAs. Interestingly, miR-141, miR-500, and let-7b individually did not have significant effects

on Treg development. However, when I overexpressed these miRNAs in combination, as observed in MS patients, there was a significant reduction in iTregs. These findings suggest that there may be specific combinations of miRNAs, or a certain level of miRNA dysregulation that enhances the probability of developing a defect in Tregs, and thus potentially makes an individual more susceptible to developing MS.

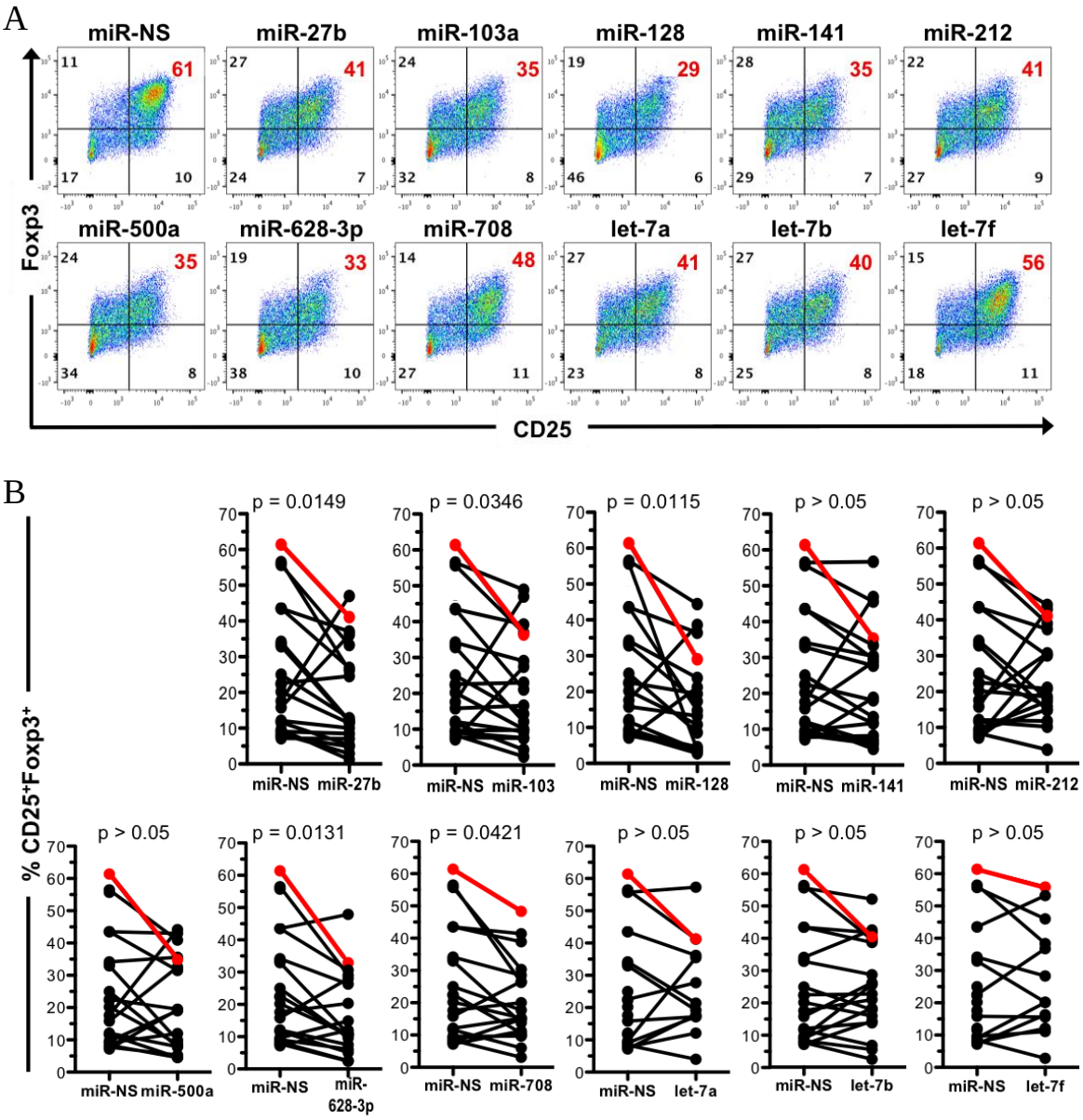


Figure 4.1 Overexpression of TGFβ-targeting miRNAs decreases Treg induction.

PBMCs from healthy controls were transfected with individual miRNA (0.05 μM) and cultured in iTreg inducing conditions. The cells were analyzed using flow cytometry and iTregs (CD4+CD45RA+CD25^{hi}Foxp3+) generated from naïve CD4 T cells were quantified. (A) Flow plots are representative data from a single individual. iTreg numbers are in red. (B) Each line represents a unique PBMC sample (n = 14-19) with the red line representing the data in panel A. A Wilcoxon matched pairs test comparing miR-NS and individual miRNA was used to calculate significance ($p < 0.05$).

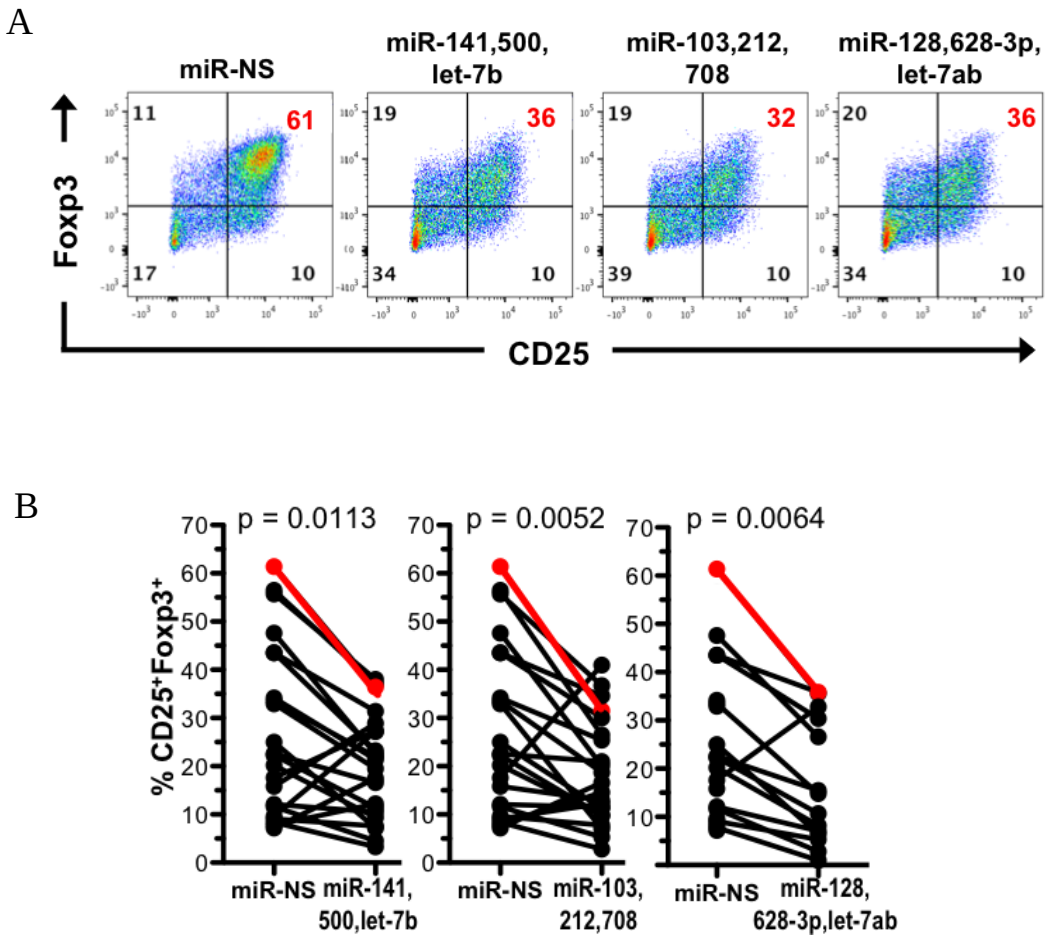


Figure 4.2 Overexpression of TGFβ-targeting miRNAs in combination exacerbate suppression of Treg induction.

