TRANSCRIPTIONAL REGULATION OF T HELPER CELL DIFFERENTIATION IN AUTOIMMUNITY

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Acknowledgements

There are many people that I would like to thank for all of their support during my years as a graduate student. First and foremost, I would like to thank my mentors Michael Racke and Amy Lovett-Racke for all of their help and encouragement. You have taught me how to think critically and work independently. I have not only learned a lot about science and immunology from you, but I have learned many of life's lessons as well. I will be forever grateful for your guidance, patience, understanding, and friendship. I would also like to thank all of the members of the Racke lab, past and present, especially Judy Choy, Rehana Hussain, Sara Northrop, Li-hong Ben, Yuhong Yang, Bob Ratts and Petra Cravens, for all of their technical support, intellectual discussion, and friendship. Additionally, I would like to acknowledge Drs. Nancy Monson, Nitin Karandikar, and Olaf Stuve, as well as the members of their labs for their advice and support throughout the years. I would also like to thank my committee members for all of their guidance and suggestions and Karen Kazemzadeh for all of her help.

I would like to give special thanks to my family, especially my parents, Louis and Susan Rocchini. You have been more supportive and understanding throughout these many educational years than any child could ever ask for. You have been wonderful role models for me. Thank you for your love, motivation, and encouragement. I would also like to thank my brother and sister, Lou and Kristen, and their spouses, Allison and Ryan, for their encouragement and

understanding, especially when I decided to move to Texas. I know I haven't always been around when I needed to be. Thanks for understanding. Additionally, I would like to thank my grandparents for all of their love and enthusiasm. Thank you also to my parents in-law, Michael and Terry Gocke, my brothers and sisters in law, Jack, Mark, Tim, Fizzah, Ryan, and Roxie, and my nephews Michael and Connor. You have also been a constant source of love and support.

I would also like to thank all of the friends that I have made during my years here at Southwestern, especially Steve, Mari Pili, Petra, Vinodh, Bob, Beth and Chris for all of our happy hours. Thank you also to my best friend Meghan for understanding when I left Charleston to move to Texas and for always being there to listen, and to my good friend Rose, for all the memories and making my life in Texas bearable.

Finally, I would like to thank my husband Christian. I could not have made it this far without you. You are my inspiration. Thank you for always being there to listen, to talk about science with me, and for making me laugh when I am discouraged. I admire you and love you very much.

TRANSCRIPTIONAL REGULATION OF T HELPER CELL DIFFERENTIATION IN AUTOIMMUNITY

By

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December, 2006

TRANSCRIPTIONAL REGULATION OF T HELPER CELL DIFFERENTIATION IN AUTOIMMUNITY

Publication No._____

Anne Elizabeth Gocke, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2006

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As a means to understand the significance of transcriptional regulation of Th cells in MS pathogenesis, we investigated the role of the transcription factors T-bet and PPARα in the differentiation and effector function of T helper cells in the EAE model. Based on its ability to induce a pro-inflammatory immune response, T-bet has been found to regulate genes that have been directly linked to pathogenicity in EAE/MS. We examined the use of T-bet specific AS oligonucleotides and siRNA to silence T-bet expression in autoreactive encephalitogenic T cells and demonstrated that this transcription factor plays a critical role in the differentiation of these cells and in the induction of EAE. siRNA and AS T-bet suppressed T-bet expression, IFN-γ production, and STAT1 expression during antigen-specific T cell differentiation. *In vitro* suppression of T-bet in myelin specific cells used for adoptive transfer, and *in vivo* administration of AS and siRNA T-bet inhibited the induction of EAE. T-bet bound the IFN-γ and STAT1 promoter regions, suggesting the ability of T-bet and STAT1 to regulate one another in a positive feedback loop, but was not found to regulate the IL-

12/STAT4 pathway. Moreover, we explored the role of T-bet in the IL-23/IL-17 pathway. IL-17 producing T cells have recently been implicated in the pathogenesis of EAE, but the potential role of T-bet in the differentiation of Th17 cells is not completely understood. We demonstrated that therapeutic administration of siRNA T-bet significantly improved the clinical course of established EAE. The improved clinical course was associated with a decrease in T-bet protein expression, reduced IFN-γ production, and a reciprocal increase in GATA3 expression. We also observed a decrease in IL-23R expression both *ex-vivo* in splenocytes from siRNA T-bet treated mice and when cells were transfected with siRNA T-bet and subsequently activated *in vitro*. When T-bet was silenced we observed an ensuing decrease in both IL-23R and IL-17 expression in the CNS. This observation was correlated by the finding that T-bet directly regulates transcription of the IL-23R and, in doing so, influences the fate of pathogenic Th17 cells, which depend on optimal IL-23 responsiveness for survival. Taken together, these data demonstrate that suppression of T-bet ameliorates EAE by limiting differentiation of autoreactive Th1 cells, as well as inhibiting pathogenic Th17 cells via regulation of the IL-23R.

In contrast to T-bet, PPARα has been demonstrated to regulate anti-inflammatory genes and the PPARα agonists gemfibrozil and fenofibrate have been associated with protection from EAE. However, the manner in which these agents ameliorate disease is not well understood. We investigated the mechanism by which gemfibrozil induces immune deviation and protects mice from EAE. We demonstrated that treatment with gemfibrozil increases GATA3

and decreases T-bet expression *in vitro* and directly *ex-vivo*. The protective effects of gemfibrozil in EAE were shown to be partially dependent on IL-4 and to occur in a receptor dependent manner. Moreover, PPARα was demonstrated, for the first time, to regulate the IL-4 and IL-5 genes and bound the IL-4 promoter in the presence the coactivator SRC-1, suggesting transactivation of the IL-4 gene. Finally, therapeutic administration of gemfibrozil and fenofibrate ameliorated clinically established EAE. The data presented herein illustrate that modulation of specific transcription factors can influence Th cell differentiation and the clinical outcome of EAE, and suggest that targeting T-bet and/or PPARα by gene silencing and administration of specific agonists could provide a novel therapy for inflammatory immune-mediated diseases such as MS.

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Chera B, Schaecher KE, **Rocchini A**, Imam SZ, Ray SK, Ali SF, Banik NL Calpain upregulation and neuron death in spinal cord of MPTP-induced parkinsonism in mice. *Ann N Y Acad Sci.* 2002 Jun;965:274-80.

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Gocke AR, Hussain RZ, Ben L, Drew PD, Lovett-Racke AE, and Racke MK. Transcriptional Modulation of the Immune Response by PPARα Agonists Ameliorates Autoimmune Encephalomyelitis. Submitted.

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Abbreviations

μg: micrograms

μl: microliters

μM: micromolar

15dPGJ$_2$: 15 deoxy-Δ prostaglandin J$_2$

Ab: antibody

Ag: antigen

APC: antigen presenting cells

APL: altered peptide ligand

AS: antisense

BSA: bovine serum albumin

cDNA: complementary DNA

CFA: complete Freund's adjuvant

ChIP: chromatin immunoprecipitation assay

CNS: central nervous system

Con A: conconavalin A

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

ECL: enhanced chemiluminescent reagent

EDTA: ethylenediaminetetra acetic acid

ELISA: enzyme linked immunosorbant assay

ETOH: ethanol

FACS: fluorescence activated cell sorter

FBS: fetal bovine serum

FDA: Food and Drug Administration

FITC: fluorescein isothiocyanate

HBSS: Hanks Balanced Salt Solution

HDAC: histone deacetylase

HRP: horseradish peroxidase

IFN-γ: interferon gamma

Ig: immunoglobulin

IkB: inhibitor kappa B

IL: interleukin

IL-12Rβ2: interleukin 12 receptor beta 2

IL-23R: interleukin 23 receptor

iNOS: inducible nitric oxide synthase

i.p.: intraperitoneally

I.P.: immunoprecipitation

i..v.: intravenous

kDa: kilodalton

LNC: lymph node cells

LPS: lipopolysaccharide

LT: lymphotoxin

LTB_4: leukotriene B4

MAP kinase: mitogen activated protein kinase

MBP: myelin basic protein

MHC: major histocompatibility complex

ml: milliliter

MMP: matrix metalloproteinase

MOG: myelin oligodendrocyte glycoprotein

mRNA: messenger RNA

MS: multiple sclerosis

NFAT: nuclear factor of activated T cells

NFkB: nuclear factor kappa B

NK: natural killer

NKT: natural killer T cells

NO: nitric oxide

NS: nonsense

OD: optical density

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phycoerythrin

PLP: proteolipid protein

PMA: phorbol myristate acetate

PP: peroxisome proliferators

PPAR: peroxisome proliferator activated receptor

PPRE: PPAR response element

PT: pertussis toxin

PVDF: polyvinylidene fluoride

r: recombinant

RA: rheumatoid arthritis

RAG: recombinase activating gene

RNAi: RNA interference

RT-PCR: reverse transcriptase polymerase chain reaction

RXR: retinoid X receptor

s.c. subcutaneous

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

siRNA: small interfering RNA

SRC-1: steroid receptor co-activator 1

STAT: signal transducer and activator of transcription

T-bet: T-box expressed in T cells

TCR: T cell receptor

TE: Tris EDTA

Tg.: transgenic

TGF-β: transforming growth factor beta

Th: T helper

Th1: T helper 1

Th2: T helper 2

Th17: T helper 17

TLR: toll like receptor

TNF-α: tumor necrosis factor alpha

TZD: thiazolidinediones

VCAM: vascular cell adhesion molecule

w/w: weight per weight

WT: wildtype

Chapter I. Introduction

An effective immune response against pathogens, such as microbes, allergens, and tumor antigens, requires the differentiation of T lymphocytes into two major subtypes known as cytotoxic T lymphocytes and T helper lymphocytes (Fig. 1). T helper cells (Th) express the co-receptor CD4 on their surface and induce immune responses that lead to macrophage and cytotoxic T cell activation as well as the elimination of intracellular pathogens. In addition, Th cells generate humoral immunity by signaling to B cells to produce antibodies and eliminate extracellular pathogens. When Th cell development and activation become dysregulated, leading to a loss in tolerance to self antigens and consequent pathogenic inflammation, autoimmune disease results. Many models of autoimmune disease focus on autoreactive Th cells and, more recently, on the role of transcription factors that regulate gene expression in Th cells. One such model is Experimental Autoimmune Encephalomylelitis (EAE), an animal model for the human demyelinating disease Multiple Sclerosis (MS).

Multiple Sclerosis

Multiple Sclerosis (MS) was first described by Jean-Marie Charcot as early as 1868. It was originally named sclerose en plaque for the appearance of plaques or lesions in the white matter of the spinal cord which are now believed

to result from immune-mediated mechanisms [1]. Inflammatory cells, composed mostly of lymphocytes and macrophages, are seen in perivascular regions and white-matter parenchyma in the central nervous system (CNS) of MS patients and are believed to enter the CNS by a breach in the blood-brain-barrier. If these infiltrating mononuclear cells contain lymphocytes that can recognize myelin antigens, a cascade of events may occur which ultimately results in the formation of a plaque [2]. It is for this reason that MS is often categorized as an organ-specific autoimmune disorder.

Approximately 2.5 million people world wide and 350,000 people in the United States have physician diagnosed Multiple Sclerosis [3]. The onset of MS typically occurs in early adulthood and can progress differently among individuals. There is believed to be a predisposing genetic component to the disease, as indicated by a concordance rate among monozygotic twins of approximately 30%, which is 6 times the rate observed in dizygotic twins [4]. Furthermore, the presence of the HLA-DR2 allele has been found to substantially increase the risk of developing MS [5]. It has also been suggested that environmental factors can contribute to disease susceptibility. This concept is supported by a trend toward an increasing prevalence and incidence of MS in certain geographical locations, such as the northern United States [6, 7].

Although progress has been made in recent years to understand the pathogenesis and underlying cause of MS, many aspects of the disease remain

poorly understood. For this reason, investigators rely heavily on animal models to elucidate pathophysiological mechanisms of this debilitating disease. There are several animal models used to study MS including the Theiler's Murine Encephalomyelitis viral model, which is induced by an enterovirus, and the prototype immune-mediated model, Experimental Autoimmune Encephalomyelitis.

Experimental Autoimmune Encephalomyelitis

Experimental Autoimmune Encephalomyelitis (EAE) resembles MS in many respects and therefore is used commonly in the laboratory to study MS as well as organ-specific autoimmune diseases in general. EAE was first described over 50 years ago and the first attempts at producing EAE were efforts to understand the pathogenesis of post rabies vaccination encephalomyelitis. The vaccine that Pasteur developed for rabies was made of a suspension of spinal cords from rabbits infected with rabies. Following administration of the vaccine, people began to develop an encephalomyelitis that was unrelated to rabies [8]. This prompted investigators to determine the cause of the side-effect. They found that the effects could be reproduced in rabbits and monkeys following repeated administration of neural tissue, indicating that the post vaccine encephalomyelitis was resulting from an unintentional induction of an immune response against neural antigens [9, 10]. Induction of disease required many injections over a period of weeks, but induction became easier following the

discovery of complete Freund's adjuvant (CFA). CFA is composed of mineral oil mixed with *Mycobacterium* and is made into an emulsion with the immunizing neural antigen. Use of CFA was found to induce a vigorous and prolonged immune response against the antigen and made the induction of EAE much more reliable [11, 12]. It was also discovered later that a separate injection of pertussis toxin in addition to antigen/CFA increased the incidence of EAE[13-15].

EAE can be induced in genetically susceptible strains of rodents and monkeys. Initially, inducing EAE in mice was difficult, but following the discovery of CFA and pertussis toxin it became much easier [13, 15, 16]. It was also found in these initial studies that certain strains of mice were more susceptible to EAE than others and this depended upon genetic background, primarily associated with MHCII genes [16-18]. Today, mice are the most common animals used for the induction of EAE. Murine EAE can result in a relapsing/remitting disease course, similar to what is seen in the early phase of MS, or it can result in a more chronic progressive form of disease, depending upon the strain of mice used to induce disease as well as the choice of antigen. In chronic EAE, the pathology of the white matter shows demyelination which is also reminiscent of the pathology seen in the CNS of MS patients [19].

Murine EAE can be divided into two major subtypes, the first being active EAE and the second being passive or adoptively transferred EAE. In active EAE, susceptible strains of mice are immunized with an appropriate myelin antigen or

peptide, such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG), emulsified in complete Freund's adjuvant (CFA) followed by administration of Pertussis Toxin (PT) on the day of immunization and 48 hours later [20-22]. PT is believed to play a role in the breakdown of the blood brain barrier, allowing autoreactive cells to pass more easily into the CNS to mediate disease pathogenesis [23]. Depending upon the strain of mouse used and the immunizing antigen, clinical signs and symptoms of EAE become apparent 10-15 days after immunization. Following immunization there is an induction phase and an effector phase of disease. In the induction phase, autoreactive cells are activated and expand in peripheral lymph nodes in response to antigen/CFA. In the effector phase, the activated cells migrate to the CNS resulting in tissue damage and clinical signs of disease.

In adoptively transferred EAE, disease is induced by injecting mice with activated, myelin-specific T cells (usually CD4+ T cells). Therefore, only the effector phase occurs in EAE animals in which disease has been induced by adoptive transfer. The activated T cells can be generated by immunizing mice with a myelin protein or peptide emulsified in CFA, removing the draining lymph nodes of these mice, activating the T cells again with the myelin antigen *in vitro*, and then transferring the activated T cells into naïve recipient mice to induce EAE [24-27]. Alternatively, passive EAE can be induced by removing lymphocytes from a T cell receptor (TCR) transgenic mouse in which the TCR preferentially recognizes a myelin peptide, stimulating these cells *in vitro* with the

appropriate myelin antigen, and injecting these activated cells i.p. into naïve recipient mice. Adoptive transfer EAE is a very reproducible model and is useful for studying the effector phase of the disease.

The clinical signs and disease course of EAE vary depending upon the strain of mice used, the immunizing antigen, the adjuvant, the timing of immunization, and the dose of antigen/adjuvant used. The first sign of clinical EAE is typically ruffled fur and weight loss. This is usually followed by the presentation of a limp tail and eventually hind limb weakness and paralysis [28]. For laboratory studies, EAE animals are given a corresponding clinical score that reflects disease state as indicated in Table 1. A score of 0 is given if an animal shows no signs of EAE. A score of 1 is given for a loss of tail tone. A clinical score of 2 is assigned for an animal with mild hind limb weakness. A score of 3 is given for moderate to severe hind limb weakness. A clinical score of 4 corresponds to hind limb paralysis and a score of 5 is given if the animal becomes quadriplegic. If an experimental animal dies from EAE, a clinical score of 6 is assigned [28, 29]. This type of disease progression is typical in mice and rats. Other species may have a different clinical course.

Table 1. Clinical Grading Scale for EAE

Clinical Score	**Clinical Deficit**
0	No abnormalities
1	Limp tail
2	Mild hindlimb weakness
3	Moderate to severe hind limb weakness
4	Hindlimb paralysis
5	Quadriplegia
6	Death due to EAE

The Role of T cells in EAE

T cells, in particular CD4+ lymphocytes, (although CD8+ lymphocytes are now believed to be involved as well), play an essential role in the induction of EAE as has been demonstrated by various adoptive transfer and blocking studies. Transfer of disease in mice was prevented by *in vitro* depletion of CD4+ T helper cells prior to transfer, but could still be transferred if CD8+ T cells were depleted [30], implicating T helper cells as playing an important role in EAE. This was supported by a study showing prevention of disease by *in vivo* administration of antibodies that depleted CD4+ T cells prior to active immunization [31]. Furthermore, EAE was demonstrated to develop following the transfer of CD4+ T cell clones that were specific for a CNS autoantigen [32]. More recently, the importance of T cells in EAE has been shown in studies where disease is prevented with antibodies that block the costimulatory interactions, such as CD28-CD80/86 and CD40/CD40L, which are crucial for T cell activation [33-35]. It is believed that the primary role of T helper cells in EAE is to instruct macrophages and microglia, by secreting cytokines, to initiate tissue destruction and demyelination [36, 37]. This macrophage activation is driven by the production of Interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) from T cells [38].