PBMCs from a healthy donor were transfected with miRNAs in combination (0.05 μM total) and cultured in iTreg inducing conditions. The cells were analyzed using flow cytometry and iTregs (CD4+CD45RA+CD25^{hi}Foxp3+) generated from naïve CD4 T cells were quantified. (A) Flow plots are representative data from the same individual shown in Figure 4.1A. iTreg numbers are in red (B) Each line represents a unique PBMC sample (n = 14-19) with the red line representing the data in panel A. A Wilcoxon matched pairs test comparing miR-NS and individual miRNA was used to calculate significance ($p < 0.05$).

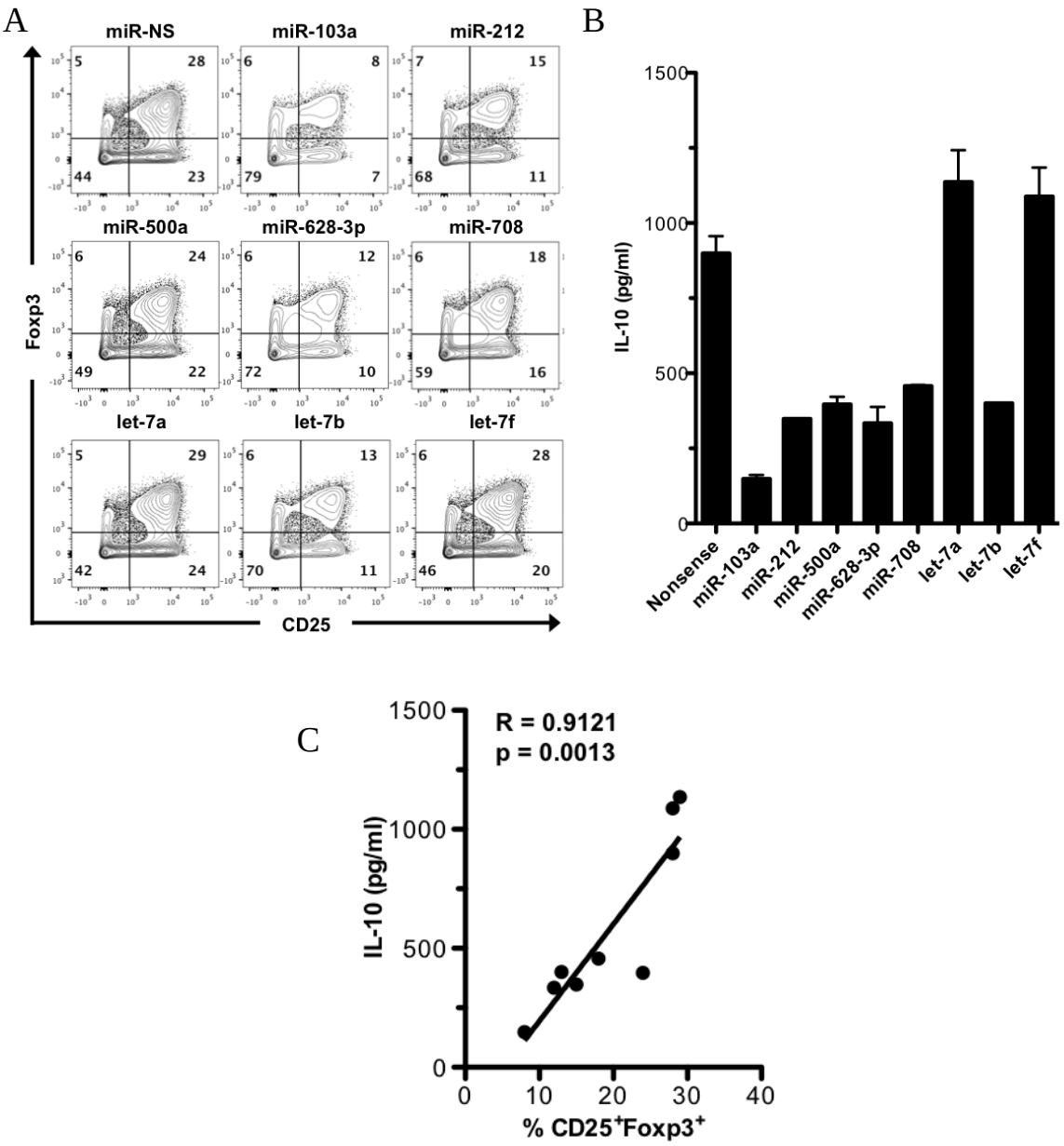


Figure 4.3 IL-10 production is not inhibited in iTregs generated from miRNA transfected Naïve CD4 T cells.

PBMCs from a healthy control (HC) were transfected with 0.05 μM of miRNA and cultured in iTreg inducing conditions. (A) iTregs (CD4+CD45RA+CD25^{hi}Foxp3+) were analyzed using flow cytometry. (B) IL-10 was measured in the supernatants of the iTreg cultures demonstrated in panel A using ELISA. (C) A non-parametric Pearson correlation test analyzed the degree of relatedness between the levels of IL-10 and percent of iTregs generated. Representative of 4 independent experiments.

Chapter 5: Altered Expression of TGFβ-Targeting miRNAs is Associated with Reduced iTregs and Enhanced Susceptibility to CNS Autoimmunity

Introduction

Despite numerous genetic and epidemiological studies, few factors have been identified and confirmed as being linked to the cause of MS. While the cause is unknown, it is evident that MS patients appear to lack sufficient immune mechanisms capable of suppressing CNS inflammation. Interestingly, adult mice deficient in TGFβ signaling develop uncontrolled multi-focal autoimmunity, also indicative of a loss in immune regulation. Several in depth investigations have demonstrated that both MS patients and TGFβ-deficient mice exhibit a Treg phenotype with normal numbers, diminished suppressive effect, a limited TCR repertoire, and low FoxP3 expression (Vandenbark, Finn *et al*. 2001, Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Kumar, Putzki *et al*. 2006). Under normal conditions, the TGFβ-signaling pathway promotes the expression of the critical transcription factor, FoxP3, thereby promoting the development and function of Tregs (Fontenot, Gavin *et al*. 2003, Hori, Nomura *et al*. 2003, Khattri, Cox *et al*. 2003, Fontenot, Rasmussen *et al*. 2005). Taken together, these studies suggest a potential link between the Treg defect observed in MS patients and TGFβ signaling deficiencies. However, no direct connection has been made between defects in TGFβ-signaling and Treg development in MS patients.

As demonstrated in Chapters 3 and 4, several of the miRNAs identified as dysregulated in the naïve CD4 T cells of MS patients have the ability to inhibit TGFβ signaling and Treg development, especially when overexpressed in combination. Given that the initial observation of TGFβ-targeting miRNA dysregulation was made in MS patients, the logical next step was to look at Treg development and disease susceptibility *in vivo*. Therefore, the ***objective*** of aim 3 was to elucidate the effect of miRNA dysregulation on Treg generation and disease susceptibility in the context of CNS autoimmunity. The ***working hypotheses*** were as follows: 1) the naïve CD4 T cells of MS patients have a reduced capacity to differentiate into Tregs and, 2) overexpression of TGFβ-targeting miRNAs *in vivo* would increase disease susceptibility via a reduced capacity to generate Tregs. The ***rationale*** for the work described in Chapter 5 was as follows: 1) the demonstration that MS patients have a defect in Treg development would verify the *in vitro* findings in the context of CNS disease, and 2) the identification of specific TGFβ-targeting miRNAs that decrease Treg development and promote disease would potentially link miRNA dysregulation to enhanced disease susceptibility.

Materials and Methods

Human Subjects

The MS patient (n = 10) and healthy control (n = 7) PBMCs in Figure 4.1 were obtained at the OSU MS Clinic. The samples were collected sequentially on two days from individuals who agreed to participate in the study following proper informed consent

protocol. There was no significant difference in age or gender between the groups. All of the MS patients were of the relapsing-remitting subtype. Of the MS patients, 5 were untreated (UMS) and 5 were currently on treatment (TMS). For the MS patients on treatment, 3 received Copaxone, 1 received Tysabri and 1 received Tecfidera. The healthy controls were a mix of individuals unrelated to MS patients (UHC) and first-degree relatives of MS patients (1°HC).

In Vitro Treg Induction

PBMCs were isolated as described in Chapter 2, counted, and plated at a density of 1 x 10^6 cells/ml on 48-well plates coated with 1 µg/ml of human anti-CD3/CD28. The PBMCs were cultured according to the *In Vitro Human Treg Induction* protocol in Chapter 2.

Flow Cytometry: Human iTregs

After the 72 hr incubation, the PBMCs were analyzed for Treg induction via flow cytometry as described in Chapter 2. In order to look at differentiation of Tregs from naïve CD4 T cells, the PBMCs were stained with the surface antibodies human anti-CD4-PB (BD Biosciences), human anti-CD45RA-APC (BioLegend), and human anti-CD25-FITC (BD Biosciences). The cells were also stained with the intracellular antibody human anti-FOXP3-PE (eBioscience). When analyzed, the cells were first subgated on naïve CD4 T cells (CD4+CD45RA+). The Tregs were defined as the CD4+CD45RA+$CD25^{hi}$Foxp3+ cells.

Mice

B10.PL mice were bred in specific pathogen-free conditions at the OSU University Laboratory Animal Resources facility.

In Vivo miRNA Injection

Neonatal mice received an intraperitoneal (i.p.) injection via the inguinal route within 24 hr of birth. Mice with injected with 5 μg of each miRNA mimic in combination (miR-103/212/708, miR-141/500a/let-7b, or miR-128/628-3p/let-7ab). miR-NS injected mice were used as the control group.

Flow Cytometry: Murine Tregs

Cells were isolated from the spleen of mice 96 hr after i.p. injection with miRNAs. The resulting thymocytes and splenocytes were analyzed *ex vivo* for Tregs as described in Chapter 2. The cells were stained with the surface antibodies mouse anti-CD4-PcP (BioLegend) and mouse anti-CD25-PE (BD Biosciences). The cells were also stained with the intracellular antibody mouse anti-FoxP3-APC (eBioscience). When analyzed, the cells were first subgated on CD4+ T cells. The Tregs were defined as the CD4+ $CD25^{hi}$FoxP3+ cells.

Experimental Autoimmune Encephalomyelitis (EAE)

Neonatal mice were injected i.p. 24 hr after birth with 5 µg of each miRNA in combination (miR-103/212/708 or miR-141/500a/let-7b). The same mice received a second i.p. injection at 3 weeks of age with 10 µg of each miRNA (miR-103/212/708 or miR-141/500a/let-7b). Mice injected with miR-NS at 24 hr and 3 weeks were used as the control group. At 7 weeks, each mouse was immunized with 50 µl of MBPAc1-11 peptide (4 mg/ml) emulsified in 50 µl of CFA supplemented with 100 µg of *Mycobacterium tuberculosis*. Mice were injected i.p. with 100 ng of pertussis toxin at the time of immunization and 48 hr later. Mice were monitored for clinical signs and assessed according to the six-point EAE scoring scale (0-6): 0, no clinical disease; 1, limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or moribund state; 6, death due to EAE.

Statistical Analysis

Statistical significance (p value < 0.05) for Figure 4.1 was calculated with an unpaired t-test comparing the HC and MS patient groups. For Figure 4.3, statistical significance (p value < 0.05) was calculated using a Mann-Whitney test.

Results

MS Patients and their First-Degree Relatives Demonstrate a Similar Reduced Capacity to Generate Tregs Ex Vivo

In Chapter 4, I demonstrated that TGFβR1 and SMAD4-targeting miRNAs are capable of causing a reduction in Treg development. Given that our initial findings of dysregulated TGFβ-targeting miRNA were made in MS patients, we wanted to verify that naïve CD4 T cells of MS patients have a diminished capacity to differentiate into iTregs. Although previous studies had demonstrated that Tregs taken directly *ex vivo* from MS patients had a decreased suppressive capacity, it had not been previously demonstrated that MS patients had a defect in the ability to generate iTregs. Naïve CD4+CD45RA+ T cells from MS patients and healthy controls (HC) were stimulated directly *ex vivo* with TGFβ, IL-2 and tRA on plate coated with anti-CD3/28 plates and analyzed for their capacity to become iTregs ($CD25^{hi}$FoxP3+) via flow cytometry (Figure 5.1). Figure 5.1A depicts representative flow plots from a treated MS patient (TMS), an untreated MS patient (UMS), their first-degree relative (1°HC), and an unrelated healthy control (UHC). The initial comparison of the total healthy controls to the MS patients was not significant (Figure 5.1B). However, it appeared that the control population was bimodal in that there were high and low iTreg percentages in the HC cohort. Therefore, we reanalyzed the data by separating UHC and controls that were 1^{o}HC of MS patients. We observed a significant difference in the percentage of iTregs generated from the MS patients compared to the UHC, but not the 1^{o}HC (Figure 4.1B). In addition, comparison of the UMS and TMS patients indicated that treatment did not enhance or normalize iTreg

development. These data suggest that MS patients have a reduced capacity to generate iTregs from naïve CD4 T cells that may be genetically linked to their first-degree relatives who demonstrate a similar level of iTreg differentiation. These findings support and drive further investigation of our working hypothesis that there are genetic components, potentially miRNAs, which are dysregulated in MS patients and contribute to their defect in Tregs.

Overexpression of TGFβ-Targeting miRNAs Enhance EAE Susceptibility and Severity

Previous studies have shown that when TGFβ signaling is defective, autoimmunity can occur due to impaired Treg development (Shull, Ormsby *et al*. 1992, Kulkarni and Karlsson 1993, Gorelik and Flavell 2000). Given the data from these studies and our *in vitro* findings that MS patients have dysregulated miRNAs that can decrease TGFβ signaling and Treg development, we hypothesized that these miRNAs can result in enhanced disease susceptibility and severity when overexpressed. To test this hypothesis, I injected healthy neonatal mice with combinations of miRNAs (miR-141/500/let-7b and miR-103/212/708) within 24 hr of their birth, a critical window of time for Treg development. A second injection of the same miRNA combinations was administered at 3 weeks of age to sustain the miRNA effects during development. The control group of mice was injected with miR-NS at both 24 hr and 3 weeks after birth. At 7 weeks, EAE was induced in these mice using a suboptimal immunization regimen so that the control mice would develop minimal EAE, allowing us to observe differences between the miR-NS and miRNA combination groups. When injected with either combination of miRNAs,

mice had a significantly earlier onset and increased severity of disease (Figure 5.2). To determine if the enhanced EAE susceptibility could potentially be a result of defective Treg development, we looked at the Tregs from mice injected with the miRNA combinations. Splenocytes were isolated and analyzed for Tregs ($CD4+CD25^{hi}FoxP3+$) via flow cytometry. Figure 5.3 shows representative flow data of one mouse from each miRNA group. This panel demonstrates that miRNA overexpression has the ability to cause decreased Treg development *in vivo*. However, due to variations in Treg frequency between litters, we have had limited success in showing statistical significance. However, we believe that the data as a whole suggest that, when overexpressed, the identified miRNAs can impair the TGFβ signaling pathway, dampen Treg development, and enhance susceptibility to CNS autoimmunity.