Cytokines in EAE

Effector CD4+ T cells are divided into T helper 1 (Th1) and T helper 2 (Th2) subtypes based on the types of cytokines they secrete following antigen stimulation and their ability to stimulate cell-mediated or humoral immune responses [39] (Fig. 2). Cytokines are soluble proteins that mediate interactions between cells of the immune system and regulate immune functions. They have either predominately pro-inflammatory or anti-inflammatory effects and in EAE, the ability of an animal to respond to immunization by producing either pro or anti-inflammatory cytokines correlates with the ability to resist or be susceptible to disease [40]. Th1 cells produce pro-inflammatory cytokines such as IFN-γ, lymphotoxin (LT), and TNF-α, where as Th2 cells produce anti-inflammatory cytokines such as IL-4, IL-5, and IL-13. The Th1 cell phenotype has been shown to induce EAE effectively, and CFA is a strong adjuvant that primes for a Th1 response [41]. In contrast, the development of a Th2 response does not lead to induction of EAE and is associated with recovery, resistance and suppression of disease. This is supported by a study showing that immunization with autoantigen in incomplete Freund's adjuvant, which lacks *Mycobacterium,* promotes a Th2-like phenotype and does not induce EAE [42]. The differentiation of naïve T cells to Th1 cells occurs in the presence of IL-12 produced by dendritic cells (DC) [43]. Furthermore, it has been shown that microbial products, such as CpG DNA, that are recognized by Toll-like receptors (TLRs) stimulate DC activation and IL-12 production which can drive Th1

differentiation and induce EAE [44, 45]. The prevention of disease by *in vivo* administration of a neutralizing antibody to IL-12 has demonstrated the important subunit specific role of this cytokine in EAE induction [46]. In addition, IL-12p40 deficient mice are resistant to EAE, where as IL-12p35 deficient mice are not [47]. It has also been shown more recently that another member of the IL-12 family, IL-23, is essential for the initiation of EAE [48]. IL-23, which is produced by macrophages and dendritic cells, is a heterodimeric cytokine comprised of the p40 subunit of IL-12 and a specific p19 subunit [49]. IL-23 has been shown recently to play a critical role in the maintenance of a newly defined population of T helper cells called Th17 cells, which have been implicated in EAE pathogenesis [50-53].

The cytokines important for the progression of EAE include the Th1 associated cytokines such as IL-12p40, IFN-γ, TNF-α, and lymphotoxin α (LT-α), as well as the recently classified Th17 associated cytokines such as IL-6, IL-1β, IL-23 and IL-17 . IFN-γ secreted by Th1 cells results in the upregulation of MHC and costimulatory molecules on antigen presenting cells (APCs), such as microgila, in the CNS [37, 54]. Gamma interferon also enhances adhesion molecule expression on endothelial cells and aids in the infiltration of mononuclear cells into the CNS as seen in EAE and MS lesions [54]. However the role of IFN-γ in EAE is somewhat controversial. IFN-γ and IFN-γ receptor deficient mice have been shown to develop a more severe form of EAE than wild-type controls [55-57]. Furthermore, the treatment of mice with neutralizing

anti-IFN-γ antibodies led to an exacerbation of disease when the antibodies were given early, but not at later time points, suggesting that this cytokine may play both a regulatory and a pathogenic role in disease, and may function differently in peripheral lymphoid organs and the CNS [58, 59]. TNF-α and LT-α have cytotoxic effects on oligodendrocytes, the cells that produce myelin, leading to apoptosis of these cells [60, 61]. TNF-α also promotes the release of nitric oxide (NO) from macrophages resulting in further damage of oliogodendrocytes and demyelination [62]. Neutralizing antibodies to both of these cytokines have been shown to reduce EAE severity [63]. Similar studies have shown that the pro-inflammatory cyokines IL-1, IL-6, IL-23p19, IL-12p40, and IL-17 are associated with pathogenesis in EAE and mice that are deficient in these cytokines have been shown to be resistant to disease induction [47, 48, 64-67].

Contrary to Th1 cytokines, Th2 cytokines are often associated with suppression and remission of EAE. IL-4, which is a critical cytokine for the development of a Th2 response, has been associated with the remission stage of EAE when expressed in the CNS [68] and most IL-4 producing Th2 cells are nonencephalitogenic [69]. Exogenous IL-4 administration has been shown to significantly ameliorate EAE [69] and immunomodulatory treatments in EAE, such as the administration of retinoids, are mediated via IL-4 and reduce disease severity [70]. Furthermore, studies using IL-4 deficient mice have shown that these mice develop more severe EAE [71], and mice deficient in Signal Transducer and Activator of Transcription 6 (STAT6), which is a transcription

factor through which IL-4 acts, also developed more severe disease [72]. Other Th2 cytokines such as IL-5 and IL-13, as well as the immunomodulatory cytokines IL-10, and TGF-β have also been shown to ameliorate EAE. More specifically, IL-10 expression in the CNS during EAE was found to correspond to the onset of the recovery phase of disease [73], and *in vivo* delivery of IL-10 inhibited EAE [74]. IL-10 deficient mice developed more severe EAE and transgenic mice overexpressing IL-10 were resistant to disease [75]. TGF-β, which inhibits T cell activation, down-regulates expression of MHCII, and induces T cell apoptosis [76, 77], is expressed in the brain during the acute and chronic phases of EAE [78]. Treatment of T cells with TGF-β prior to adoptive transfer prevents passive EAE [79] and systemic administration of TGF-β suppresses acute and chronic EAE [80, 81]. These studies support the concept that Th2 responses are beneficial in EAE where as pro-inflammatory Th1 or Th17 responses are detrimental.

Whether a T cell differentiates into a pro-inflammatory, EAE initiating, Th1/Th17 cell or an anti-inflammatory Th2 cell during development and activation is ultimately dependent on cytokine milieu and is tightly regulated by intracellular signaling pathways that culminate in the induction of one or more transcription factors. These transcription factors, which can be subset specific, play an important role in controlling further cytokine production and lineage commitment of the T helper cell. Figures 3 and 4 depict signaling pathways that are involved in Th1 and Th2 cell differentiation respectively.

Transcriptional Regulation of T helper Cells

The analysis of T cell specific transcriptional regulatory elements has led to the identification of several families of transcription factors that appear to be important regulators of T helper cell development and activation. These factors include, but are not limited to, GATA zinc-finger proteins, T-box family proteins, members of the NFkB/Rel/NFAT family, and STAT proteins. In recent years, transcription factors belonging to the nuclear hormone receptor superfamily, such as peroxisome proliferator activated receptors (PPARs), have also been shown to be expressed by lymphocytes and are now believed to play an important immunomodulatory role in T helper cells [82]. A better understanding of these transcription factors that regulate the differential expression of cytokine genes and the differentiation of Th1 and Th2 cells could further elucidate the role of T helper cells in destructive autoimmune diseases such as MS.

T-box Expressed in T cells (T-bet)

T-bet is a member of the T-box family of transcription factors that contain a highly conserved DNA binding domain, called the T-box, and bind to a core DNA sequence in promoter regions of a diverse set of genes. It was originally cloned based on its ability to bind the Th1-specific IL-2 promoter [83]. T-bet is expressed in Th1, but not Th2 cells and overexpression of T-bet in primary T

cells can force Th precursor cells, differentiating Th2 cells, and even fully polarized Th2 cells to a Th1 phenotype [83]. This Th1 phenotype is indicated by the production of IFN-γ and the repression of Th2 cytokines such as IL-4 and IL-5 [83]. T-bet expression has been found to correlate directly with IFN-γ expression and T-bet has been shown to transactivate the IFN-γ gene and induce chromatin remodeling of individual IFN-γ alleles [83, 84]. For these reasons, T-bet is believed to activate Th1 genetic programs, at least in part, by regulating IFN-γ gene transcription. Furthermore, *in vivo* studies using T-bet deficient mice demonstrated that these mice have normal lymphocyte development, but displayed overwhelming defects in initiating Th1 responses [85]. T-bet deficient Th and natural killer (NK) cells were found to produce little, if any, IFN-γ [85], and DCs as well as CD8+ T cells have been shown to be dependent on T-bet for optimal IFN-γ production [86-88]. IFN-γ production by B effector 1 cells has also been demonstrated to depend on T-bet [89]. In addition, a corresponding increase in Th2 cytokines has been shown in T-bet deficient mice. More specifically, T-bet-deficient mice spontaneously develop physiological and inflammatory changes characteristic of asthma, a Th2-mediated disease, orchestrated by Th2 lymphocytes [90], and the number of T-bet expressing cells was found to be reduced in the airways of asthmatic patients [90]. Taken together this data has led to the conclusion that T-bet is required for Th1 lineage commitment and is a master regulator of the pro-inflammatory immune response.

T-bet and Th1-mediated Autoimmune Disease

Because Th1 responses are often associated with the development of autoimmune disease, studies in recent years have focused on the pathogenic role of T-bet in autoimmunity. Loss of T-bet expression has been shown to correlate with the development of tolerance in a Th1 TCR transgenic model of autoreactive T cells [91], supporting the notion that T-bet is a critical element in the pathogenesis of Th1 mediated autoimmunity.

T-bet has been extensively studied in models of inflammatory bowel disease, and both Celiac and Crohn's diseases, which are considered to be Th1 related syndromes, have demonstrated enhanced T-bet activity and expression [92, 93]. In an inflammatory bowel disease mouse model, deficiency in T-bet was protective and overexpression of T-bet promoted disease [94]. In inflammatory arthritis models the role of T-bet is somewhat conflicting. T-bet deficiency has been shown to protect mice from arthritis in the passive collagen antibody induced model due to a requirement for T-bet in dendritic cells to express inflammatory mediators, such as IL-1α [95]. However, in other models of arthritis, T-bet deficient mice are more susceptible to disease, and in humans with rheumatoid arthritis (RA) T-bet expression has been shown to correlate with lower disease activity [96, 97]. In animal models of type 1 diabetes, the role of T-bet has also been found to be conflicting, but in humans the disease has been

found to be associated with two T-bet polymorphisms, one of which results in increased IFN-γ transactivation, suggesting that T-bet expression may correlate with disease pathogenesis in at least a subset of diabetic patients [98].

Evidence also suggests that T-bet might contribute to pathogenesis in MS. T-bet deficient mice have been shown to be protected from MOG induced EAE [99], and we have shown that mice are protected from EAE when T-bet is silenced using RNA interference, as shown in chapters III and IV of this document. Furthermore, the severe EAE that develops in mice that are deficient in matrix metalloproteinase 12 (MMP 12) has been shown to correlate with enhanced T-bet expression and Th1 activity [100]. Indeed, agents that have been shown to be effective in ameliorating EAE, such as lovastatin and γ-secretase inhibitors, have been demonstrated to reduce T-bet expression [101, 102]. Therefore, therapies that target T-bet may be effective in ameliorating Th1-mediated autoimmune disease.

GATA3

GATA3 is a member of the GATA family of zinc finger proteins that contains six members, GATA1-GATA6 [103]. GATA proteins bind as monomers to consensus GATA sequences, (A/T)GATA(A/G), and to related nonconsensus GATA motifs such as CGATGG and AGATTA [104]. GATA1,2, and 3 belong to

the same subfamily of GATA proteins and are expressed in hematopoietic cells [105].

GATA3 was originally cloned as a T cell-specific transcription factor that bound to the enhancer of the TCR α gene [106]. Expression of GATA3 in hematopoietic cells appears to be restricted to T and NK cells [106] and studies done in RAG2-/- mice named GATA3 the earliest known transcription factor required specifically for T cell lineage commitment [107]. It is rapidly induced by IL-4/STAT6 and has also been demonstrated to regulate both Th2 cytokine expression and the differentiation of naïve T helper cells into Th2 cells [108]. Upregulation of GATA3 in primary T cells has been shown to be dependent on TCR signals and is blocked by the NFkB inhibitor, SN50, implicating NFkB in the regulation of GATA3 [109]. Using representational difference analysis, GATA3 was found to be selectively upregulated during the differentiation of Th2 but not Th1 cells [108]. GATA3 was also found to bind the IL-5 promoter and to activate the expression of an IL-5 promoter construct after cotransfection into Th1 and nonlymphoid cells [110]. In addition to directly binding the IL-5 and IL-13 promoters, GATA3 has been demonstrated to play an important role in establishing changes in chromatin structure that occur within the IL-4 locus during Th2 differentiation [111]. Naïve T cells express low levels of GATA3, but these levels are upregulated quickly following stimulation under Th2 conditions. Furthermore, GATA3 is believed to undergo a process of autoactivation to enhance its own expression, and this occurs independently of STAT6 [112].

GATA3 and Immune-Mediated Diseases

Several studies have illustrated the importance of GATA3 in Th2 immune-mediated diseases. Transgenic mice that overexpress a dominant negative mutant of GATA3 have been shown to be more resistant to allergic asthma, a disease mediated by Th2 cells [113]. Th2 cells derived from these animals expressed lower levels of Th2 cytokines following rechallenge with antigen. In addition, GATA3 anti-sense oligonucleotides added during the differentiation of Th2 cells *in vitro* significantly reduced levels of GATA3 protein and subsequent production of IL-4, and local administration of GATA3 antisense oligonucleotides attenuated airway hyperresponsiveness, mucus production, and infiltration of eosinophils in an animal model of allergic asthma [90]. Data from these studies strongly suggests that GATA3, similar to T-bet in Th1 cells, is a master regulator of the Th2 immune response, and taken together with studies in T-bet deficient mice, it is apparent that maintaining a Th1/Th2 balance is essential for a controlled immune response. Therefore, in designing therapies for Th1 mediated diseases, such as EAE/MS, it is important to consider the possible deleterious effects of skewing the immune response too heavily to a Th2 response.

Peroxisome Proliferator Activated Receptors (PPARs)

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors belonging to the nuclear hormone receptor superfamily, which includes vitamin D, retinoid, and thyroid hormone receptors [114]. Nuclear hormone receptors consist of an N-terminal activation domain, a highly conserved DNA-binding domain, and a conserved C-terminal ligand binding domain [115]. The nuclear receptors bind as monomers, homodimers, or heterodimers to DNA response elements in the promoter or enhancer regions of target genes [116]. Following activation by ligand, PPARs form heterodimers with another member of the nuclear hormone receptor superfamily, retinoid X receptors (RXR), and bind to a DNA response element known as a PPAR response element (PPRE) in the regulatory regions of target genes [117, 118]. PPREs contain repeats of the sequence AGGTCA separated by one or two nucleotides [119]. Ligand induced activation of PPARs is accompanied by minor structural changes to the receptor resulting in the ability to dissociate from co-repressor complexes and associate with transcriptional co-activators. More specifically, in an unliganded state, PPARs bind various transcriptional co-repressors and histone deacetylases (HDACs) in a DNA-independent manner. In the presence of agonist, these co-repressors become dissociated allowing transcriptional co-activators, such as CREB-binding protein and steroid receptor coactivator-1 (SRC-1), to bind to the ligand binding domain of activated PPARs.

These co-activators have histone acetyltransferase activity, which enables the remodeling of chromatin structure and promotes initiation of transcription [120].

The initial cloning of PPARs occurred while searching for a molecular target of peroxisome proliferating agents in the liver of rodents. These peroxisome proliferators (PP) were named for their ability to increase the number and activity of liver peroxisomes after administration in rodents [121]. Three isoforms of PPARs have been identified, PPARα, PPARγ, and PPARβ/δ, and they are encoded by distinct genes on different chromosomes. Although the different isoforms have some sequence and structural homology, they vary in tissue distribution and exhibit differences in function [122, 123]. PPARs have been most extensively studied in the regulation of genes involved in lipid metabolism. Various types of naturally occurring fatty acids can bind to and activate PPARs. Some eicosanoids derived from the metabolism of arachidonic acid, including leukotrienes, have also been identified as ligands for certain PPAR isoforms. More specifically, PPARγ is activated by endogenous agonists composed of polyunsaturated fatty acids such as α and γ linolenic acids, arachidonic acid and eicosapentaenoic acid [124]. PPARα can be activated by polyunsaturated acids as well, but can also be activated by some medium-chain saturated and monounsaturated fatty acids such as palmitic and oleic acids [124]. Similar to PPARα, PPARβ/δ can be activated by some saturated, monounsaturated, and unsaturated fatty acids [124]. In terms of activation by eicosanoids, PPARγ is stimulated by 15-deoxy-Δ prostaglandin J_2 ($15dPGJ_2$),

PPARα is activated by leukotriene B_4 (LTB_4), and PPARβ/δ responds to prostaglandin A_1 and prostaglandin D_2 [125, 126]. Activation of PPARs that is induced by binding of endogenous ligands contributes to the regulation of lipid homeostasis. PPARα regulates fatty-acid metabolism by regulating genes that transport intracellular fatty acids into peroxisomes and mitochondria for β oxidation. PPARγ regulates adipogenesis and plays a role in insulin sensitization and cellular differentiation, and activation of PPARβ/δ increases high density lipoprotein (HDL) and decreases serum triglycerides. Synthetic ligands or agonists also exist for the PPAR isoforms. The most well characterized of these synthetic ligands are probably the anti-diabetic thiazolidinediones (TZD), which were developed based on their insulin-sensitizing effects in animals. It was concluded, following a series of studies, that TZDs, such as rosiglitazone, pioglitazone, and ciglitazone, were ligands for PPARγ [127, 128]. Furthermore, a class of FDA approved drugs called fibrates, which have been proven to be safe and effective for the treatment of hypertriglyceridemia, are PPARα agonists. Examples of fibrates include clofibrate, ciprofibrate, fenofibrate, and gemfibrozil [129].

More recently, PPARs have been shown to play an important role in the regulation of the inflammatory response. PPARα and PPARγ are known to be expressed by macrophages, dendritic cells, and B and T lymphocytes [130], leading to the hypothesis that PPARs could be involved in various aspects of immunoregulation. Consistent with this, we have found that PPARα agonists

induce immune deviation to a Th2-like phenotype and protect mice from EAE. Results from this study will be presented in Chapter V.

The Role of PPARs in Inflammation and Immunity

PPARs are believed to play an important role in various aspects of the immune response, including both innate and adaptive immunity. For example, both PPARα, and PPARβ/δ are believed to contribute to the process of wound healing [131] and, PPARγ, via its ability to upregulate the scavenger receptor CD36, has been shown recently to enhance the phagocytosis of malarial parasites by macrophages, which is mediated by the innate immune system [132].

Furthermore, recent data demonstrates the important roles of PPARs in the regulation of the adaptive immune response at various stages of the response and in various cell types, including DCs, T cells and B cells. It is possible that depressed expression or activity of the PPAR isoforms could lead to an inability to mount an effective immune response or could contribute to the exacerbation of autoimmune disease.