Discussion

Taken together, my findings demonstrate that dysregulation of TGFβ-targeting miRNAs can result in enhanced disease susceptibility in the context of CNS autoimmunity. Furthermore, as shown by the data in Figure 5.1, first-degree relatives of MS patients appear to have a similar decrease in their capacity to generate iTregs, suggesting a genetic component to the observed defect in Treg development. However, given the low sample number, additional studies are needed to verify these findings. It would be of interest to explore whether the Treg defect observed in the individuals from Figure 5.1 was due to a dysregulation of TGFβ-targeting miRNAs.

My initial approach to addressing whether MS patients have a reduced capacity to generate iTregs involved using the same PBMCs as used in the miRNA profiling study. This would have potentially allowed me to directly correlate miRNA dysregulation with a reduction in Treg differentiation. However, when performing flow analysis on these cells I found the samples to have very low percentages of naïve CD4 T cells. I believe that the lack of healthy naïve CD4 T cells available to be differentiated into Tregs was a possible consequence of the freezing process used to store the cells. Therefore, in an effort to overcome this issue, we began using fresh PBMCs obtained from the OSU MS Clinic. The pitfall with utilizing new samples was that we lacked miRNA profiles for these individuals. Additionally, the volume of blood collected varied greatly between individuals, limiting the analyses that could be performed. However, given that these samples were obtained from patients visiting the clinic, we had an increased likelihood that family members would accompany MS patients. It is important to note that we have obtained additional clinic samples other than those recorded in Figure 5.1. However, when analyzing the data, I noticed a significant increase in overall Treg generation after using a new aliquot of IL-2. This was an important finding in terms of designing my future experiments. While this prevented us from being able to pool all our data, we were still able to calculate significance for the first group of samples obtained.

Despite showing that first-degree relatives have a similar defect in their capacity to generate Tregs, we do not know why these individuals remain healthy. One possibility is that the first-degree relatives have MS, but are currently undiagnosed. The other and most

likely scenario is that the defects in Treg development observed in the first-degree healthy controls predisposes them to developing MS, but they have not been exposed to the necessary environmental triggers needed to initiate disease. While outside the scope of my study, investigating the miRNA profiles and Treg induction of MS patients and their first-degree relatives could provide valuable information regarding miRNAs as genetic risk factors for MS.

The next step to investigate miRNAs *in vivo* was the use of the animal model for MS, EAE. However, several challenges emerged when I was designing the experiment. Since the miRNA binding sites are not completely conserved between mice and humans, I had to be selective when choosing which miRNAs to use. I chose miRNAs that had binding sites in murine *SMAD4* and *TGFBR1* genes. The miRNA combinations administered to mice were specifically chosen based on their *in vitro* success and presence in MS patients. Delivery of the miRNAs into mice was not without its own challenges.

The time period surrounding birth is a critical window in the development of T cells. Therefore, we wanted to administer the miRNAs to mice as early in development as possible. Initially, we proposed to do tail vein injections on pregnant mothers with the hopes of delivering miRNAs to pups *in utero*. However, this form of delivery was stressful on mothers late in their pregnancies, and thus an alternative method was needed. To overcome this issue, we chose to deliver miRNAs to pups within 24 hr of their birth via i.p. injection. Again, this was also not without its complications. Given that neonatal

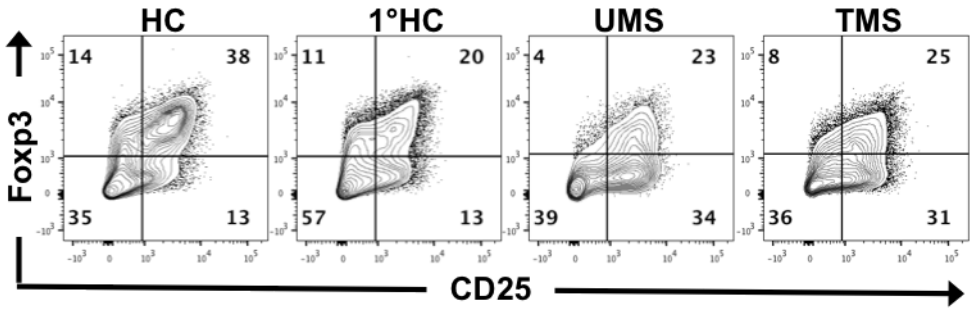
pups have very low fluid capacity in their peritoneal cavities, I observed severe injection site leakage. In order to prevent leakage, I tried, unsuccessfully, resting the pups on ice in order to limit their movement. After several attempts to prevent leakage, I was trained on how to deliver i.p injections through an inguinal route. This provided a longer injection route and less chance for immediate leakage, allowing the miRNA time to be absorbed. While this method has significantly improved injection efficiency, I still occasionally observe volume loss. This potentially could result in lower miRNA concentrations in those mice and diversity in the data.

I also observed data diversity when looking at Tregs in mice 4 days after miRNA injection. After comparing several experiments, I noticed that there is a large amount of diversity in Treg frequency between different litters. The logical solution was to randomize litters. However, due to the fact that I was using neonatal mice, rejection of the pups by the mothers was a concern. In attempt to prevent this issue, we injected mice from the same litter with different miRNA combinations and marked their tails. Due to the foreign scent caused by the markings, the pups were frequently being rejected by their mothers. To completely overcome this issue, we chose to do timed pregnancies. These pregnancies involved priming cages with breeder males, exposing females to the same cages, and subsequently housing the male and multiple female breeders together for 24 hr. We did this with the hopes that multiple females would get pregnant and give birth at the same time, allowing us to randomize litters. While this approach has been moderately successfully, I believe that litter diversity is still an issue. When analyzing the data, I

observed heterogeneity among mice within the same miRNA group. I believe this could still be an artifact of litter variation. I am currently attempting randomized injections within the same litter, using tattoo ink to marker the pups. To avoid rejection, I plan to tattoo the mothers during pregnancy to acclimate them to the scent of the ink.

Despite the pitfalls of neonatal i.p. injections, we were able to limit injection site leakage and maternal rejection, allowing us to use these mice for further *in vivo* experiments. The top priority was to induce EAE in these mice with the hypothesis that they would have earlier onset of disease and increased disease severity. Since we were looking for increases in disease severity, we had to modify our existing EAE protocol to be suboptimal. Consequently, our control mice developed mild disease allowing us to observe increases in disease severity in the other miRNA groups. EAE experiments have allowed us to test miRNA combinations commonly observed in MS patients as potential risk factors for developing CNS autoimmunity. I think it is important for us to elucidate the effect of individual miRNAs *in vivo*, in order to determine if the miRNAs are having a synergistic effect as observed in the *in vitro* miRNA transfection studies. I believe that this would facilitate identification of the miRNAs with the most significant effect on the pathogenesis and susceptibility of MS. Despite experimental challenges, our *ex vivo* and *in vivo* data support our hypothesis that miRNAs are regulators of TGFB signaling, suppressors of Treg induction, and contributors to disease susceptibility.

A



B

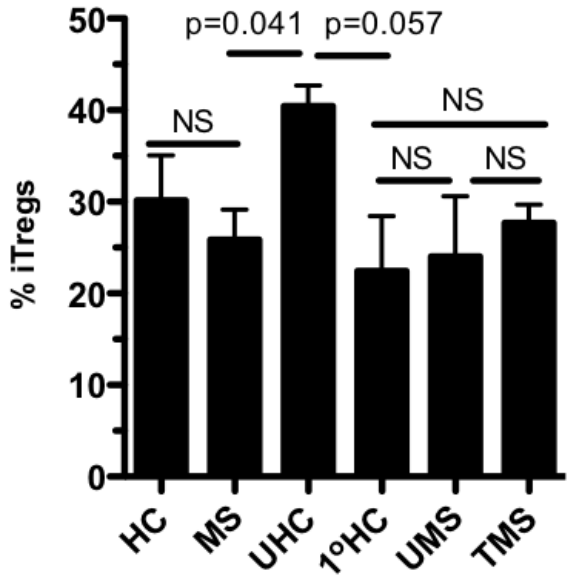


Figure 5.1 MS patients and their first-degree relatives demonstrate a similar reduced capacity to generate Tregs *ex vivo*.

Fresh PBMCs were collected sequentially from MS patients and healthy controls (HC) and iTregs were generated simultaneously from both populations. (A) Representative plots of an unrelated HC (UHC), a HC who is the first-degree relative of an MS patient (1^{o}HC), an untreated MS patient (UMS), and a treated MS patient (TMS) are shown. (B) The differentiation of naïve CD4 T cells into iTregs (CD4+CD45RA+$CD25^{hi}$Foxp3+) was compared between total HC (n = 7) and MS (n = 10) groups, using an unpaired t-test. There was no significant difference between the HC and MS patients. There was a significant difference between the UHC and MS patients, but not the 1^{o}HC and MS patients.

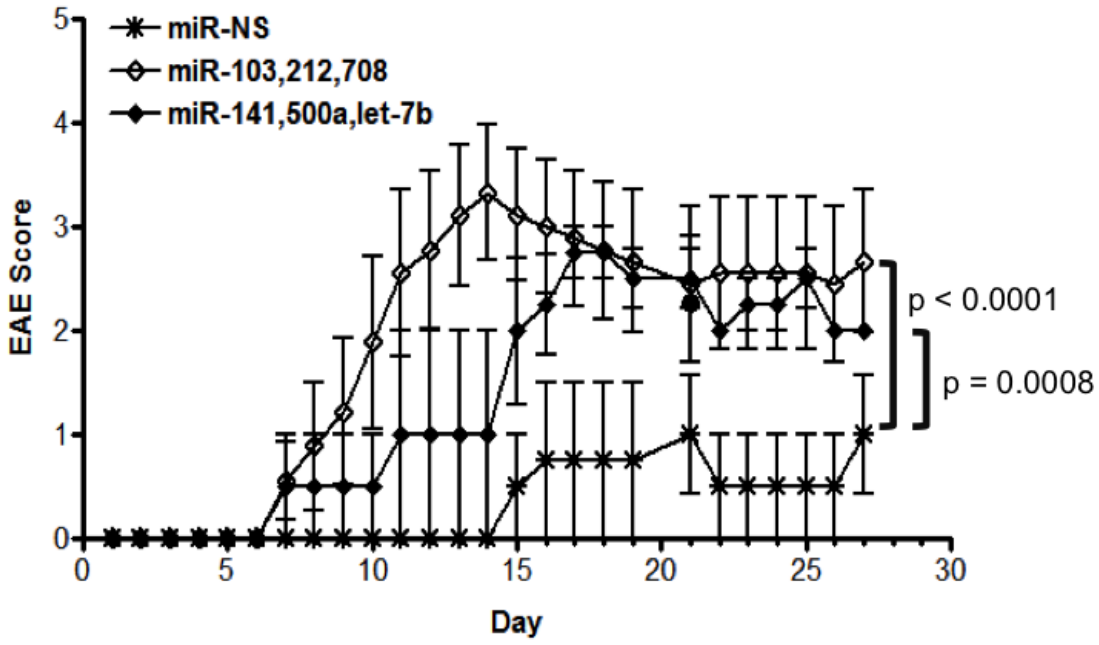


Figure 5.2 Altered expression of TGFβ-targeting miRNAs is associated with susceptibility to CNS autoimmunity.

Mice were i.p. injected 24 hr and 3 wk after birth with miR-NS (n = 4; incidence 2/4), miR-103/212/708 (n = 9; incidence 8/9), or miR-141/500a/let-7b (n = 4; incidence 4/4). EAE was induced using suboptimal conditions, and disease course was monitored for 4 wk. A Mann-Whitney test calculated significance ($p < 0.05$). Both miRNA combination groups exhibited earlier onset and significantly increased disease severity. Representative of 3 independent experiments.

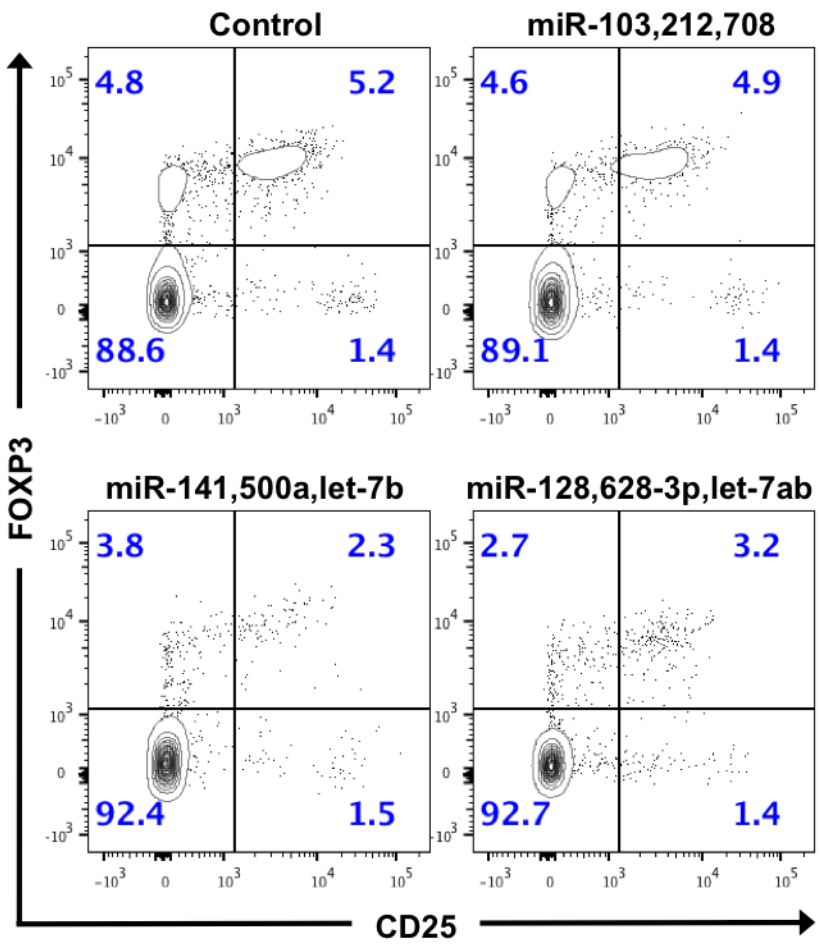


Figure 5.3 Altered expression of TGFβ-targeting miRNAs is potentially associated with reduced iTregs development.

Mice were i.p. injected 24 hr after birth with miR-NS, miR-103/212/708, or miR-141/500a/let-7b, or miR-128/628-3p/let-7ab. Splenocytes were isolated and analyzed for frequency of Tregs ($CD4^+CD25^{hi}Foxp3^+$) via flow cytometry. Representative flow plots are shown for one mouse from each miRNA group. When comparing miRNA combination groups to miR-NS control, statistical significance was not observed.