PPARγ and the Immune Response

Many recent studies indicate PPARγ in the regulation of the inflammatory immune response. PPARγ specific ligands have been shown to inhibit the production of many inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, the inducible production of nitric oxide, and the expression of matrix metalloproteinase 9 (MMP9) on macrophages, monocytes, and epithelial cells [133]. It has also been demonstrated that DCs express PPARγ and agonist-induced activation of this isoform can influence the maturation of DCs [134]. PPARγ has been shown to be expressed in T cells and its activation was demonstrated to inhibit the expression of IL-2 following T cell activation [82]. This decrease in IL-2 was believed to be mediated by blocking the downstream effects of the transcription factors NFAT and NFkB and by an increase in the apoptosis of T cells that are activated in the presence of PPARγ ligands [135, 136]. More recent studies have shown the ability of ligand-activated PPARγ to inhibit IL-12 production by DCs and IFN-γ production by Th1 cells [137], suggesting a role for this nuclear hormone receptor in Th cell differentiation. In addition, PPARγ +/- mice have been shown to be more susceptible to experimentally induced arthritis and inflammatory bowel disease, and PPARγ agonists can inhibit the clinical signs of EAE [138-140], supporting the idea that PPARγ has anti-inflammatory properties, and agonists for PPARγ could be beneficial in the treatment of Th1 inflammatory-mediated diseases.

PPARα and the Immune Response

PPARα, the first PPAR to be cloned, was first proposed to be a modulator of inflammation when Leukotriene B_4, which is a chemotactic factor for neutrophils and an inflammatory mediator, was identified as one of its natural ligands. This was supported by the finding that PPARα deficient mice have more prolonged inflammatory responses than wildtype littermates in response to LTB_4 [141]. In addition, PPARα agonists have been shown to decrease the expression of the pro-inflammatory cytokine IL-6 and the adhesion molecule VCAM, as well as decrease NF-kB activation, IL-12, and IL-6 production in aged mice [142, 143]. PPARα ligands are believed, in part, to inhibit the functional expression of NF-kB by sustaining the expression of the negative regulator IkBα [144]. More recently, the PPARα agonist WY14,643 was demonstrated to inhibit IgG responses in mice immunized with the MOG peptide 35-55 in combination with CFA [145]. In this study, splenocytes from agonist treated mice were found to have impaired production of IFN-γ, IL-6, and TNF-α. PPARα agonists have also been shown to induce apoptosis in macrophages [146]. Moreover, the fibrates gemfibrozil and ciprofibrate, which are synthetic PPARα agonists, have been demonstrated to induce IL-4 secretion by splenocytes, resulting in the development of a Th2-like phenotype [137], and a recent study concluded that unliganded PPARα can suppress T-bet expression and decrease IFN-γ production in T cells via the p38 MAP kinase pathway [147]. The PPARα agonists gemfibrozil and fenofibrate have also been shown to be effective in

preventing the induction of EAE [148], further supporting the concept that these agents are immunomodulatory and could provide an effective treatment for Th1-mediated diseases.

Transcriptional Regulation in EAE and Autoimmunity

In recent years, transcription factors have been investigated for their contribution to autoimmune disease. It is believed that their varied patterns of activity can account for differences in gene expression that are seen in autoimmune versus nonautoimmune cells [149]. Several transcription factors involved in Th cell differentiation, including T-bet and PPARs, have been studied specifically for their role in the pathogenesis of EAE and MS. Based on its ability to induce a proinflammatory Th1 response, T-bet has been found to regulate genes that have been directly linked to increased pathogenesis in EAE and possibly MS. In contrast, PPARs, specifically PPARα and PPARγ have been demonstrated to regulate anti-inflammatory genes and are believed to be associated with protection from EAE and could play a role in MS as well. Although the role of transcription factors in EAE and MS has become increasingly more defined, there is still much to be discovered regarding specific mechanisms of transcriptional activation that occur during disease pathogenesis. This increased understanding of transcriptional activation of T helper cells in EAE and MS could lead to the development of novel and more specific targets for therapeutic intervention. This study sought to examine the molecular

mechanisms associated with transcriptional activation of T-bet and PPARα in the EAE model.

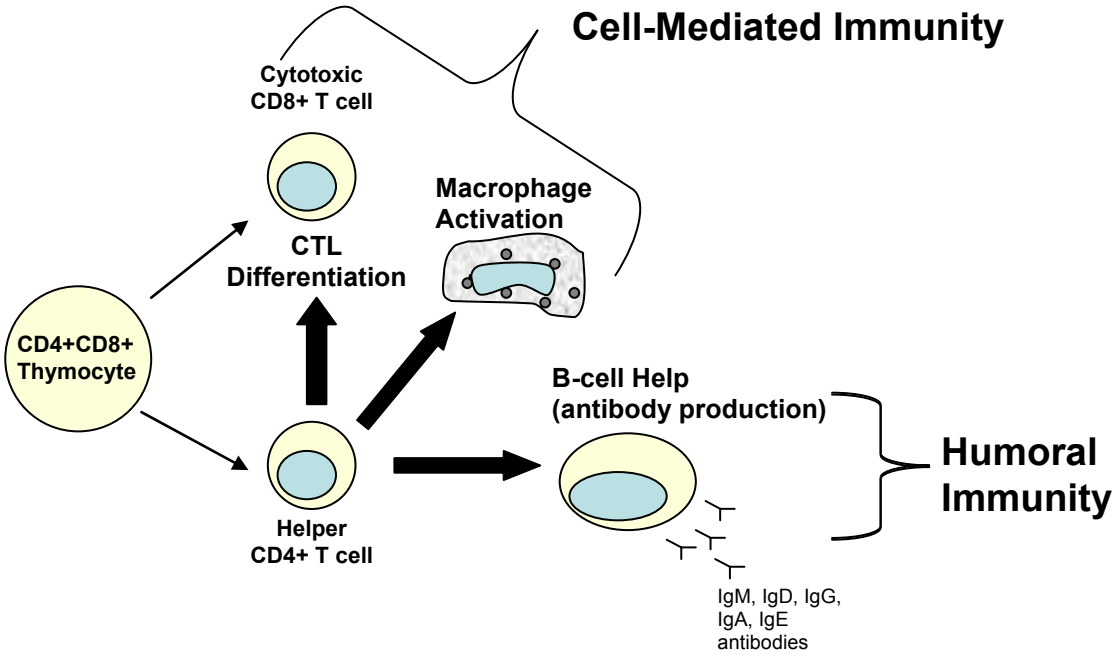


Figure 1. T cell Differentiation
CD4+ and CD8+ T cells arise from a common lymphoid progenitor cell in the thymus. CD4+CD8+ thymocytes undergo selection and become single positive CD8+ T cells or CD4+ T helper cells. Cytotoxic T cells are essential for the elimination of cells containing intracellular pathogens, such as viruses. Helper T cells secrete cytokines and function to activate macrophages and CD8+ T cells, resulting in a cell-mediated immune response. Alternatively, T helper cells stimulate B cells to produce antibodies, resulting in a humoral immune response.

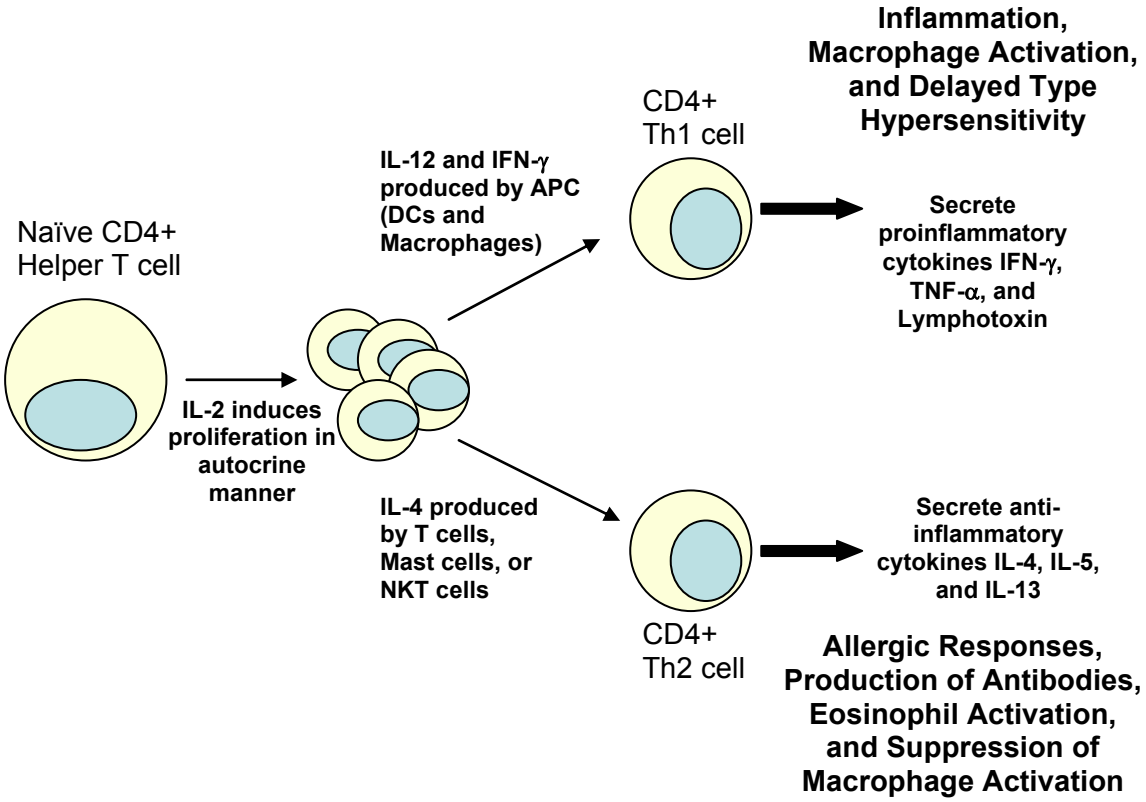
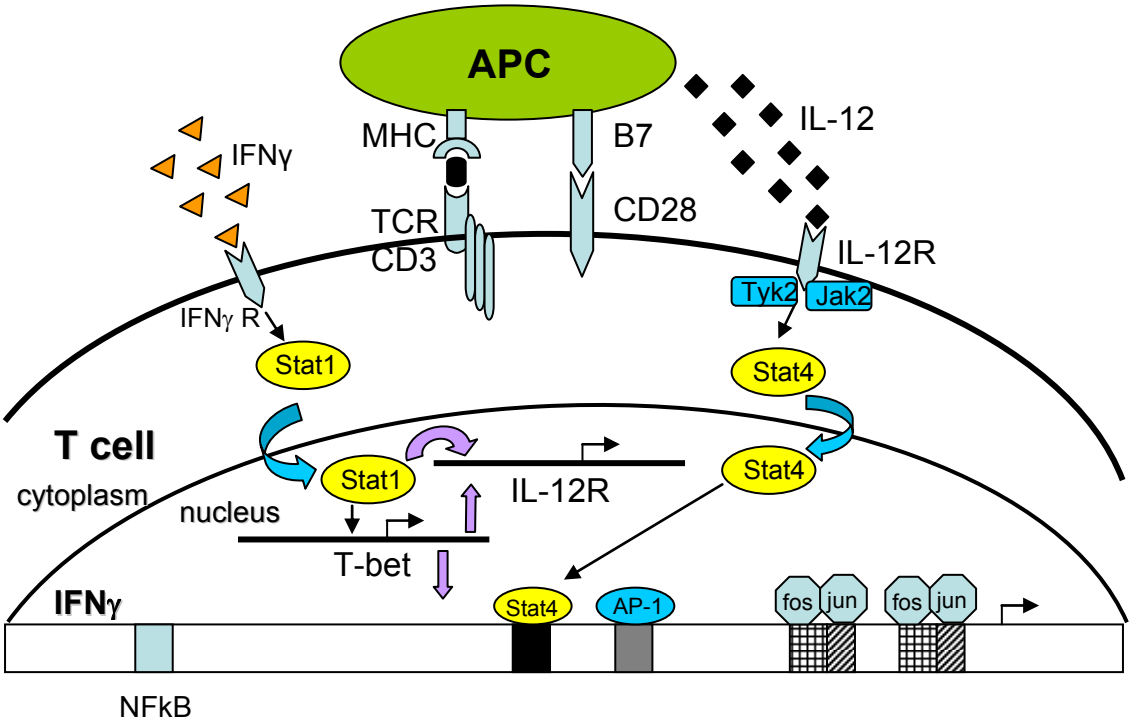
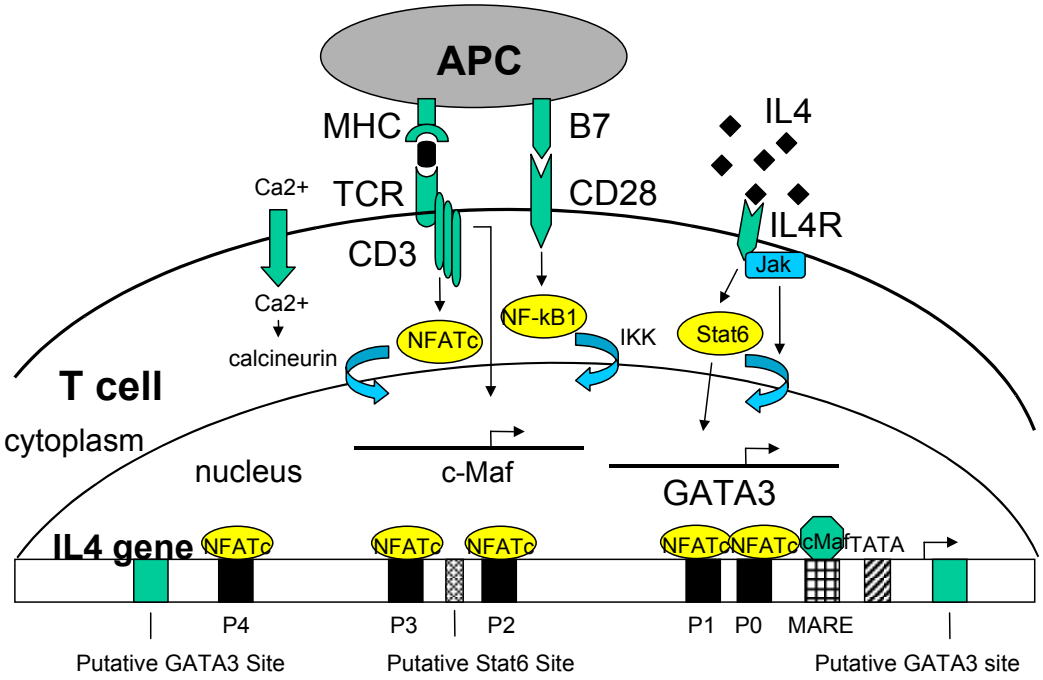


Figure 2. Differentiation of T helper cells into Th1 and Th2 subsets
Cytokines that are produced during the innate immune response influence the differentiation of naïve CD4+ T helper cells into Th1 or Th2 cells. IL-12 and IFN-γ are produced by macrophages, dendritic cells, and NK cells in response to microbes. These cytokines stimulate the differentiation of Th1 cells which produce the proinflammatory cytokines IFN-γ, TNF-α and lymphotoxin. Th1 cells mediate inflammation, macrophage activation, and delayed type hypersensitivity. Alternatively, IL-4, which may be secreted in small amounts by activated CD4+ T cells in response to helminthic parasites or allergens, by mast cells, or by NKT cells induces differentiation of Th2 cells. Th2 cells produce the cytokines IL-4, IL-5, and IL-13 and mediate allergic responses, production of antibodies, eosinophil activation, and the suppression of macrophage activation.



Adapted from A.E. Lovett-Racke, Ph.D.

Figure 3. Transcriptional Regulation of Th1 Differentiation
IFN-γ produced in response to TCR signaling, or during the innate immune response by NK cells, can bind to the IFN-γ receptor on CD4+ T cells and activate the transcription factors STAT1 and T-bet. T-bet can transactivate the IFN-γ gene and induce upregulation of the IL-12R. IL-12 that is produced by activated APCs interacts with the IL-12R on antigen-stimulated CD4+ T cells. This activates the transcription factor STAT4 which can translocate to the nucleus and transactivate the IFN-γ gene resulting in amplification of the Th1 immune response.



Adapted from A.E. Lovett-Racke, Ph.D.

Figure 4. Transcriptional Regulation of Th2 Differentiation
IL-4 that is produced by T cells, mast cells, or NKT cells in response to parasites or allergens interacts with the IL-4R on the surface of antigen sitmulated CD4+ T cells. This activates the transcription factor STAT6, which translocates to the nucleus to induce Th2 differentiation. STAT6 activation and the production of IL-4 leads to induction of the transcription factor GATA3. GATA3 can transactivate the Th2 cytokine genes IL-5 and IL-13 and induce chromatin remodeling of the IL-4 gene, allowing transactivation of IL-4 by the transcription factor c-Maf. STAT6 dependent IL-4 production is believed to enhance GATA3 resulting in amplification of the Th2 immune response. The transcription factors NFAT and NFkB1, which are induced to translocate to the nucleus following antigen/APC engagement and TCR signaling, also contribute to Th2 differentiation via regulation of IL-4, IL-5, IL-13, and GATA3.

Chapter II. Materials and Methods

Antibodies

Antibodies used for all experiments are commercially available. The T-bet, STAT1, IL-12Rβ2, GATA3, IL-12, and actin antibodies used for western blotting and/or chromatin immunoprecipitation assays were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse IgG HRP, goat anti-rabbit IgG HRP, goat anti-rat IgG HRP, and rabbit anti-goat IgG HRP secondary antibodies were also purchased from Santa Cruz Biotechnology. IL-23R, IL-23p19, and IL-17 antibodies were purchased from R&D Systems (Minneapolis, MN). PPARα and SRC-1 antibodies used for western blotting and chromatin immunoprecipitation were purchased from Affinity Bioreagents (Golden, CO). Capture and biotinylated antibodies that were used for IFN-γ, IL-4, and IL-5 ELISAs were purchased from BD Pharmingen (San Diego, CA). The IL-17 antibodies used for ELISA were purchased from R&D Systems. The anti-CD4-PE and anti-T-bet-FITC antibodies that were used for flow cytometry were purchased from BD Pharmingen and Santa Cruz Biotechnology.