Chapter 6: Discussion

MS is a devastating disease that affects approximately 2.5 million people worldwide (Hersh and Fox 2010). While the pathology of the disease is not completely understood, it is characterized by inflammation, demyelination, and neurodegeneration in the CNS. Depending on the location and severity of damage within the CNS, patients diagnosed with MS suffer from a wide range of neurological symptoms such as pain, coordination impairment, cognitive changes, and emotional distress. Unfortunately, MS is the leading cause of non-traumatic disability in young adults, with diagnosis typically occurring between the ages of 20-40. Given the unpredictable and progressive nature of the disease, diagnosis with MS can greatly impact a patient's quality of life from an early age, threatening their autonomy and life planning. Currently, there is no cure for MS, and available treatments are limited, with only 1 of 12 DMTs indicated for SPMS and none indicated for PPMS, resulting in minimal chance for recovery especially in patients with progressive forms of the disease. Studies have demonstrated early use of DMTs in patients presenting with a CIS have a delayed conversion to CDMS (Jacobs, Beck *et al*. 2000, Comi, Filippi *et al*. 2001, Kappos, Polman *et al*. 2006, Comi, Martinelli *et al*. 2009, Miller, Wolinsky *et al*. 2014). Therefore, diagnosis of patients early during the disease process while treatments are available is essential for delaying progression of the disease. However, diagnosis is challenging, as there is no single test for MS. Additionally, despite

the ability of existing drugs to slow down disease progression, the eventual worsening of symptoms is inevitable. Identification of individuals susceptible to developing MS may allow these patients to be proactive by submitting to routine screening and to prepare themselves for possible diagnosis. Therefore, I believe that research aimed at the identification of novel biomarkers and therapeutic targets is imperative for understanding disease activity, measuring treatment efficacy, and stopping disease progression.

Both genetic and environmental studies have attempted to find causal biomarkers associated with MS, but identification of single markers with high sensitivity and specificity has been limited at best. For instance, genome-wide association studies (GWAS) have identified HLA-DRB1*1501 as a genetic marker for individuals with high risk to developing MS (Haegert and Marrosu 1994); however, this marker lacks sensitivity. This overall lack of success potentiates the need for innovative strategies for elucidating factors of disease susceptibility. Additionally, the complex nature of the disease makes treatment discovery and selection challenging. Given that both beneficial and adverse responses vary among MS patients, modification of treatment plans is often necessary. However, the current methods for treatment selection primarily rely on trial and error, with no means of predicting whether a patient will respond to a particular therapy prior to prescription. In order to enhance personalized therapeutic strategies, it is necessary to identify predictive markers for treatment response. At the very least, markers indicative of treatment efficacy are needed to allow for immediate adjustment in treatment if a patient is a non-responder. However, these are long-term and big picture

goals, and at this time, any insight into the mechanisms underlying the differential immune responses observed in MS patients would enhance the field's understanding of this multi-factorial disease.

To this end, my lab began investigating the potential of miRNAs as biomarkers for MS. In recent years, miRNAs have emerged as important regulators that affect various biological processes. Even more recently, studies have begun to successfully show the clinical applications of miRNAs in various diseases such as cancer and rheumatoid arthritis (Lu, Getz *et al*. 2005, Calin and Croce 2006, Pers and Jorgensen 2013, Redova, Sana *et al*. 2013). In addition, the accessibility and relatively low number of miRNAs make them ideal potential biomarker candidates. In contrast to the MS studies performed by other labs looking at miRNA levels in total PBMCs, we specifically looked at miRNA expression in specific cellular subsets within the blood. Given that MS is thought to involve a misdirected attack against the CNS mediated by myelin-specific T cells, the lab chose to specifically analyze the CD4 T cells of MS patients and healthy individuals. It is important to note that both memory and naïve CD4 T cells were profiled. While there is no direct evidence from MS patients, we believe that naïve CD4 T cells are unaffected by disease as they have not encountered antigen and experienced activation. To address this question, my lab previously performed a miRNA profiling study on the naïve CD4 T cells from mice with EAE and their non-EAE littermates. There were very few differences in miRNA levels between the EAE and non-EAE groups, and no differences were observed in miRNAs found differentially expressed in the MS patients' naïve CD4

T cells. Therefore, it is unlikely that inflammation is causing the miRNA changes in naïve CD4 T cells in MS patients. Thus, we primarily focused on miRNA data from the naïve CD4 T cells with the aim of identifying differences in MS patients that were endogenous, rather than consequences of disease. With this rationale in mind, 85 miRNAs were identified as being differentially expressed in the naïve CD4 T cells of MS patients, and analysis began to explore their potential role in the patholophysiology of MS. I highlight these findings and rationale because they served as the foundation for my graduate research, and were taken into account when analyzing the potential importance of my work.

Further analysis of the profiling data revealed 19 differentially expressed miRNAs predicted to target genes of the TGFβ signaling pathway (TGFβR1, TGFβR2, SMAD2, and SMAD4), which were potentially capable of inhibiting TGFβ signaling (Figure 3.1). This possible connection of the identified miRNAs to TGFβ signaling in naïve CD4 T cells was of interest to the lab because TGFβ is known to be essential for the differentiation and function of Tregs (Yamagiwa, Gray *et al*. 2001, Zheng, Gray *et al*. 2002, Chen, Jin *et al*. 2003). Interestingly, Tregs have the ability to directly suppress autoreactive T cells, a population of cells that appears to lack regulation in MS patients. These uncontrolled autoreactive T cells are hypothesized to be one of the main contributors to the excessive inflammation observed in individuals with MS. One of the main elements of regulation that is lacking in MS patients is a fully functional Treg population. The Tregs of MS patients have diminished suppressive effect on the

autoimmune response promoted by myelin-specific T cells (Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Kumar, Putzki *et al*. 2006). Even though the effect of dysfunctional Tregs has been partially elucidated, the mechanism by which Tregs become defective and their exact role in MS pathogenesis have not yet been determined. Importantly, a link between a disruption of the TGFβ signaling pathway and the Treg dysfunction in MS patients has not previously been established. Additionally, the miRNAs identified in my studies have not previously been defined to play role in the development of Tregs or CNS autoimmunity. Therefore, my project provided an innovative strategy for identifying potential mechanisms for the Treg defects observed in MS patients.

Before discussing the implications of my data, it is important to note that preliminary studies were performed in order to identify the most promising gene target/ miRNA pairs and narrow the scopes of my studies. To validate that TGFβ signaling was actually being affected in MS patients, we analyzed the levels of TGFβR1, TGFβR2, SMAD2, and SMAD4 in the naïve CD4 T cells of MS patients and healthy donors. These studies demonstrated that TGFβR1 and SMAD4 were the only genes that were significantly reduced in MS patients (Figure 3.2). Therefore, we focused my project on the miRNAs predicted to target these genes. However, we should not dismiss TGFβR2 completely, as it trended towards a decrease in MS patients (Figure 3.2). Additionally, several of the miRNAs predicted to target TGFβR2 are also predicted to target TGFβR1 and SMAD4. Therefore, I think future studies are needed to assess the levels of TGFβR2 in additional

patients samples, as well as miRNA-transfected samples. Notably, SMAD7, a negative regulator of the TGFβ signaling pathway, was also excluded from initial analysis as another means of narrowing our focus. However, having now established our experimental system, I propose that it would be beneficial to investigate the effects of SMAD7-targeting miRNAs on Treg development, allowing us to further enhance our knowledge of the extent to which miRNAs regulate the TGFβ signaling pathway and affect T cell differentiation.

Taken together, my *in vitro* data suggest that enhanced levels of the TGFβ-targeting miRNAs identified in the naïve CD4 T cells of MS patients decrease capacity to generate Tregs and potentially enhance susceptibility to developing MS. When using suboptimal Treg inducing conditions, I was able to demonstrate a reduction in the percent of MS and miRNA transfected naïve CD4 T cells that were able to differentiate into iTregs, appearing to indicate a defect in Treg number (Figure 4.1 and 4.2). Although there has been some controversy as to whether MS patients have a reduced number of Tregs, the majority of studies suggest that the defect is not in number but in function (Vandenbark, Finn *et al*. 2001, Viglietta, Baecher-Allan *et al*. 2004, Haas, Hug *et al*. 2005, Huan, Culbertson *et al*. 2005, Kumar, Putzki *et al*. 2006, Haas, Fritzsching *et al*. 2007). Therefore, my data is seemingly contradictory to these findings. Interestingly, mice with TGFβ deficiencies have normal Treg numbers as adults, however the number of Tregs postnatally is significantly reduced (Marie, Letterio *et al*. 2005, Liu, Zhang *et al*. 2008, Ouyang, Beckett *et al*. 2010). This limited pool of Tregs, which are CD25+, proliferate in

response to IL-2 in the periphery, resulting in normal numbers of Tregs. While normal in number, this expanded pool of Tregs has a diminished capacity to suppress effector T cells as evidenced by the uncontrolled autoimmunity observed in mice with deficiencies in TGFβ signaling (Gorelik and Flavell 2000, Marie, Letterio *et al*. 2005, Liu, Zhang *et al*. 2008). The inability of Tregs from TGFβ signaling-impaired mice to suppress autoimmunity has been attributed to decreased Foxp3 expression and a decreased repertoire of Tregs. Similarly, MS patients have also been found to have a decreased TCR repertoire in their Tregs (Haas, Fritzsching *et al*. 2007), indicating that they may have insufficient diversity to respond to self-antigens and prevent autoimmunity. In addition, thymic output of Tregs was shown to be reduced in pediatric and adult MS patients, and differences in suppressive capacity of Tregs in MS patients and controls were dependent on the number of new thymic emigrant Tregs, not total number of Tregs (Haas, Fritzsching *et al*. 2007, Balint, Haas *et al*. 2013). Taking into account these studies, I postulate that the reduced frequency of Tregs generated from naïve CD4 T cells of MS patients and miRNA transfected samples represent a defect in TCR diversity not number, thus mimicking early events in the Treg development of both MS patients and mice with TGFβ signaling deficiencies. Therefore, my data suggests that the miRNAs identified in this study may limit Treg development, resulting in the generation of a less diverse population of Tregs with diminished suppressive capacity, but capable of expanding in response to IL-2. Importantly, my *in vitro* findings provide a novel link between dysregulation of miRNAs, disruption of TGFβ signaling, and suppression of Treg

development; providing a foundation for addressing whether these defects can be replicated *in vivo* and cause enhanced susceptibility to developing MS.

To this end, my *in vivo* findings provide evidence that early dysregulation of TGFβ-targeting miRNAs may result in defective Treg development and enhanced disease susceptibility, in the context of EAE. In attempt to most closely mimic what we believe to be occurring in MS patients, I injected neonatal mice within 24 hr of birth with miRNA combinations that are commonly observed in MS patients. *Ex vivo* analysis of thymic and splenic Tregs was performed four days after injection with the intention of detecting differences in Treg numbers prior to their expansion in response to peripheral IL-2. Taken together, my data indicate a reduction of Treg numbers in mice injected with the TGFβ-targeting miRNAs (Figure 5.3). However, this observed decrease in Treg numbers was not statistically significant. I attribute this lack of consistency to the natural fluctuation of Treg numbers between litters. I attempted to randomize the injection conditions within individual litters, however maternal rejection was high. My only option was to perform timed pregnancies and mix pups from several litters prior to injection. Therefore, further experimentation using single litters and an alternative form of pup labeling is needed to determine if Treg development is significantly reduced in the miRNA-injected mice. I hypothesize that when the miRNA conditions are randomized among littermates, we will find that both the development of nTregs and the capacity to generate iTregs are significantly inhibited in the miRNA-injected mice. Additionally, while outside the scope of my graduate work, I postulate that these mice also have less

diverse TCR repertoires, resulting in reduced capacity to recognize the myelin-reactive T cells responsible for driving EAE. In order to address whether overexpression of TGFβ-targeting miRNAs could also enhance disease susceptibility, EAE was induced in mice that received postnatal miRNA injections. My EAE data indicate that overexpression of TGFβ-targeting miRNAs results in earlier onset and enhanced severity of disease (Figure 5.2). Taking into account both my *in vitro* and *in vivo* findings, I propose that dysregulation of the TGFβ-targeting miRNAs in these mice is causing Treg defects, which result in a lack of regulation responsible for the expedited onset and enhanced progression of disease. My findings support the hypothesis that the differential expression of TGFβ-targeting miRNAs could potentially be used as susceptibility markers for MS.

Taken together, my data support further investigation of the identified miRNAs as potential biomarkers. However, there are several factors to take into account. In of themselves, miRNAs posses several qualities that make them desirable biomarkers, such as their accessibility, relatively low number, and successful application in other immune-related diseases. However, I do not want to use the term biomarker loosely and prematurely label the identified miRNAs as such. Simply stated biomarkers are characteristics that can be objectively measured as an indicator of biological processes, pathogenic mechanisms, and therapeutic responses (Group. 2001). However, several pre-clinical and clinical validation steps after the initial discovery of candidate markers are needed to truly label something as a "biomarker." Two of the most challenging but also most essential features of a good clinical biomarker are specificity and sensitivity. The

identification of a single biomarker capable of both is challenging, especially in multi-factorial diseases such as MS. For example, some of the healthy controls analyzed in the initial miRNA profiling study demonstrated expression levels of single miRNAs similar to MS patients, suggesting elevated levels of these individual miRNAs may not be good indicators of susceptibility. Interestingly, in contrast to healthy individuals, MS patients typically had several of these miRNAs overexpressed, suggesting a potential role for panels of miRNAs in the identification of individuals predisposed to developing MS. While my data highlight two combinations of miRNAs commonly found in MS patients, additional studies using larger cohorts of patients and more in depth analysis of miRNA patterns would be required to identify such a panel. These types of studies are outside the scope of my graduate research, however my data supports further investigation of miRNA combinations as potential indicators of MS risk. Additionally, it is important to note that while my observations have been made in the context of MS, it is possible that patients with other immune-mediated diseases associated with Treg defects may also differentially express TGFβ-targeting miRNAs. To ensure sensitivity of these miRNAs as biomarkers for MS, it is necessary to compare their expression levels between MS patients and patients with other immune-related diseases. If individuals with other immunologic diseases are found to have differential expression of these miRNAs, these miRNAs would not be exclusively indicative of MS susceptibility. However, they could still serve as predictive markers for therapeutic response, active markers for treatment efficacy, or even therapeutic targets.