Peptides

Two different myelin peptides were used for EAE induction and *in vitro* cell culture. These were myelin basic protein (MBP) Ac1-11 and myelin

oligodendrocyte glycoprotein (MOG) 35-55. Both peptides were generated for our laboratory by CS Bio (Menlo Park, CA) and were reconstituted in PBS according to manufacturer's instructions. For induction of EAE, MBP Ac1-11 was administered at a 50μg dose and MOG 35-55 was administered at a 200μg dose. For *in vitro* experiments and culturing of cells for adoptive transfer, both peptides were used at a final concentration of 2μg/ml.

Mice

B10.PL mice transgenic for the MBP Ac1-11-specific TCR chains Vα2.3 or Vβ8.2 were kindly provided by Dr. J. Goverman (University of Washington, Seattle, WA). These mice were bred and maintained in a federally approved animal facility at the University of Texas Southwestern Medical Center (Dallas, TX) in accordance with the regulations of the Institutional Animal Care and Use Committee. B10.PL and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in our barrier animal facility at UT Southwestern. C57BL/6 IL-4-/- mice were purchased from Jackson Laboratory and backcrossed onto the B10.PL background in our animal facility. These mice were then backcrossed again for six generations with the Vβ8.2 TCR transgenic mice to obtain B10.PL IL-4-/- Vβ8.2 TCR transgenic mice. All mice were between 7-10 weeks of age when experiments were initiated.

EAE Induction and Murine Cell Culture

For actively induced EAE, B10.PL and B6 mice were injected subcutaneously (s.c.) at four sites over the flanks and shoulders with 50 μg MBP Ac1-11 or 200 μg MOG 35-55 (CS Bio, Menlo Park, CA) in an emulsion with complete Freund's adjuvant (CFA) (Difco, Detroit, MI). Pertussis toxin (200 ng/mouse) (List Biological Laboratories) was injected i.p. at the time of immunization and 48 hours later to enhance the upregulation of adhesion molecules and facilitate blood brain barrier breakdown. Mice were scored on a scale of 0-6: 0, no clinical disease; 1, limp/flaccid tail; 2, moderate hind limb weakness; 3, severe hind limb weakness; 4, complete hind limb paralysis; 5, quadriplegia or premoribund state; 6, death.

EAE was induced in IL-4-/- Vβ8.2 B10.PL mice and IL-4+/+ Vβ8.2 B10.PL mice by s.c. injection as described above with 50μg of MBP Ac1-11 in an emulsion with CFA (Difco, Detroit, MI). No pertussis toxin was administered because it is not necessary to induce disease in these TCR transgenic mice due to the high frequency of MBP-specific T cells.

For adoptive transfer experiments, spleens from Vα2.3/Vβ8.2 TCR transgenic mice (5-10 weeks old) were removed and single cell suspensions were prepared. Splenocytes were cultured in 24-well plates at $2X10^6$ cells/well in complete RPMI. Cells were activated with MBP Ac1-11 (2 μg/ml), rIL-12 (0.5

ng/ml) and irradiated B10.PL splenocytes ($8X10^6$ cells/well). After 72 h, the cells were washed with PBS and $5X10^6$ cells were injected i.p. into naïve B10.PL mice. The mice were evaluated daily for clinical signs of EAE as previously described.

For adoptive transfer of siRNA transfected splenocytes, the spleens from Vα2.3/Vβ8.2 TCR transgenic mice (5-10 wk old) were removed and single cell suspensions were prepared. The splenocytes were cultured in 24-well plates at $2X10^6$ cells per well in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, HEPES buffer, $5 X 10^{-5}$ M 2-ME and non-essential amino acids. The cells were transfected with AS oligonucleotides and siRNA as described below. Splenocytes from wild-type B10.PL mice were irradiated with 3000 rads and cultured in 24-well plates at $6X10^6$ cells per well in RPMI 1640 supplemented as described above except the fetal bovine serum concentration was increased to 10% and pencillin/streptomycin were added at 100 U/ml. MBP Ac1-11 peptide was added to the cultures at 2 µg/ml. After 24 hr transfection of the transgenic splenocytes and 24 hr incubation of the irradiated wild-type splenocytes with peptide, the transfected cells were washed with HBSS and added to the irradiated wild-type splenocytes. The cells were incubated for an additional 72 hr, then the cells were washed with PBS twice, and $10X10^6$ activated cells were injected i.p. into wild-type B10.PL mice. The mice were evaluated daily for clinical signs of EAE as described previously.

Preparation of Cell Lysates

Cell lysates were prepared to be used for western blotting. For the preparation of nuclear protein extracts, cells were collected 24 or 48 hours post stimulation with antigen (MBP Ac1-11) (15-20X10^6 cells/condition) and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL) according to manufacturer instructions. In brief, the cells were spun down and resuspended in reagent CER I. The cells were vortexed for 15 sec. and incubated on ice for 10 min. CER II was added to the cells which were then vortexed for 5 sec., incubated on ice for 1 min., and then vortexed again for 5 sec. The tubes were then centrifuged for 5 min at 20,000g. The supernatants were removed, the reagent NER was added to the pellets, vortexed for 15 sec., and incubated on ice for 10 min. The tubes are vortexed, and incubated on ice 3 more times for a total of 40 minutes, and then centrifuged for 10 min at 20,000g. The supernatants, containing the nuclear extract, were transferred to chilled clean tubes and flash frozen along with cytoplasmic extracts at -80 degrees Celsius.

For preparation of total cell lysates, cells were collected, spun down, and resuspended in SDS-lysis buffer. Cells were lysed on ice for 30 min. and spun down to remove cell debris. Total cell lysates were also made from the brains of mice using a tissue homogenizer and lysing with SDS-lysis buffer. Protease inhibitors (aprotinin, leupeptin, and pepstatin, Pierce Biotechnology, Rockford, IL)

were added to all lysates at time of preparation. Prior to use, the protein concentration of all lysates was determined using the BioRad Protein Assay (BioRad, Hercules, CA) according to manufacturer's instructions.

Western Blotting

To evaluate protein expression in various experiments, western blots were performed using nuclear or whole cell lysates. The nuclear or whole cell lysates were diluted in 5X or 10X SDS-loading buffer and boiled for 3 min to denature the protein. 10-30 µg of the extracts were electrophorectically separated on 10% or 4-20% SDS-PAGE gels (Criterion gels purchased from BioRad Laboratories, Hercules, CA) and transferred to polyvinylidene fluoride (PVDF) or nitrocellulose membranes (Millipore Limited, Bedford, MA) using a semi-dry transfer system (BioRad Laboratories, Hercules, CA). The membranes were blocked on a rocker overnight at 4°C with 5% non-fat dry milk dissolved in TBS/Tween. Primary antibodies were diluted 1:100 – 1:10,00 (depending upon the antibody used) in blocking buffer and added to the membranes for 1-2 hr., rocking at room temperature. The membranes were then washed 3X in TBS/Tween for a total of 30 minutes. Secondary antibodies conjugated to horse radish peroxidase (HRP) were diluted 1:1000 – 1:20,000 (depending upon the antibody used) and added to the membranes for 45 min -1 hr., rocking at room temperature. The membranes were washed 3X for a total of 30 minutes in TBS/Tween and an enchanced chemiluminescent substrate (ECL) (Amersham, Pistacaway, NJ) was

added for 1 min. The blots were exposed to film for various times (.05-10 min) and developed. Antibodies used are commercially available and described previously.

Densitometry

To quantitate relative protein expression on western blots, densitometry was performed. The density of the bands was determined using the spot density option on an AlphaImager™ (Alpha Innotech Corp.). The data was normalized by dividing the average density (actual density – background) of the experimental band by the density of the actin control band.

Chromatin Immunoprecipitation Assay

To identify genes that may be directly regulated by T-bet or PPARα, chromatin immunoprecipitation assays (ChIP) were performed. All buffers used for this procedure are listed in Table 2.

Table 2. Chromatin Immunoprecipitation Assay Buffers

Buffer Name	Contents
Cell Lysis Buffer	5mM PIPES, pH 8.0; 85mM KCL; 0.5% NP40; protease inhibitors (leupeptin, pepstatin,and aprotinin)
Nuclei Lysis Buffer	50mM Tris-Cl, pH 8.1; 10mM EDTA; 1% SDS
IP Dilution Buffer	0.01% SDS; 1.1% Triton X 100; 1.2 mM EDTA; 16.7 mM Tris-Cl, pH 8.1; 167 mM NaCl
TSE-150	0.1% SDS; 1% Trition X 100; 2mM EDTA; 20mM Tris-Cl, pH 8.1; 150 mM NaCl
TSE-500	0.1% SDS; 1% Triton X 100; 2mM EDTA; 20mM Tris-Cl, pH 8.1; 500 mM NaCl
LiCl Detergent	0.25 M LiCl; 1% NP40; 1% DOC; 1mM EDTA; 10 mM Tris-Cl, pH 8.1
Elution Buffer	100 mM NaHCO3; 1% SDS
5X Proteinase K Buffer	50 mM Tris-Cl, pH 7.5; 25 mM EDTA; 1.25% SDS
Re-ChIP Buffer	1% Triton x 100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-Cl, pH 8.1

For ChIP assays, splenocytes from Vβ8.2TCR transgenic mice were purified and plated at 5 x 10^6 cells per well in a 24 well plate and activated with 2μg/ml MBP Ac1-11 or left unactivated for 24 or 48 hours. Approximately 1 x 10^7 cells per condition were collected for ChIP assay. The cells were cross-linked with 37% formaldehyde (Sigma, St. Louis, MO) to a final concentration of 1% for a total of 20 minutes with agitation. After 20 minutes, 1 ml of 2.5M glycine was added to stop the reaction and the cells were agitated for 10 additional minutes. The cross-linked cells were then centrifuged at 300g for 10 minutes at 4°C. The supernatant was decanted and the cell pellet, containing cross-linked protein/DNA complexes, was washed twice with 1 ml 1X PBS and transferred to eppendorf tubes. The washed cell pellets were then snap frozen with liquid nitrogen and stored at -80°C until use.

The following day, the cells were thawed on ice and resuspended in 200µl of cell lysis buffer + protease inhibitors (0.6µg/ml of leupeptin, aprotinin, and pepstatin, Pierce Biotechnology, Rockford, IL). The cells were then chilled on ice for 10 minutes and centrifuged at 300g for 5 minutes at 4°C in a microcentrifuge to pellet the nuclei. The nuclei (cell pellet) were then resuspended in 200µl of nuclei lysis buffer plus protease inhibitors and incubated on ice for 10 minutes. Following this incubation, the cells were sonicated using a 15 second pulse followed by 30 second rest for a total of 4 times with a microtip sonicator. All sonication and resting steps were performed with the cells on ice. Sonication breaks the protein/DNA complexes into fragments ranging in size from 200-1000 base pairs. Following sonication, the cells were centrifuged at 2400g for 2-3 minutes in a microcentrifuge. The supernatant was then transferred to a new tube and the cell debris (pellet) was discarded. To confirm that the sonication generated appropriate sized DNA fragments, the DNA was run on a 1% agarose gel with a DNA ladder. A smear on the gel that begins at the well and concentrates around 400-600 base pairs indicates that the DNA fragments are an ideal size for the assay. The volume of sonicated protein/DNA complexes was increased to 1400 µl using I.P. dilution buffer and divided into 3 tubes per condition. 200µl was aliquoted for input DNA control, 600 µl was aliquoted for a no antibody or isotype control, and the final 600 µl was aliquoted for immunoprecipitation with an antibody specific for the transcription factor of interest. The input controls were frozen at –20°C until reverse cross-linking was performed. To the remaining samples, 1-2 µl of non-specifc DNA (salmon or

herring sperm DNA) was added, and to experimental samples (IgG isotype control Ab and +Ab), 15μl of IgG isotype control Ab or 15μl of antibody specific for the transcription factor of interest (T-bet, STAT1, or PPARα) was also added. The samples were then incubated overnight at 4°C with agitation.

The following day, the immune complexes were harvested. Forty microliters of Protein A Sepharose beads (Immunopure Plus beads, Pierce, Rockford, IL) were added to the samples and incubated for 1-2 hours at room temperature with agitation. The beads were then centrifuged at 2400g for 2-3 minutes, the supernatant was decanted, and 500μl of wash buffer TSE-150 was added. The samples were then agitated for 5 minutes at room temperature, spun down at 2400g for 2-3 minutes, and the supernatant was decanted. This process was repeated using wash buffer TSE-500, LiCl/detergent, and finally with TE. Following the I.P. washes, the immune complexes were eluted from the beads. Then, 500 μl of elution buffer (1%SDS/0.1M NaHCO3) was added to the beads, the samples were vortexed briefly, incubated for 15 minutes with agitation, and centrifuged at 2400g for 2-3 minutes. The supernatant was then transferred to a new tube. To achieve a higher yield of immune complexes, this process was repeated.

Following the elution, the cross-linking was reversed. To reverse cross-linking 10% of the final volume of 3M NaOAc was added to all the samples, they were vortexed briefly, and incubated overnight at 65°C. The input DNA controls

were added at this step. The input controls were removed from the freezer and thawed on ice. Then the volume was increased to 500 µl with elution buffer before the 3M NaOAc was added and cross-linking was reversed as stated above.

The next day, DNA purification steps were initiated with the addition of 1 ml of 100% ETOH to all samples. The samples were then frozen at –20°C overnight. After freezing, the samples were centrifuged for 30 minutes at 2400g at 4°C. The supernatant was discarded and the pellets were resuspended in 1 ml 70% ETOH. The samples were then centrifuged again for 15 minutes at 4°C. The supernatant was discarded and the pellets were resuspended in 100 µl TE buffer. Following the ETOH precipitation, 25 µl of 5X proteinase K buffer and 1.5 µl of 10mg/ml proteinase K were added to all samples to digest the protein. The samples were incubated at 42°C in a water bath for 2 hours. Following incubation, the volume was increased to 400µl with TE buffer and a phenol/chloroform extraction was performed. Briefly, 400µl of 25:24:1 phenol:chloroform:isoamyl alcohol was added to each tube, vortexed, and centrifuged at 2400g for 10 minutes. Following centrifugation, the aqueous layer was removed and transferred to a new tube. Approximately 2-5µg of glycogen, 10% of the final volume of 3M NaOAc, and 1 ml of 100% ETOH were then added to the aqueous phase, and the samples were ethanol precipitated overnight at -20°C.

The next day, all samples were centrifuged for 30 minutes at 4°C, the supernatant was discarded, and 1 ml of 70% ETOH was added. The samples were spun down at 2400g for 15 minutes at 4°C, the supernatant was discarded again, and the pellets were dried using a speed vac. Finally, the pellets containing the DNA previously bound to the transcription factor of interest were resuspended in 150μl of TE buffer and the samples were analyzed using PCR to detect transcription factor binding in promoter regions of genes of interest. PCR was performed using the following conditions: 55^0C annealing for 1 min., 72^0C elongation for 1 min and 94^0C denaturing for 1 min for a total of 30 or 35 cycles. A schematic of the ChIP assay is shown in figure 5. and a list of all primer sets used for ChIP and re-ChIP assays are listed in Table 3.

Table 3. Gene promoter-specific primer sets used for ChIP assay.

Gene	Forward Primer	Reverse Primer
IFN-γ #1	CCATACGCAGACACCATTG	TAGAAACACGAGCTCTGGG
#2	TCTCTTGAGGTCCTCCATGC	GCACATTCTGCTACGCTTG
#3	GAACATAGAACGGTCCCCGT	TCCTGGTCTACAGAGTGAG
#5	CTGGGTCAAGATAACTGGG	TCAGCCAAAGGCTCAACCA
#7	CACGTTGACCCTGAGTGAT	GAGGAAACTCTTGGGCTTC
STAT1 #1	CAGGATGGAGGTTCTCAACCTG	GTGAACGGATATCTGCAGCTCC
#2	GGAAGTGCTTGTGAGCTATC	CCATGCTAACCATCTCTGCC
#3	CAGTGGGTAGAAGGTCTTGCTG	AGTGCATTGGAAAGCTGG
IL-23R #1	ACCTGTCCTGTGGTCCGGAA	GCCAAGCTAAGAACCACAGG
#2	GGCGAGCATTTAACCTACTAAGCC	CCCTTATGTCAGAGCTCTGACT
#3	GTTTTTCCTTGTAGGGGAGG	GCTTGCTTCCAGTCGTAATG
#4	CAAGGCAGCTCACTTTCAG	TCCTGGCTGTGCTGTCTTCT
#5	GCAGCGGCTTGATTGTGAA	ACCAGAGAGGTGAGCATCA
IL-4	CGGACACCTGTGACCTCTTCCTTC	CCAATCAGCACCTCTCTTCCAGGAG
IL-5	CCTGAGTTTCAGGACTCGCC	CCAGACACAGCTGAGAGTCA
GATA3	GGAAAGCTGGTTCGGAGGCA	GCCGATTCATTCGGGCTCAG
cMaf	CGAGGGATCCGGAGAGAGAA	GCGCTTTGCATAAGGAGGGC

ChIP re-ChIP

For ChIP re-ChIP assay, cross-linking, lysis, sonication, and immunoprecipitation with a PPARα specific antibody were performed per normal ChIP assay as described above. Following the initial immunoprecipitation, eluted immune complexes were diluted 10X with re-ChIP buffer and then immunoprecipitated again using an antibody specific for the coactivator SRC-1. Following this step, the assay was continued as routine ChIP assay as described in detail above and PCR amplification of the IL-4 promoter was performed. The primer set used for amplification of the IL-4 gene is listed in Table 3.

Transient Transfection of Cells for Overexpression Assay

Transient transfection of EL4 cells was performed using the AMAXA nucleofection system according to manufacturer's instructions. EL4 cells are a mouse thymoma cell line that have been shown to express low levels of endogenous T-bet. These cells were kindly provided by Dr. James Forman and Dr. Christoph Wuelfing (UTSW, Dallas, TX) and were maintained in our lab using complete RPMI 1640 media. Cells were grown at 37°C in humidified air containing 5-8% CO_2. Two million cells per transfection were resuspended in nucleofection solution L. Setting C-009 on the Nucleofector machine was used

for nucleofection with the standard protocol. Full-length human T-bet cDNA was purchased from ATCC and cloned into a pCS2 mammalian expression vector using standard procedure and sequenced for verification. EL4 cells were transfected with the pCS2-T-bet expression construct or an empty vector control. At 24 hours post-transfection, the cells were collected and RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was then synthesized using the 1st strand reaction mix from Invitrogen. RT-PCR was performed using primers for T-bet, IL-23R, and HPRT (control) to examine the effect of T-bet overexpression on mRNA expression levels for the endogenous target gene IL-23R. The primers sets used are listed in Table 4.

Table 4. Primers used for mRNA analysis following overexpression of T-bet

GENE	FORWARD PRIMER	REVERSE PRIMER
T-bet	GCACCGCTACTTCTACCCGG	CCTCAGTTTCCCCGACACCT
IL-23R	CAGCCTGCAACGCTTCCAAC	GAACCACATCCTTCGCCTGG
HPRT	GATTCAACTTGCGCTCATCTTA	GTTGGATACAGGCCAGACTTTG

Transfection with siRNA and AS oligonucleotides

AS oligonucleotides generated on a phosphorothioate backbone, which increases their resistance to degradation by endonucleases, were purchased from Life Technologies. The siRNA were purchased as purified duplexes from Dharmacon RNA Technologies. Genes targeted were T-bet, STAT1, PPARα, and GATA3. The sequences are shown in Table 5.

For *in vitro* transfection of splenocytes, 2 µl TransIT-TKO® transfection reagent (Mirus) was diluted in 50 µl serum-free/antibiotic-free RPMI 1640 media per well to be transfected. After a 10 minute incubation at room temperature, 1 µl 40 µM siRNA or 2 µl 20 µM AS oligonucleotide was added to 52 µl diluted transfection reagent. The AS oligonucleotides and siRNA were then incubated with the diluted transfection reagent at room temperature with gentle agitation for 30 minutes. The AS oligonucleotides or siRNA were added to the Vβ8.2 transgenic splenocyte cultures containing $5X10^6$ cells in 500 µl media per well of a 24-well plate and incubated overnight at 37^0C. On the following day, the non-adherent cells were collected and washed with fresh media. The adherent cells in the plates were also washed once with fresh media. The non-adherent cells were resuspended in 2 ml media and placed back in their original wells. MBP Ac1-11 peptide was added at 2 µg/ml and the cells were stimulated for 48 hours. For Vα2.3/Vβ8.2 transgenic splenocytes, $2X10^6$ splenocytes were placed in each well of a 24-well plate. The transfection protocol was the same, except the cells were placed with wild-type splenocytes ($6X10^6$ cells/well) that had been irradiated and cultured with MBP Ac1-11 after the 24 hr transfection. A schematic of the transfection protocol is shown in figure 6.

For PPARα experiments, splenocytes from a Vβ8.2 TCR transgenic mouse were transfected *in vitro* with siRNA PPARα or siRNA NS as described above and stimulated in the presence of 100µM gemfibrozil with or without MBP Ac1-11 for 48 hours.

In vivo siRNA administration

Synthetic siRNA specific for T-bet, PPARα, and a NS control were purchased from Dharmacon and stocks were prepared in the manufacturer's buffer at 160 μM and diluted to 20 or 50 μg/100 μl PBS (2 mg/kg/mouse) for i.v. administration via the tail vein. Similarly, AS oligonucleotides specific for T-bet, GATA3, and a NS control were purchased from Life Technologies and injected via the tail vein into mice. The sequences of the siRNA and AS oligonucleotides are listed in Table 5.

Table 5. Sequences of AS Oligonucleotides and siRNA

Gene	**AS sequence 5'->3'**	**siRNA**
T-bet	CTCCACGATGCCCATC	5' UGAUCGUCCUGCAGUCUCUdTdT 3' 3' dTdTACUAGCAGGACGUCAGAGA 5'
PPARα		5'UCACGGAGCUCACAGAAUUUU3' 3'AAUUCUGUGAGCUCCGUGAUU5'
Non-sense (NS)	CTATGTCATCCGCTCCAC	5' CGAACGAGUACCGUACACUdTdT 3' 3' dTdTGCUUGCUCAUGGCAUGUGA 5'
GATA3	AGTCACCTCCATGTCCTC	
STAT1		5'AUUCCAUCGAGCUCACUCAdTdT 3' 3'dTdTTAAGGUAGCUCGAGUGAGU 5'

Preparation and Administration of PPARα Agonists

The PPARα agonists gemfibrozil and fenofibrate (Sigma-Aldrich Co., St. Louis, MO) were administered orally to mice either by gavage or as a food

supplement. For administration of gemfibrozil and fenofibrate, stock solutions (50mg/ml) were prepared by dissolving these agents in ethanol (for Gemfibrozil) or DMSO and then ETOH (for Fenofibrate). When mice were fed by gavage, the stock solution was diluted again in PBS so that the indicated dose (500 μg) of agonist was administered in a total of 200μl. A 500μg dose administered to a mouse weighing approximately 25g is equivalent to a 20mg/kg dose per day. Patients prescribed gemfibrozil for elevated triglycerides are recommended to take two 600mg tablets daily. In an average person weighing 75kg, this is a 16mg/kg dose daily, which is close to the dose administered to mice in the experiments presented herein. Alternatively, mouse chow was supplemented with gemfibrozil or fenofibrate by adding the stock solution to the chow (0.25% w/w), allowing the ethanol to evaporate, and then using this chow as the source of food. This is the recommended dose and method of administration found in lipid and metabolism literature, and allows the animals continuous access to the drug. The control animals were fed a diet in which the same amount of ethanol was added to the chow without the PPARα agonist.

Intracellular Staining and Flow Cytometry

Splenocytes from a Vα2.3/Vβ8.2 TCR transgenic mouse were purified and plated at 5 x 10^6 cells/well in triplicate (3 wells/condition). There were 6 wells/condition and the conditions were as follows: Media alone, Ac1-11 at 2μg/ml, or Ac1-11 at 2μg/ml + IL-12 at 1 ng/ml. The cells were activated for 24

hours. Following 24 hours of activation, the cells were collected and placed into 6 tubes per condition with approximately 0.5 to 1 million cells per tube. Each condition received 3 separate stains: unstained, IgG1-FITC isotype control stain, or IgG1 T bet FITC stain. There were a total of 18 tubes plus 3 tubes (unactivated cells) for compensating the machine. In addition, all of the cells were stained with CD4 PE allowing us to gate on CD4+ T cells. The compensation tubes were Vα2 FITC, CD4 PE, and unstained.

The cells were collected in the appropriate tubes and washed with FACS Buffer. The cells were then stained with CD4-PE (10ul per 1million cells, BD Pharmingen, San Diego , CA) and the appropriate markers for the compensation tubes (Vα2 FITC, CD4 PE, and unstained) for 30 minutes. Following the 30 minute incubation, cells were washed with FACS buffer, centrifuged for 10 minutes at 25g, decanted and resuspended as described above. The compensation tubes were then fixed in 250-500μl of PFA, and the remaining experimental tubes were permeabilized with 750μl of 2X FACS Lysing Solution (Becton Dickinson) supplemented with 0.025% tween 20 for 10 minutes at room temperature. Following the permeablilization, cells were washed twice. The cells were then stained with T-bet-FITC or IgG1-FITC as a control (10μl per 1 million cells, BD Pharmingen, San Diego, CA) and incubated for 30 minutes. Following the incubation, the cells were washed with FACS buffer and fixed in 250-500μl of PFA. Cells were gated on viable CD4+ lymphocytes and the data was analyzed using Cell Quest Pro software.

ELISA

Cytokine expression was determined in the supernatants of lymph nodes and splenocytes taken from siRNA T-bet or siRNA NS mice and of splenocytes transfected *in vitro* with siRNA/AS T-bet or NS cultured at $4X10^6$ splenocytes/ml in 24-well plates activated with MOG 35-55 or MBP Ac1-11 (2 μg/ml). Similarly, cytokine expression was determined in the supernatants of splenocytes taken from siRNA PPARα or siRNA NS treated mice that were fed gemfibrozil or ETOH and were restimulated at $5X10^6$ splenocytes/ml in 24-well plates by activating with MBP Ac1-11 (2 μg/ml). Mouse IFN-γ, IL-4, IL-5, and IL-17 were quantified using a sandwich ELISA based on noncompeting pairs of antibodies. Purified anti-mouse IFN-γ, IL-4, IL-5 (BD Pharmingen, San Diego, CA), and IL-17 (R&D Systems, Minneapolis, MN) were diluted in 0.1 M $NaHCO_3$, pH 8.2, at 2 μg/ml. Immunolon II plates (Dynatech Labs) were coated with 50 μl per well and incubated overnight at 4^0C. The plates were washed 2X with PBS/0.05% tween 20 and blocked with 200 μl 1% BSA in PBS per well for 2 hr. The plates were then washed 2X with PBS/tween and 100 μl of supernatants (collected at 24, 48, and 72 hours) were added in duplicate. The plates were incubated overnight at 4^0C and washed 4X with PBS/tween. Biotinylated anti-IFN-γ, anti-IL-4, anti-IL-5 (BD Pharmingen) and anti-IL-17 (R&D Systems) were diluted in PBS/1% BSA, 100 μl of 1 μg/ml was added to each well and the plates were incubated at room temperature for 1 hr. The plates were washed 6X with PBS/tween and 100 μl

avidin-peroxidase was added at 2.5 µg/ml and incubated for 30 min. The plates were washed 8X with PBS/tween and 100 µl ABTS substrate containing 0.03% H_2O_2 was added to each well. The plates were monitored for 15-20 min for color development and read at 405 nm (Emax precision microplate reader, Molecular Devices, Sunnyvale, CA), . A standard curve was generated from recombinant IFN-γ, IL-4, IL-5, and IL-17 standards and the concentration of each cytokine in the samples was calculated using SOFTmax PRO software.

For IL-12Rβ2, STAT1 and T-bet, whole cell lysates were generated from $2X10^6$ Vα2.3/Vβ8.2 TCR transgenic splenocytes that had been activated with MBP Ac1-11 plus $6X10^6$ irradiated feeder cells from wild-type B10.PL mice. The protein concentration was determined using the BioRad Protein Assay. Immulon II plates were coated with 20 µg/ml protein in 0.1 M $NaHCO_3$, pH 8.2, overnight at 4^oC. The plates were washed 2X with PBS/tween, blocked with PBS/BSA for 2 hr, and anti-IL-12Rβ2, anti-STAT1 and anti-T-bet were added at 5 µg/ml in duplicate wells for 2 hr. The plates were washed 6X with PBS/tween and a biotinylated secondary antibody was added at 2 µg/ml for 1 hr. The remainder of the assay was the same as the cytokine ELISA. The relative change in each protein was determined by subtracting the background and determining the ratio of the OD of the activated cells to the OD of the no antigen cells.

Proliferation Assay

Draining lymph nodes and splenocytes were removed from mice after immunization and *in vivo* administration of siRNA T-bet or siRNA NS and single-cell suspensions were obtained by pressing the tissue through a mesh screen. The cells were plated at 10^5 cells per well in quadruplicate in 96 well plates. Proliferative responses of lymph nodes and splenocytes were determined using MOG 35-55 (2 μg/ml) or no antigen. Cultures were maintained in 96-well flat-bottom plates for 96 hr to measure antigen-induced proliferation at 37° C in humidified 5% CO_2/air. The wells were pulsed with 50 μCi of ^3H thymidine for the final 18h of culture. Cells were harvested on glass filters and incorporated ^3H thymidine was measured with a Betaplate counter (Wallac, Gaithersburg, MD). Results were determined as means from quadruplicate cultures.

Statistics

Non-parametric Mann-Whitney analysis was used to determine the statistical significance between groups for EAE experiments because a Guassian distribution could not be assumed. Student's two-tailed T test was used to determine statistical significance of cytokine production between different experimental groups. All statistics were done using GraphPad Prism 3.0 software (San Diego, CA).

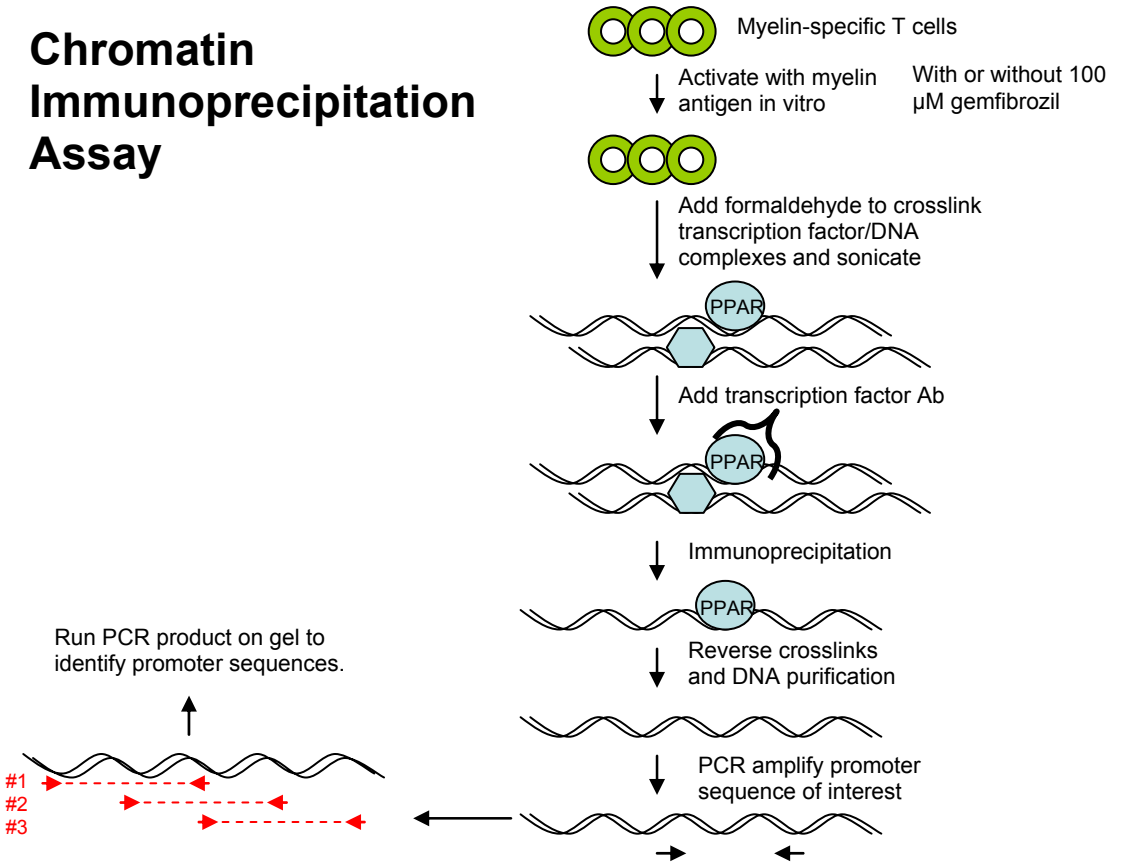


Figure 5. Schematic Representation of a ChIP Assay
Cells are activated *in vitro* and protein/DNA complexes are crosslinked using formaldehyde and sonicated. The immune complexes are then immunoprecipitated using an antibody specific for the transcription factor of interest, which in this example is PPARα. The crosslinking is then reversed and the DNA is purified using ETOH precipitation and phenol chloroform extraction. Finally, the DNA, which was bound by PPARα in the cell, can be used as a template in a PCR reaction to detect genes whose promoter/enhancer regions are specifically regulated by PPARα.

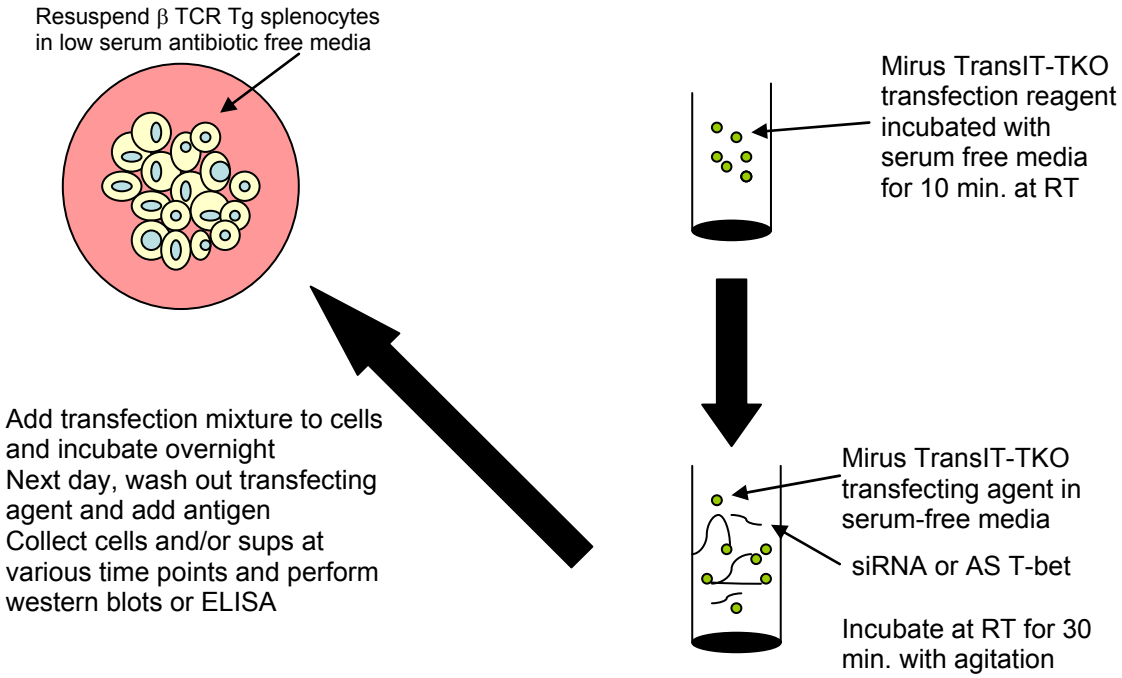


Figure 6. Transfection of Cells with siRNA or AS Oligonucleotides
Splenocytes are isolated and plated in 500μl of low serum media per well of a 24 well plate. For transfection with siRNA or AS oligonucleotides, the transfection reagent TKO is incubated in serum free media for 10 minutes at room temperature. Either 1 or 2 μl of siRNA or AS oligo is then added to the transfection reagent mixture and incubated at room temperature for 30 minutes with agitation. The transfection mixture is added to the cells and incubated overnight in a 37° incubator. The following day, the cells are washed and stimulated with antigen. Cells and supernatants can then be collected at various timepoints for desired experiments.