Although my investigation of the identified TGFβ-targeting miRNAs has focused on implications in Treg development, it is important to note that TGFβ is a pleiotropic cytokine that has potent immunologic effects other than promoting Treg differentiation and function. Therefore, my findings may have broader implications on CD4 T cell development and differentiation. For instance, TGFβ has been shown to promote the development of Th17 cells in the presence of IL-6 in mice (Bettelli, Carrier *et al*. 2006, Mangan, Harrington *et al*. 2006, Veldhoen, Hocking *et al*. 2006), suggesting that diminished TGFβ signaling may negatively modulate Th17 cells. However, in the case of encephalitogenic T cells, members of the lab and others have found that myelin-specific Th17 cells differentiated with IL-6 and TGFβ are not encephalitogenic (Yang, Weiner *et al*. 2009, Ghoreschi, Laurence *et al*. 2010, Lee, Yang *et al*. 2015). In fact, TGFβ is a negative regulator of T-bet, a key transcription factor in encephalitogenic T cells, irrespective of whether it has a Th1 or Th17 phenotype (Gocke, Cravens *et al*. 2007, Yang, Liu *et al*. 2010, Lee, Yang *et al*. 2015). Thus, suppression of the TGFβ pathway enhances T-bet expression, as well as promotes the differentiation of pathogenic Th17 cells, suggesting that miRNAs that suppress TGFβ signaling may promote the development of autoreactive effector T cells. Furthermore, differentiation of human Th17 cells occurs independently of TGFβ (Acosta-Rodriguez, Napolitani *et al*. 2007, Wilson, Boniface *et al*. 2007), and TGFβ induces IL-10 expression in autoreactive Th1 cells, promoting a self-regulation mechanism (Huss, Winger *et al*. 2010). IL-10 production has been shown to be impaired in MS patients (Cao, Goods *et al*. 2015), but positively associated with therapeutic benefit (Miller, Shapiro *et al*. 1998, Rudick, Ransohoff *et al*.

1998, Kim, Ifergan *et al*. 2004). Thus, diminished TGFβ signaling would potentially allow encephalitogenic Th1 cells to mediate pathology more robustly due to a lack of inherent regulation. Additionally, I hypothesize that therapeutic benefits associated with TGFβ induced IL-10 may be reduced in patients with overexpression of TGFβ-targeting miRNAs, suggesting a potential role for these miRNAs as predictive indicators of therapeutic response.

In addition to providing novel insight into the Treg defect observed in MS patients, my findings also shed some light on past studies as well. Given its immunosuppressive properties, TGFβ was used as a successful therapy to ameliorate EAE (Racke, Dhib-Jalbut *et al*. 1991). Consequently a clinical trial using TGFβ as a therapy for MS patients was performed, but failed (Calabresi, Fields *et al*. 1998). Taking into account my findings, I believe that the dysregulation of miRNAs targeting TGFβR1 and SMAD4 in CD4 T cells of MS patients may make these patients less responsive to TGFβ therapy. Therefore, they are not able to respond as robustly to TGFβ therapy as mice with fully function TGFβ-signaling pathways. Interestingly, my lab has previously demonstrated that systemic administration of small RNAs can have therapeutic benefits in CNS autoimmunity (Lovett-Racke, Rocchini *et al*. 2004, Gocke, Cravens *et al*. 2007, Yang, Liu *et al*. 2010). This poses the question of whether miRNAs could be targeted in order to restore the responsiveness of MS patients to TGFβ. In attempt to answer this question, I have started preliminary studies using inhibitors of the TGFβ-targeting miRNAs. In these studies, I used MS patient cells and transfected them with miRNA inhibitors in the hopes

of restoring their capacity to generate iTregs. It is important to note that the cells used were from the same MS patients as the initial miRNA profiling study. This is significant because I was able to individualize the miRNA inhibitor combinations used based on each patient's miRNA profile. However, I was only able to restore the Treg induction in 1/5 patients. Opposite to what I expected, 4/5 patients actually exhibited decreased Treg development in response to miRNA inhibitors. Given that these miRNAs have the capacity to regulate critical genes outside of the TGFβ signaling pathway, therapeutically targeting miRNAs may cause unforeseen side effects. While directly targeting these miRNAs may not be the best therapeutic approach, I think my findings still support that alternative forms of enhancing TGFβ-signaling may result in more robust Treg development and provide therapeutic benefits.

While my research has facilitated a better understanding of the lack of regulation observed in MS patients by providing a novel link between miRNA-driven inhibition of TGFβ signaling and defective Treg development, my findings also opened to the door for additional questions and potential implications. One question not readily answered by my project is whether miRNA dysregulation can also affect Treg function. My working hypothesis is that Tregs generated from naïve CD4 T cells overexpressing the TGFβ-targeting miRNA will be completely functional, however they will lack the TCR diversity need to recognize and suppress myelin-reactive T effector cells. While outside the scope of my graduate work, I propose that dissecting the TCR repertoires of patients with MS and mice with dysregulated TGFβ-targeting miRNAs could provide a critical

link between the miRNA-mediated TGFβ deficiencies and Treg defects in MS patients. Another question remaining is the extent to which the Treg defects observed *in vivo* are caused by the differential expression of TGFβ-targeting miRNAs. In the EAE experiments, it is unclear whether the miRNA-induced effects on disease onset and severity are directly linked to defective Treg development. Similarly, we have not established if the Treg defect observed in MS patients is a direct effect of the dysregulation of TGFβ-targeting miRNAs in these individuals. For both the EAE and MS studies, correlation analyses need to be performed comparing miRNA levels to Treg defects. If Treg defects are shown to correlate with miRNA levels, additional analysis can be done to compare Treg defects and disease severity in mice with EAE. A similar study in MS patients would be challenging and require significant additional information such as the disease status (i.e. phase of disease) of the patients at the time of blood draw. Despite these unanswered questions, I have provided the lab with the foundation necessary for immediate investigation of these questions, as well as opened the door to the potential long-term application of these miRNAs as markers of susceptibility, treatment efficacy, and treatment response.

While my observations have been made in the context of CNS autoimmunity, my project positively impacts the science community as a whole by providing new insight into how miRNAs regulate TGFβ-signaling. Given that TGFβ-signaling is essential for both normal and pathogenic processes, my findings may have broader implications on CD4 T cell development and differentiation than those observed in MS. Additionally, it is well

established that miRNAs play key roles in the development and function of Tregs (Dooley, Linterman *et al*. 2013, Josefowicz 2013), and differential expression of miRNAs have been found in MS patients (Du, Liu *et al*. 2009, Keller, Leidinger *et al*. 2009, Otaegui, Baranzini *et al*. 2009, De Santis, Ferracin *et al*. 2010, Guerau-de-Arellano, Smith *et al*. 2011, Guerau-de-Arellano, Alder *et al*. 2012, Smith, Guerau-de-Arellano *et al*. 2012, Gandhi, Healy *et al*. 2013, Ridolfi, Fenoglio *et al*. 2013, Søndergaard, Hesse *et al*. 2013, Keller, Leidinger *et al*. 2014). Therefore, it is reasonable to infer that the TGFβ-targeting miRNAs identified in MS patients may also play a role in normal Treg development and function. Notably, none of the miRNAs identified in my studies have been previously defined to play a role in normal Treg development. Furthermore, MS is not the only disease where Treg regulation is lacking, resulting in inappropriate immune responses. Therefore, it is possible that patients with other immune-mediated diseases associated with Treg defects may also differentially express TGFβ-targeting miRNAs. As such, my findings may have broader implications on the maintenance of immune homeostasis.

Overall, my project has led to identification of differentially expressed TGFβ-targeting miRNAs in the naïve CD4 T cells of MS patients that can individually or synergistically disrupt the TGFβ-signaling pathway, inhibit Treg development, and enhance susceptibility to CNS autoimmunity. These findings are of significance as they provide novel potential mechanisms for the inherent defects in immune regulation observed in MS patients and support my hypothesis that TGFβ-targeting miRNAs may be

susceptibility factors for MS. Furthermore, my observations are novel, as no link between the disruption of the TGFβ-signaling pathway and the Treg dysfunction in MS patients has previously been established. Additionally, the miRNAs identified in my studies have not previously been defined to play role in the development of Tregs or CNS autoimmunity. Therefore, I believe that the novelty of using miRNAs as a means to dissect out differences between MS patients and healthy individuals, provides an opportunity to identify candidate biomarkers and therapeutic targets, as miRNAs posses characteristics desirable for both. Importantly, the potential clinical application of these miRNAs would allow for a novel approach to patient care, allowing for a more personalized health plan for patients with MS.

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