Chapter III. Silencing T-bet Defines a Critical Role in the Differentiation of Autoreactive T Lymphocytes

Introduction

Cytokines play a major role in the differentiation of CD4+Th cells into Th1 and Th2 subsets. Following T cell receptor engagement of peptide/MHC, there are two cytokine receptors that aid in the differentiation of naïve T cells into Th1 effector cells. These are the IFN-γ and IL-12 receptors. The IFN-γ receptor activates a signaling pathway involving STAT1, while the IL-12 receptor activates a signaling pathway via STAT4. In contrast, the differentiation of Th2 cells is mediated primarily by IL-4, which induces the expression of the transcription factor GATA3. GATA3 is believed to be a master regulator of Th2 cell differentiation because of its ability to transactivate Th2 cytokine genes. More recently, the transcription factor, T-bet, has been found to be a key regulator of the IFN-γ gene in Th1 cells. T-bet is a member of the T-box family of transcription factors that contain a highly conserved DNA binding domain, called the T-box, and bind to a core DNA sequence in promoter regions of a diverse set of genes. T-bet has been found to be expressed in Th1 cells but not Th2 cells [83, 150, 151]. However, ectopic expression of T-bet in Th2 lymphocytes was shown to result in IFN-γ production and suppression of Th2 cytokines [83], leading to speculation that T-bet may play a critical role in the differentiation of

Th1 cells. In addition, mice that are genetically deficient in T-bet fail to generate Th1 cells and spontaneously develop physiological and inflammatory changes characteristic of asthma, a Th2-mediated disease [90]. Initial studies suggested that T-bet mediates its effects on Th1 differentiation via the IL-12/STAT4 pathway [83, 152], but more recent studies have demonstrated that its role in the production of IFN-γ appears to be mediated by the IFN-γ/STAT1 pathway [84, 151, 153]. Although it was originally speculated that T-bet may be the master regulator of cytokine expression in Th1 cells, similar to GATA3 in Th2 cells, a subsequent study has questioned the precise role of T-bet in Th1 cells [151]. Thus, the exact role of T-bet in the differentiation of naïve antigen-specific T cells into Th1 cells has not been clearly established. Since many autoimmune diseases are mediated by Th1 cells, we investigated the role of T-bet, and the genes it regulates, in the differentiation of myelin-specific T cells into classical Th1 cells that are capable of inducing EAE.

EAE is an inflammatory, demyelinating disease of the central nervous system that serves as a model for the human disease, multiple sclerosis (MS). EAE can be induced in rodents by immunization with myelin proteins or peptides, such as MBP, PLP, and MOG, emulsified in complete Freund's adjuvant, or by the transfer of activated CD4+ myelin-specific Th1 lymphocytes into naïve recipient animals. Shifting the phenotype of myelin-reactive T cells from a Th1 to a Th2 phenotype has been shown to be advantageous in the treatment of EAE [69, 70]. However, many reagents, such as retinoids and cyclophosphamide,

that have been used to induce immune deviation can affect many different cell types and signaling pathways, resulting in undesirable side effects. We have been attempting to develop therapeutic agents that specifically target a gene to alter the encephalitogenic potential of autoreactive T cells, minimizing the effects on other cell types and other signaling pathways within T cells.

To determine if suppression of T-bet may be beneficial in altering the development of encephalitogenic T cells, we developed an antisense oligonucleotide (AS oligo) and small interfering RNA (siRNA) specific for T-bet. AS oligonucleotides are small single-stranded DNA sequences that are usually complementary to the translation initiation site of the gene of interest. Hybridization to their target mRNA inhibits translation of the transcript by preventing the ribosomal complexes from binding to the now double-stranded translation initiation site or by promoting the degradation of the mRNA by RNase H. siRNA silence genes utilizing an evolutionarily conserved mechanism of degrading mRNA complementary to any double-stranded RNA in a cell. RNA interference is thought to play a critical role in cellular responses to RNA viruses and in stabilizing the genome by sequestering repetitive sequences [154]. These gene silencing techniques offer several advantages over gene knock-out and monoclonal antibody technologies for studying *in vitro* and *in vivo* roles of specific genes. First, mice that have a specific gene deleted may have altered the normal requirements for that protein during development and the observed phenotypes may not be the same as in a normal animal which has a gene

suppressed *in vitro* or *in vivo*. Second, in knockouts the gene is deleted from all cell types, which may make it difficult to determine the relevance of that gene in particular cell types that play a role in the disease or pathway of interest. Third, monoclonal antibodies do not inhibit the expression of the protein, but interfere with the function of the protein, and their use is typically limited to membrane-bound and secreted proteins, not intracellular proteins such as transcription factors. In the present study, we demonstrate that an AS oligo and siRNA specific for T-bet can be utilized to target T-bet both *in vitro* and *in vivo* to define the role of T-bet in the differentiation of autoreactive T cells. Furthermore, the results of this study suggest that silencing T-bet could be a potential therapy for Th1-mediated diseases, such as MS.

Results

Generation of T-bet-specific Antisense Oligonucleotides and Small Interfering RNA

To determine if suppression of T-bet may alter the development of encephalitogenic T cells, we developed an AS oligo and siRNA specific for T-bet (Table 3). Two control AS oligonucleotides were also generated, one specific for GATA3 which may increase encephalitogenicity and one containing a nonsense sequence [155]. The AS oligonucleotides were generated on a phosphorothioate backbone to increase their stability and minimize degradation. The siRNA specific for T-bet contains a 19 base sequence within the open reading frame generated with AA overhangs at the 5' ends. The nonsense siRNA was a random sequence that had no significant sequence similarity to other genes.

We investigated whether these T-bet-specific AS oligonucleotides and siRNA were capable of silencing T-bet expression in MBP Ac1-11-specific T cells. Splenocytes were isolated from B10.PL mice that were transgenic for the Vβ8.2 T cell receptor chain that recognizes MBP Ac1-11 when paired with a specific Vα2.3 T cell receptor chain [156]. Naive Vβ8.2 transgenic mice have a precursor frequency of MBP Ac1-11-specific T cells of 1 in 10^3-10^4 splenocytes, which is more physiologically relevant in terms of the number of T cells in a population specific for a particular antigen compared to using the MBP Ac1-11-

specific Vα2.3/Vβ8.2 transgenic splenocytes in which the vast majority of T cells recognize the peptide. The splenocytes were transfected with the AS oligonucleotides or siRNAs overnight. Transfected splenocytes were activated with MBP Ac1-11 and nuclear extracts were prepared. Western blot analysis demonstrated that T-bet protein expression was induced upon activation with MBP Ac1-11 and that this induction was inhibited in the activated splenocytes that were transfected with the siRNA and AS oligo specific for T-bet (Fig. 7A and 7C). Densitometry of the T-bet and actin blots was performed and the relative protein expression of T-bet was determined by normalizing the density of the T-bet bands to the actin bands (Fig.7B and 7D). The level of T-bet expression in mock-transfected TCR Vβ8.2 cells varies slightly between mice in the absence of antigen as seen in Figure 7.

To verify that the inhibition of T-bet resulted in the suppression of IFN-γ production, Vβ8.2 transgenic splenocytes were transfected with T-bet-specific AS oligo and siRNA, and activated with MBP Ac1-11. The supernatants were collected at various timepoints and IFN-γ production was measured by ELISA. As seen in Figure 8A, IFN-γ production was suppressed, but not totally inhibited, in the splenocytes that were transfected with the T-bet-specific AS/siRNA, indicating that silencing T-bet significantly diminished IFN-γ production. IFN-γ production was found to increase over time in the cells in which T-bet was silenced, but it is not known whether this IFN-γ was expressed by CD4+ T cells or other immune cells in the splenocyte population. Furthermore, it is possible

that as the cells divided the AS/siRNA was degraded, allowing T-bet to be induced and transactivate the IFN-γ gene. There was no IL-4 produced when T-bet was silenced during the primary activation (data not shown).

Silencing T-bet Alters the Encephalitogenicity of MBP-specific T Cells

To determine if suppressing T-bet could alter the encephalitogenic capacity of MBP Ac1-11-specific T cells, naïve Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS T-bet , siRNA T-bet and NS controls prior to activation with MBP Ac1-11 *in vitro*. In addition cells were transfected with AS GATA3 as a control. Inhibition of GATA3, which is a transcriptional regulator of Th2 differentiation, in contrast to inhibition of T-bet, should result in an increased incidence and severity of EAE. These cells were then transferred into wild-type B10.PL mice. Only 1 of 6 mice in the AS T-bet group and 1 of 6 mice in the siRNA T-bet group developed clinical signs of EAE (Fig. 8B). In contrast, 8 of 10 mice in the control groups (non-transfected, siRNA NS, AS GATA3) developed EAE. This demonstrates that silencing T-bet in myelin-reactive T cells, not only alters their cytokine production, but diminishes their ability to induce EAE as well.

Since it had been previously demonstrated that AS oligonucleotides and siRNAs could be administered intravenously and induce effects *in vivo* [155, 157-160], the ability of our T-bet-specific AS oligonucleotides and siRNA to alter actively induced EAE when injected *in vivo* was evaluated. C57BL/6 mice were

given 50 μg of siRNA T-bet, AS T-bet, AS GATA3 or PBS via the tail vein at the time of immunization with MOG35-55/CFA. All of the PBS and AS GATA3-treated mice developed EAE, but disease was suppressed in the siRNA T-bet and AS T-bet-treated mice (Fig. 9). To verify that i.v. administration of the T-bet-specific siRNA was suppressing T-bet *in vivo*, the draining lymph nodes were removed at 72 hr post immunization/siRNA administration. Nuclear extracts were prepared from the cells directly ex-vivo and western blot analysis was used to determine the level of T-bet expression. As seen in Figure 10, T-bet expression was suppressed >85% in the lymph node cells (LNC) from the mice that received the siRNA T-bet compared to the mice that received the siRNA NS, demonstrating that i.v. administration of siRNA T-bet could effectively silence T-bet *in vivo*.

Similarly, we examined LNC and splenocytes at day 13 post immunization/siRNA or AS oligo treatment. None of the mice had developed clinical signs of EAE at this time. At this time point, one would anticipate that some of the activated antigen-specific T cells would have migrated into the spleen. T-bet expression directly ex-vivo in the splenocytes from the mice that received AS T-bet or siRNA T-bet was reduced by 60% and 98%, respectively, compared to the T-bet expression in the mice that received the AS NS or siRNA NS (Fig. 11). Antigen-specific IFN-γ production was also examined. As seen in Figure 12, both the splenocytes and LNC from the AS NS-treated mice produced significant amounts of IFN-γ in response to the antigen. In contrast, there was no

antigen-induced IFN-γ production in the splenocytes of the AS T-bet or siRNA T-bet-treated mice, and only a modest amount of IFN-γ produced in LNC at 48 hr compared to the amount produced in LNC from the AS NS-treated mice. LNC from the siRNA T-bet-treated mice were not evaluated in this experiment because there were not enough cells recovered from the lymph nodes in these mice. However, in similar experiments not shown, there was no significant production of IL-4, IL-5, IL-10 or IFN-γ in LNC or splenocytes in the mice that received siRNA T-bet or AS T-bet.

The Role of T-bet in the Differentiation of Encephalitogenic T Cells Can Be Bypassed by Exogenous IL-12

To determine the role that T-bet was playing in the differentiation of encephalitogenic T cells, we examined whether the differentiation of MBP Ac1-11-specific T cells in the presence of IL-12 altered the level of T-bet expression. Naïve Vα2.3/Vβ8.2 transgenic splenocytes were activated with Ac1-11 in the presence or absence of IL-12. Nuclear extracts were prepared from activated cells and T-bet expression was determined by western blotting. The splenocytes that were activated in the presence of IL-12 had similar levels of T-bet expression as the splenocytes that were activated with antigen only (Fig. 13A). To confirm this observation, splenocytes were activated in the presence or absence of IL-12 and stained intracellularly with anti-T-bet. Flow cytometric analysis showed similar intracellular levels of T-bet in CD4+ T cells, regardless of the addition of

IL-12 (Fig. 13B). This data indicates that T-bet expression is not altered in the presence of exogenous IL-12 and suggests the possibility that T-bet does not function in the IL-12/STAT4 segment of the Th1 differentiation pathway.

To further explore this observation, naïve Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS T-bet and siRNA T-bet prior to activation with MBP Ac1-11 and IL-12. Supernatants were collected from the cultured cells and IFN-γ production was determined by ELISA. Significant IFN-γ production was seen for all transfection conditions (Fig. 13C). At 72 hr post activation, the splenocytes were transferred into B10.PL mice and all cells activated in the presence of IL-12 could transfer disease into naive recipients, regardless of whether T-bet was silenced prior to activation (Fig. 13D). This data indicates that T-bet functions independently of IL-12 and/or upstream of IL-12 in the differentiation of the encephalitogenic T cells.

T-bet Regulates the IFN-γ and STAT1 Genes

To determine which genes T-bet may directly regulate in the Th1 differentiation pathway, chromatin immunoprecipitation (ChIP) assays were performed. Naïve Vβ8.2 transgenic splenocytes were activated *in vitro* with MBP Ac1-11 and then fixed with formaldehyde to crosslink T-bet/DNA complexes. The cells were sonicated and an antibody specific for T-bet was used to immunoprecipitate the DNA that was specifically bound to T-bet. The immunoprecipitated DNA was purified and PCR amplification was used to identify

promoter/enhancer regions of genes that were bound by T-bet. As seen in Figure 14A, T-bet was found to bind the IFN-γ promoter, verifying that the IFN-γ gene may be directly regulated by T-bet in these autoreactive T cells. Similarly, we immunoprecipitated DNA bound to STAT1 from antigen-activated Vβ8.2 cells and amplified the IFN-γ gene with two primer sets (Fig. 14B). We also used STAT1 and STAT4 promoter/enhancer region specific primers to determine if these genes may be regulated by T-bet. As expected, the STAT4 gene was not amplified from the DNA precipitated with the T-bet antibody (data not shown). However, a primer set specific for the STAT1 promoter region amplified a segment from T-bet immunoprecipitated DNA (Fig. 15A). Interestingly, this primer set amplified a sequence within the STAT1 gene that contained a T-box consensus sequence. This was a surprising observation because a previous study demonstrated that STAT1-deficient T cells could not express T-bet mRNA upon activation, suggesting that STAT1 was regulating the expression of T-bet [151]. To determine if T-bet suppression affected STAT1 protein expression, Vβ8.2 splenocytes were transfected with siRNA T-bet, siRNA NS, AS T-bet, AS NS or mock-transfected and nuclear extracts were prepared after activation with MBP Ac1-11. A western blot was performed to determine the level of STAT1 expression when T-bet was silenced. As seen in Figure 15B, STAT1 expression was decreased in the splenocytes in which T-bet had been silenced, suggesting that T-bet may be able to upregulate STAT1 expression in Th1 cells. To verify this observation, T-bet was knocked down in splenocytes with siRNA T-bet and a ChIP assay was performed to determined if the STAT1 promoter could still be

immunoprecipitated from activated splenocytes as seen in Figure 15A. When the splenocytes were transfected with siRNA NS, T-bet specifically bound the STAT1 promoter, but when the cells were transfected with siRNA T-bet the STAT1 promoter could no longer be amplified from the T-bet-specific immunoprecipitated DNA (Fig. 15C). This data confirms that T-bet directly binds the STAT1 promoter and regulates STAT1 expression in activated splenocytes.

To determine if STAT1 may also regulate T-bet in a positive feedback loop to amplify and optimize IFN-γ production, a ChIP assay was performed using activated Vβ8.2 splenocytes and immunoprecipitating with an antibody specific for STAT1. The 5' end of the T-bet gene could not be amplified in these experiments (data not shown). However, due to the lack of data on the promoter/enhancer region of the T-bet gene, the primer sets that were used may not have been optimal. Therefore, we developed an siRNA specific for STAT1 and investigated whether silencing STAT1 altered T-bet expression. Naïve Vβ8.2 splenocytes were transfected with siRNA-STAT1 and activated with MBP Ac1-11 *in vitro*. Nuclear extracts were prepared and STAT1 and T-bet expression were evaluated by western blotting. As seen in Figure 15D, STAT1 expression in activated cells was reduced by 47% by the siRNA, which is significant given that STAT1 is constitutively expressed by a variety of cell types in the spleen. In addition, T-bet expression was reduced by 50% when the splenocytes were transfected with siRNA-STAT1, supporting the previously published observation that STAT1 regulates T-bet expression [151].

Since it had been reported that IL-12Rβ2 mRNA was not upregulated in T-bet-deficient mice, suggesting that T-bet regulated the IL-12Rβ2 gene [151], we investigated whether we could confirm this observation in our experimental system. First, we performed ChIP assays on activated Vβ8.2 cells with a T-bet specific antibody and attempted to PCR amplify the promoter region of the IL-12Rβ2 gene, but were not successful (data not shown). Furthermore, a scan of the IL-12Rβ2 gene revealed no T-box consensus sites within the promoter region. However, given that a negative result in this experiment does not definitively preclude the possibility of T-bet regulating this gene, we analyzed IL-12Rβ2 protein expression in whole cell lysates from Vα2.3/Vβ8.2 TCR transgenic splenocytes that had been transfected with AS T-bet, siRNA T-bet or controls by western blot. We found very little change in IL-12Rβ2 expression when T-bet was silenced (Fig. 16A). This was confirmed by ELISA in which the same whole lysates were used as the antigen, and antibodies for IL-12Rβ2, T-bet and STAT1 were used to determine the relative change in protein expression following antigen activation. A ratio of approximately 1 was seen for IL-12Rβ2 which reflects no significant change in protein expression in the cells transfected with siRNA T-bet or siRNA NS (Fig. 16B). In contrast, there was a 3-fold increase in STAT1 and T-bet expression in the cells transfected with siRNA NS compared to the cells transfected with siRNA T-bet, verifying that STAT1 and T-bet levels were significantly suppressed when the T-bet gene was silenced, but IL-12Rβ2 expression was not affected.

Discussion

This study demonstrates the important role of T-bet in the differentiation of Th1 cells in a pathogenic antigen-specific system using two gene silencing techniques, and implicates T-bet as a potential therapeutic target in EAE. We utilized MBP Ac1-11-specific TCR transgenic T cells that preferentially differentiate into Th1 cells upon activation with antigen. For the majority of the *in vitro* experiments, splenocytes from mice that expressed only the Vβ8.2 chain of the transgenic TCR were used because the precursor frequency of MBP Ac1-11-specific T cells in these mice is more physiologically relevant than that of Vα2.3/Vβ8.2 TCR transgenic mice in which >90% of the splenic T cells respond to MBP Ac1-11. In addition, exogenous cytokines and antibodies to cytokines that are often added to T cell cultures to drive the differentiation of the cells were not used in the majority of these studies. This allowed us to assess the extent of T-bet's role in Th1 differentiation when the source of cytokines was limited to the antigen presenting cells and responding T cells. T-bet expression in ovalbumin-specific T cell receptor transgenic cells differentiated *in vitro* demonstrated varied levels of T-bet expression under different Th1-inducing conditions [151]. Since the primary sources of IFN-γ and IL-12 during the differentiation of Th1 cells *in vivo* are the antigen-presenting cells, NK cells and CD8+ T cells, we thought that stimulation of splenocytes with antigen alone provided the most accurate assessment of T-bet's role in the differentiation of naïve cells because all of these cell populations are present in primary splenocyte cultures. T-bet

expression was induced upon activation of naïve Vβ8.2 TCR splenocytes with MBP Ac1-11, as originally observed in T cell clones activated with PMA/ionomycin or anti-CD3 [83]. Both the AS oligo and siRNA specific for T-bet were able to effectively suppress T-bet protein expression in MBP Ac1-11 stimulated Vβ8.2 TCR transgenic splenocytes, and IFN-γ levels were substantially reduced in the splenocyte cultures that had been transfected with T-bet-specific AS oligo or siRNA without any reciprocal induction of Th2 cytokines.

To determine if silencing T-bet could effectively suppress EAE induction *in vivo*, mice were given an i.v. injection of AS T-bet or siRNA T-bet at the time of immunization which reduced the incidence of disease by as much as 75% depending on the dose administered. Furthermore, ex vivo analysis of the draining LNC and splenocytes demonstrated suppressed T-bet expression and IFN-γ production. In contrast to T-bet-deficient mice, no increase in Th2 cytokines was seen in mice that received a single dose of AS T-bet or siRNA T-bet.

The role of T-bet in experimental models of inflammatory diseases has been studied previously. T-bet-deficiency was found to protect from Th1-mediated experimental colitis in an adoptive transfer model and T-bet-deficient mice were found to be more susceptible to an actively-induced Th2-mediated colitis, suggesting that T-bet plays a critical role in these T cell mediated inflammatory diseases [92]. Similarly, Con-A-induced liver injury was

ameliorated in T-bet-deficient mice [153]. However, T-bet-deficient Balb/c mice immunized with an insulin peptide develop diabetes-related phenotypes similar to wild-type Balb/c mice, indicating that T-bet does not play a critical role in this specific inflammatory model [161]. It has been shown recently that T-bet-deficient mice are resistant to actively-induced EAE and develop a Th2 phenotype [99]. In contrast to these previous studies, we studied the role of T-bet in T-bet competent mice using gene silencing techniques. Suppression of T-bet during the *in vitro* differentiation of myelin-reactive T cells diminished the capacity of these cells to induce EAE when transferred into naïve mice. Moreover, *in vivo* administration of siRNA or AS oligo specific for T-bet inhibited the onset of actively induced EAE, suggesting that T-bet may be a viable target for therapeutic intervention in Th1-mediated diseases.

Although it was originally reported that T-bet may play a role in the IL-12/STAT4 signaling pathway in Th1 cells [83], other studies have implicated T-bet in the transactivation of IFN-γ via the IFN-γ/STAT1 pathway [84, 151, 153]. In our MBP Ac1-11-specific T cells, T-bet expression was not increased when the cells were differentiated in the presence of IL-12. In addition, when T-bet expression was silenced in MBP Ac1-11-specific T cells prior to antigen stimulation in the presence of IL-12, IFN-γ production and the encephalitogenic capacity of the cells was not diminished, suggesting that T-bet was not necessary for the differentiation of Th1 cells when the IL-12/STAT4 pathway was directly induced. It is well established that the IL-12/STAT4 pathway plays a

significant role in the differentiation of naïve T cells into Th1 cells [162]. However, induction of the IL-12Rβ2 chain is dependent on the IFN-γ/STAT1 signaling pathway, establishing a dependence of the IL-12/STAT4 pathway on the IFN-γ/STAT1 pathway. In this study, we found no evidence that T-bet directly regulated the expression of the IL-12Rβ2 gene.

To identify genes in the Th1 differentiation pathway that may be directly transactivated by T-bet, chromatin immunoprecipitation (ChIP) assays were performed. Previously, using ectopic expression of T-bet, it has been shown that IFN-γ and IL-12Rβ2 expression were upregulated in T-bet expressing cells, but whether this effect resulted by direct transactivation of these genes or indirectly via transactivation of other genes that can subsequently regulate IFN-γ and IL-12Rβ2 gene expression was not addressed [151]. To confirm that the IFN-γ gene is directly regulated by T-bet in activated T cells as recently observed [163], Vβ8.2 transgenic splenocytes were activated and a ChIP assay was performed using an antibody specific for T-bet. The IFN-γ promoter was amplified by PCR following ChIP assay, confirming that T-bet binds to the IFN-γ promoter. Analysis of the 3500 bases upstream of the IFN-γ gene translation initiation site identified several potential T-bet binding sites based on the T-box consensus DNA binding sequence, emphasizing the potential of T-bet as a strong transactivator of the IFN-γ gene. The T-bet-immunoprecipitated DNA was also used as a template for amplification of the STAT1 and STAT4 promoter regions to determine if T-bet could potentially regulate either of these genes which are

known to play significant roles in the IFN-γ receptor and IL-12 receptor signaling pathways, respectively. As anticipated, the STAT4 promoter sequence was not amplified, but a STAT1 promoter sequence was amplified. This STAT1 promoter sequence contained a T-box consensus sequence, verifying the potential for T-bet to regulate STAT1. This was a surprising observation in light of the previous finding that T-bet expression was absent in STAT1-/- cells, suggesting that STAT1 expression was necessary for T-bet expression [151]. Furthermore, suppression of T-bet with siRNA resulted in suppressed STAT1 and suppression of STAT1 with a STAT1 siRNA resulted in suppression of T-bet, implying that STAT1 and T-bet may regulate each other in a positive feedback/amplification loop. In addition, decreased expression of IL12Rβ2 in the absence of T-bet that has been seen by others may be due to a failure to upregulate STAT1 in T-bet deficient mice, and may not be indicative of direct regulation by T-bet.

The roles of various cytokines, cytokine receptors and transcription factors in the generation of encephalitogenic Th1 cells using gene knock-out mice and monoclonal antibodies has generated conflicting data. Mice lacking the IL-12 p40 subunit are protected from EAE, whereas mice lacking the IL-12 p35 subunit or the IL-12Rβ2 chain remain susceptible to EAE [47, 164, 165]. The myelin-reactive T cells in the IL-12Rβ2-deficient mice still produced significant amounts of IFN-γ, although less than wild-type mice, suggesting that the IFN-γ/STAT1 pathway was sufficient to activate encephalitogenic Th1 cells. IL-12 may not be a critical cytokine in EAE, but IL-23, which shares the p40 subunit with IL-12,

may actually be mediating the effects originally attributed to IL-12 in EAE [48]. STAT4-deficient mice are also protected from EAE, but it is uncertain whether this is due to impaired signaling in the IL-12/STAT4 pathway in the T cells or due to the loss of STAT4 in antigen presenting cells that produce IFN-γ leading to the activation of the IFN-γ/STAT1 pathway [72]. Since it has been well established that myelin-specific CD4+ Th1 cells are the lymphocyte population that transfer EAE to naïve recipient animals, it was not anticipated that IFN-γ and IFN-γ receptor-deficient mice would develop more severe EAE than wild-type mice [55, 57, 166]. Similarly, administration of anti-IFN-γ to wild-type mice exacerbates EAE [58, 59, 167-169]. These mice have a massive expansion of myelin-specific CD4+ cells, suggesting that the complete loss of IFN-γ results in a diminished capacity to regulate the autoreactive T cells [166] and reinforces the notion that loss of a particular gene from all cell types can have unforeseen side effects. Although these observations indicate that IFN-γ is not essential for the induction of EAE, they do not negate the fact that encephalitogenic T cells generated *in vivo* in wild-type mice produce significant amounts of IFN-γ and *in vitro* suppression of IFN-γ production during the stimulation of myelin-specific T cells reduces the encephalitogenic capacity of these cells [170]. Taken together, these studies would suggest that suppressing IFN-γ production in the encephalitogenic Th1 cells, while preserving IFN-γ expression in other cell types may provide therapeutic benefit. Although T-bet is expressed in CD4+ Th1 cells, CD8+ T cells, NK cells, B cells, and dendritic cells upon activation, the requirement for T-bet for the expression of IFN-γ varies somewhat between cell

types. In T-bet deficient mice, IFN-γ is not expressed by CD4+ T cells [85]. In contrast, CD8+ T cells and B cells were originally shown to express normal levels of IFN-γ in T-bet-deficient mice [85]. However, IFN-γ has more recently been shown to be reduced in CD8+ T cells during antigen-specific activation of TCR-transgenic splenocytes deficient in T-bet [88] and T-bet has been shown to play a role in the differentiation of B effector 1 cells [89]. Similarly, splenic dendritic cells and NK cells from T-bet-deficient mice have reduced IFN-γ production [85, 86]. Murine macrophages do not express T-bet, indicating that IFN-γ transcription in these cells is regulated by other factors [86]. Therefore, it seems that T-bet may be a target for suppressing the development of CD4+ Th1 cells, while maintaining some level of IFN-γ production in other cell types that play a role in EAE. In addition, T-bet may affect other genes yet to be identified, directly or indirectly, that may alter the program of the T cells during activation resulting in reduced encephalitogenicity.

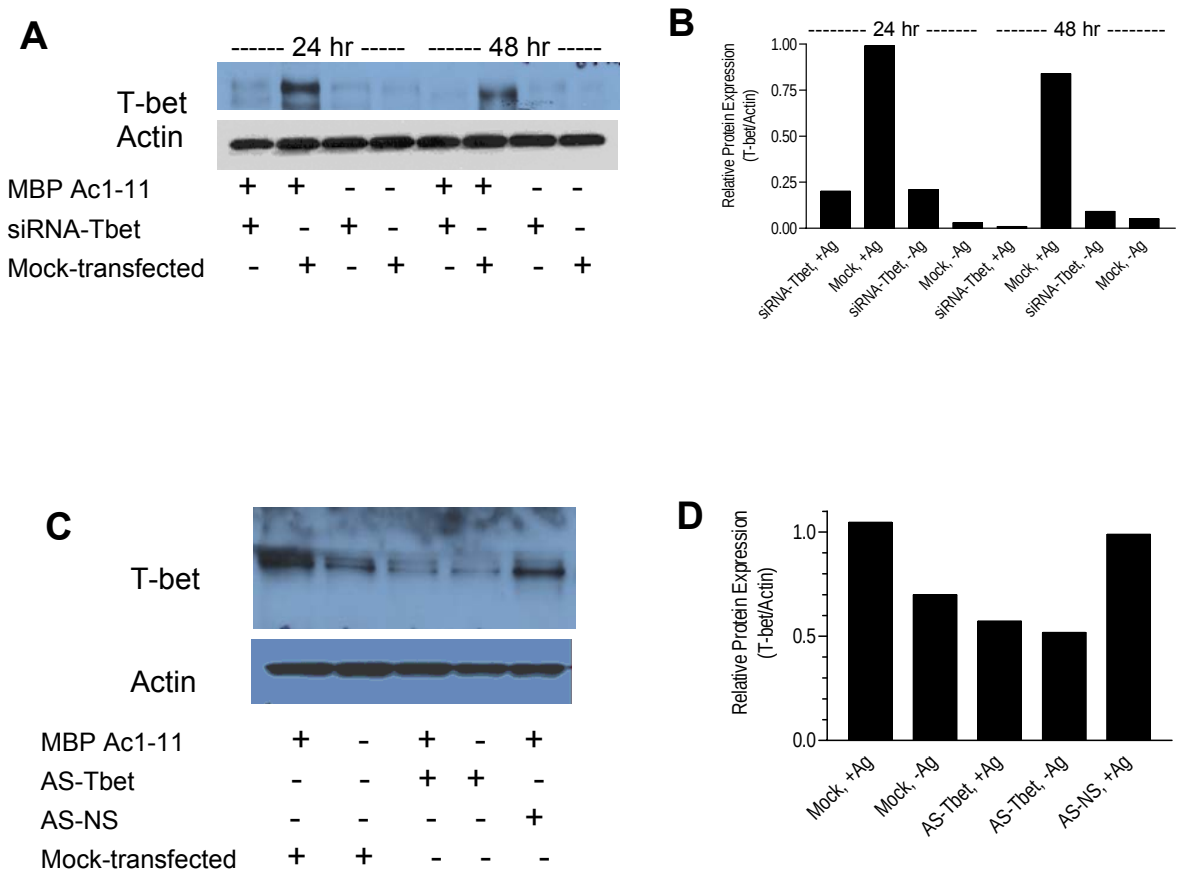


Figure 7. Suppression of T-bet expression with siRNA and AS oligonucleotides. Splenocytes from a Vβ8.2 transgenic B10.PL mouse that have a precursor frequency for MBP Ac1-11-specific T cells of 1 in 10^3 to 10^4 splenocytes were transfected with an siRNA specific for T-bet (A-B) or an antisense oligonucleotide specific for T-bet (C-D). The cells were subsequently activated with MBP Ac1-11 and nuclear extracts were prepared at 24 and/or 48 hr post activation. The protein levels were quantitated in the nuclear extracts and 30 µg was loaded per lane on a 10% SDS/PAGE gel. The proteins were transferred to nitrocellulose membranes and probed with anti-T-bet or actin antibody (A and C). Densitometry was performed on the bands and relative T-bet expression was determined by normalizing the T-bet to actin levels (B and D).

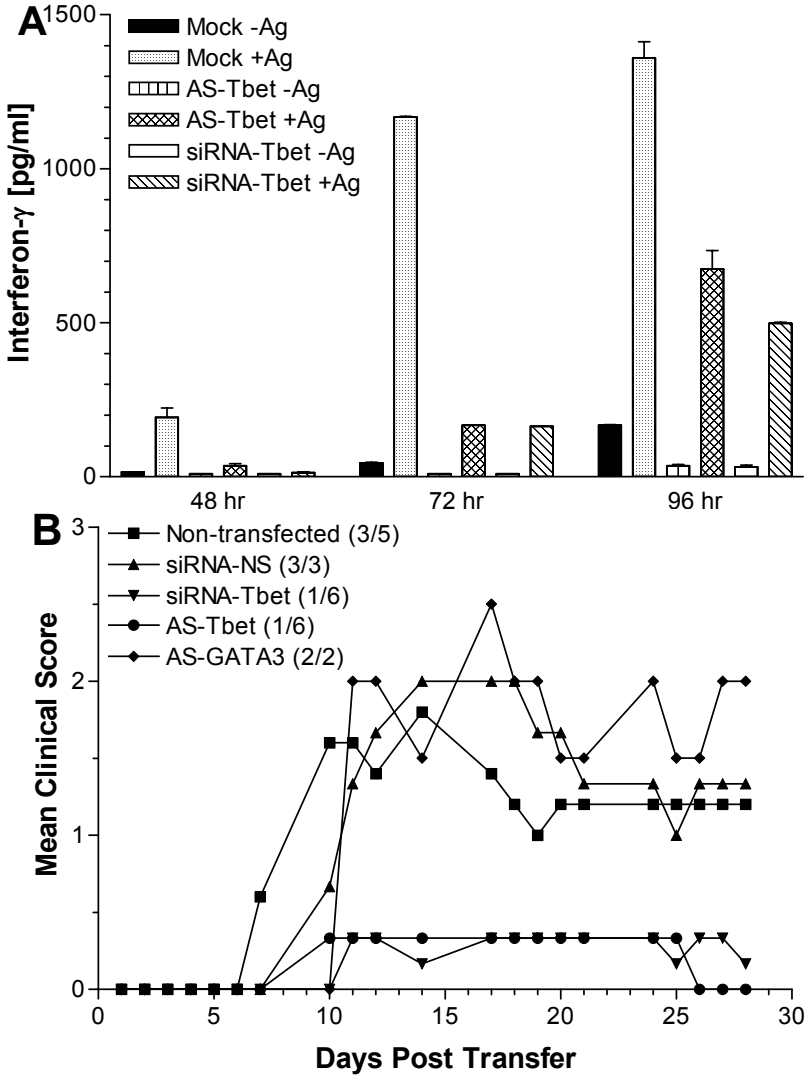


Figure 8. Silencing T-bet alters the phenotype of MBP Ac1-11-specific T cells. Splenocytes from a Vβ8.2 transgenic mouse were transfected with AS T-bet or siRNA T-bet and after 24 hr, activated with Ac1-11. (A) Supernatants from the cultured cells were collected at 24, 48 and 72 hr post activation and IFN-γ levels were measured by ELISA. (B) Vα2.3/Vβ8.2 transgenic splenocytes were transfected with siRNA T-bet, siRNA NS, AS T-bet, AS-GATA3 or nothing prior to *in vitro* activation with Ac1-11, and after 72 hr, 10 X 10^6 cells were transferred into wild-type B10.PL mice. The mice were monitored daily for clinical signs of EAE and the siRNA T-bet and AS T-bet groups had a significantly reduced incidence of EAE compared to any of the control groups. (siRNA NS vs. siRNA T-bet, $p<0.0001$; AS-GATA3 vs. AS T-bet, $p<0.0001$; siRNA T-bet vs. non-transfected, $p<0.0001$; AS T-bet vs. non-transfected, $p<0.0001$).

*Technical assistance from Rehana Hussain, Sara Northrop and Judy Choy

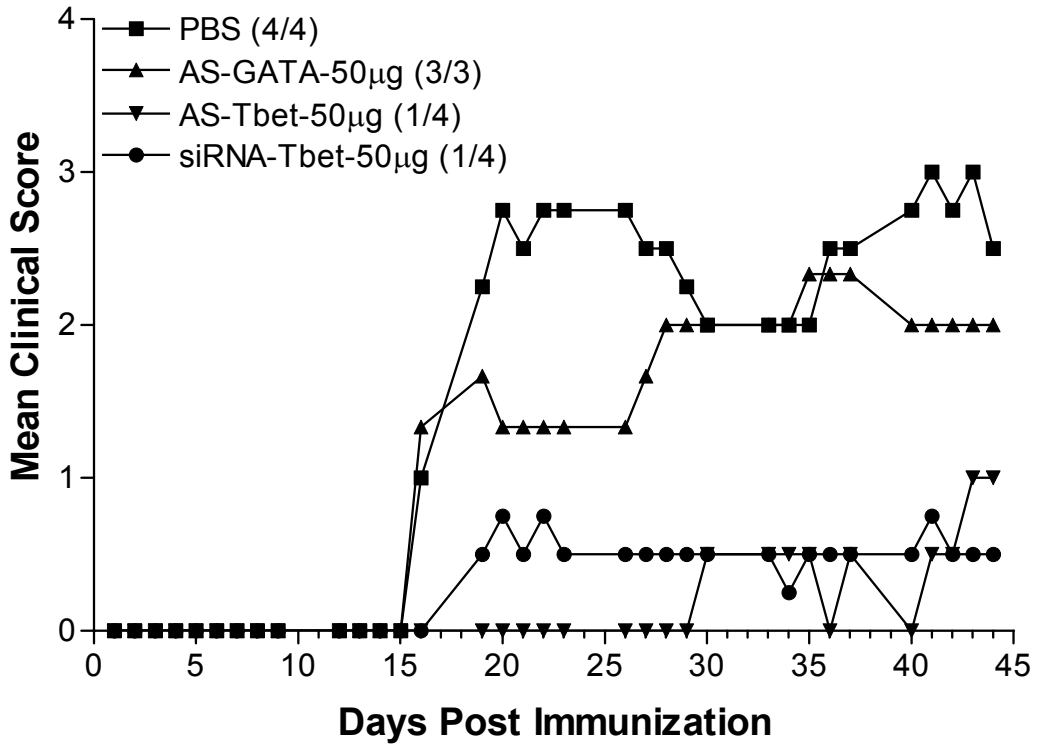


Figure 9. In vivo silencing of T-bet suppresses the incidence of actively induced EAE. EAE was actively induced in B6 mice with MOG35-55/CFA and AS T-bet and siRNA T-bet were given via the tail vein at the time of immunization. AS T-bet and siRNA T-bet (50 μg/mouse) significantly reduced the incidence of EAE compared to the control groups (PBS vs. AS T-bet, $p<0.0001$; PBS vs. siRNA T-bet, $p<0.0001$; AS-GATA3 vs. AS T-bet, $p<0.0001$; AS-GATA3 vs. siRNA T-bet, $p<0.0001$).

*Technical assistance from Rehana Hussain and Sara Northrop

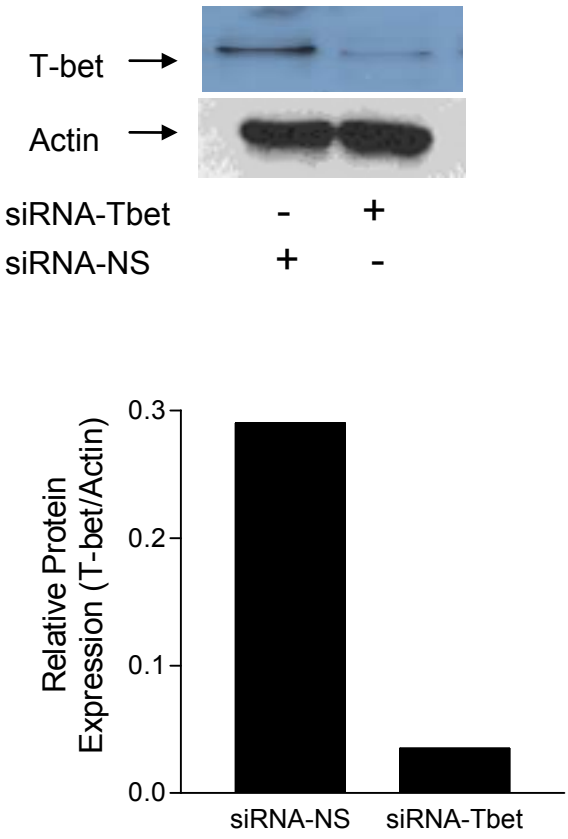


Figure 10. In vivo silencing suppresses T-bet 3 days post-immunization. The draining lymph nodes were removed from mice 3 days after they had been immunized and given 50 µg siRNA NS or siRNA T-bet. Nuclear extracts were prepared from the lymph node cells, 30 µg protein was loaded per lane on a 10% SDS/PAGE gel, and western blot analysis using anti-T-bet antibody demonstrated an inhibition of T-bet expression in the siRNA T-bet-treated mouse. Normalization of T-bet to actin demonstrated a >85% reduction in T-bet expression in the siRNA T-bet-treated mouse.

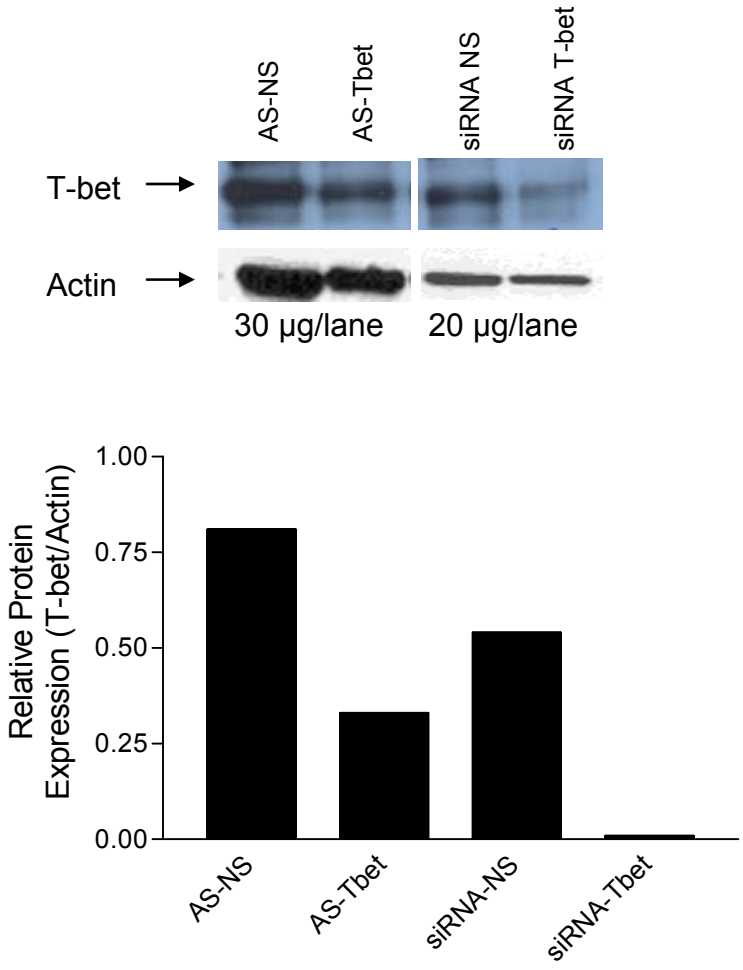


Figure 11. In vivo silencing suppresses T-bet 13 days post-immunization. The spleens were removed from mice 13 days after they had been immunized and given 50 µg AS NS, AS T-bet, siRNA NS, or siRNA T-bet. Nuclear extracts were prepared from splenocytes and 20 (siRNA) or 30 (AS) µg/ml was loaded per lane on a 10% SDS-PAGE gel. Western blots were performed using an antibody specific for T-bet, illustrating the reduced expression of T-bet in the spleens of mice that received AS T-bet or siRNA T-bet. Normalization of T-bet to actin quantitates the change in T-bet expression.

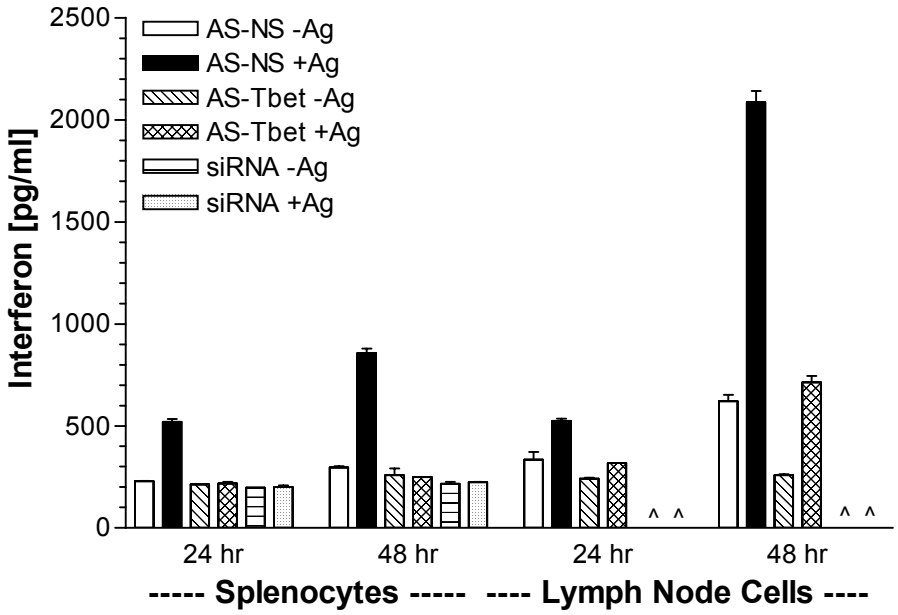


Figure 12. AS and siRNA T-bet treated mice produce less IFN-γ. The draining lymph nodes and spleen were removed from mice 13 days after they had been immunized and given 50 µg AS NS, AS T-bet, siRNA NS or siRNA T-bet. The splenocytes and lymph node cells (4 X 10^6 cells/well) were cultured in 24-well plates with or without antigen. Supernatants were collected at 24 and 48 hr, and IFN-γ levels were measured by ELISA, demonstrating the inhibition of antigen-induced IFN-γ production in the siRNA T-bet and AS T-bet-treated mice.

*Technical assistance from Rehana Hussain and Judy Choy

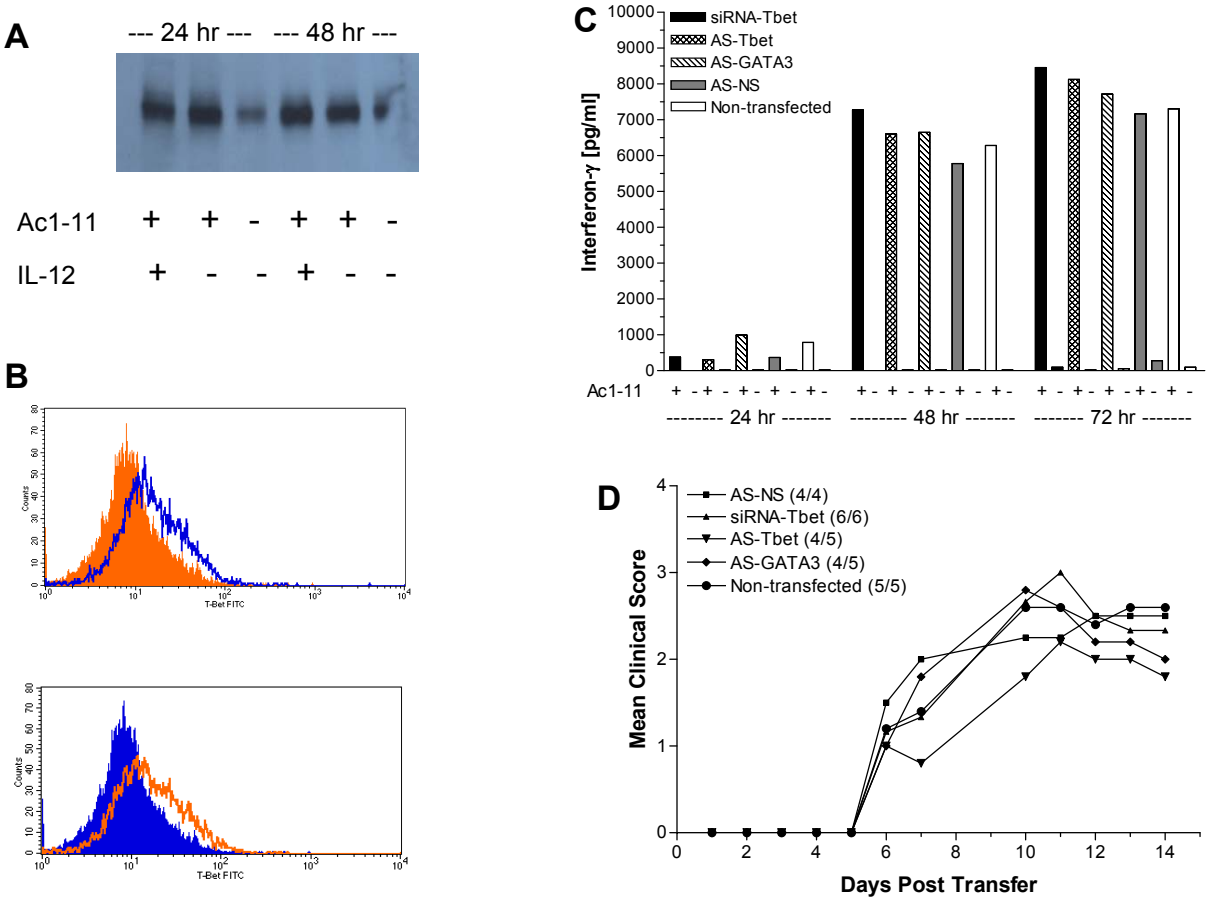


Figure 13. Differentiation of T cells in the presence of exogenous IL-12 is not dependent on T-bet. (A-B) Vα2.3/Vβ8.2 transgenic splenocytes were activated with Ac1-11 in the presence or absence of 1 ng/ml IL-12. (A) After a 24 or 48 hr stimulation, nuclear extracts were prepared, 30 µg protein was loaded per well, and western blotting was performed to determine the amount of T-bet expression. There was no difference in T-bet expression in the Ac1-11-activated cells in the presence or absence of IL-12. The background expression of T-bet in these nuclear extracts was higher than was seen in Vβ8.2 transgenic cells (Figure 7), because there are more spontaneously activated cells in the Vα2.3/Vβ8.2 mice. (B) The activated cells were also stained intracellularly with anti-T-bet antibody and evaluated by flow cytometry, demonstrating no difference in intracellular T-bet level in the cells cultured with or without IL-12. (C-D) Vα2.3/Vβ8.2 transgenic splenocytes were transfected with AS T-bet, AS-GATA3, AS NS, siRNA T-bet or nothing 24 hr prior to activation with Ac1-11 in the presence or absence of 1 ng/ml IL-12. (C) The cells were cultured at 2 X 10^6 transfected cells/well with 6 X 10^6 irradiated, wild-type B10.PL splenocytes, supernatants were collected at 24, 48, and 72 hr post activation, and ELISA was used to determine IFN-γ production, which was not altered by transfection with AS T-bet or siRNA T-bet. (D) The transfected splenocytes that had been activated in the presence of IL-12 were injected i.p. into wild-type B10.PL mice at 10 X 10^6 cells/mouse and clinical signs of EAE were monitored daily. There was no difference in the ability of AS or siRNA T-bet to alter the encephalitogenic capacity of the cells differentiated in the presence of exogenous IL-12.

*Technical assistance from Rehana Hussain and Sara Northrop for panels (C) and (D)

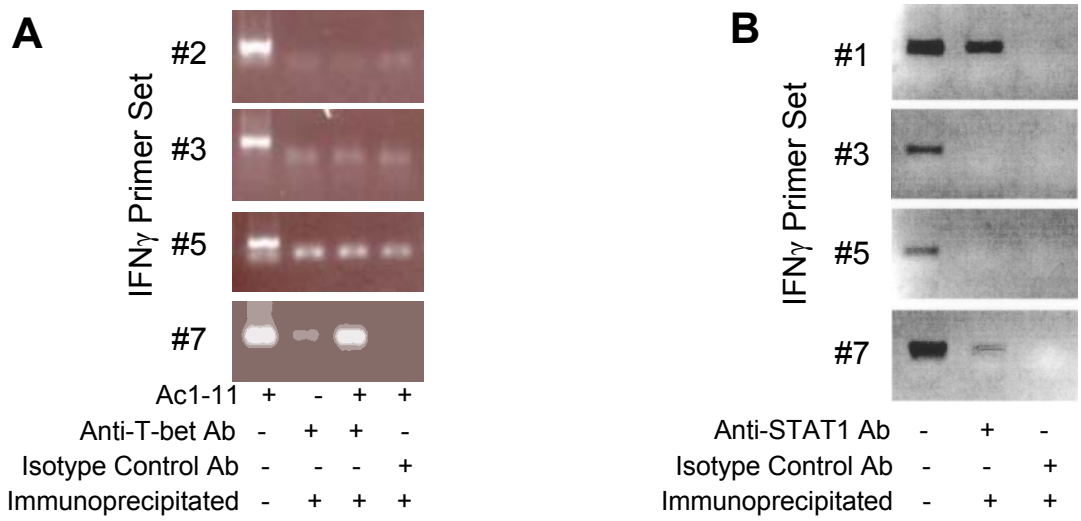


Figure 14. T-bet and STAT1 regulate the IFN-γ gene. Vβ8.2 transgenic splenocytes (15 X 10^6 per condition) were cultured with and without Ac1-11 for 24 hr. The cells were crosslinked with 1% formaldehyde, resuspended in RIPA buffer, sonicated and the supernatant containing the DNA/protein complexes was collected. Ten percent of the supernatant containing the total DNA was used as a positive control. The remaining supernatant was divided and immunoprecipitated with anti-T-bet antibody, anti-STAT1 antibody or an isotype control antibody using Protein A Sepharose beads. The immune complexes were eluted from the beads and proteinase K was used to digest the protein bound to the DNA. The DNA was purified and multiple primer sets within the promoter sequence of several genes were used to PCR amplify the gene of interest. (A) DNA immunoprecipitated with anti-T-bet antibody from Ac1-11 activated cells was used as the template in PCR reactions using 8 primer sets for the IFN-γ promoter region. One of the 4 primer sets (#7) shown specifically amplified the IFN-γ gene. (B) DNA immunoprecipitated with anti-STAT1 antibody from Ac1-11 activated cells was used as the template in PCR reactions using 4 primer sets for IFN-γ promoter region. Two of the 4 primer sets (#1 and #7) specifically amplified the IFN-γ gene.

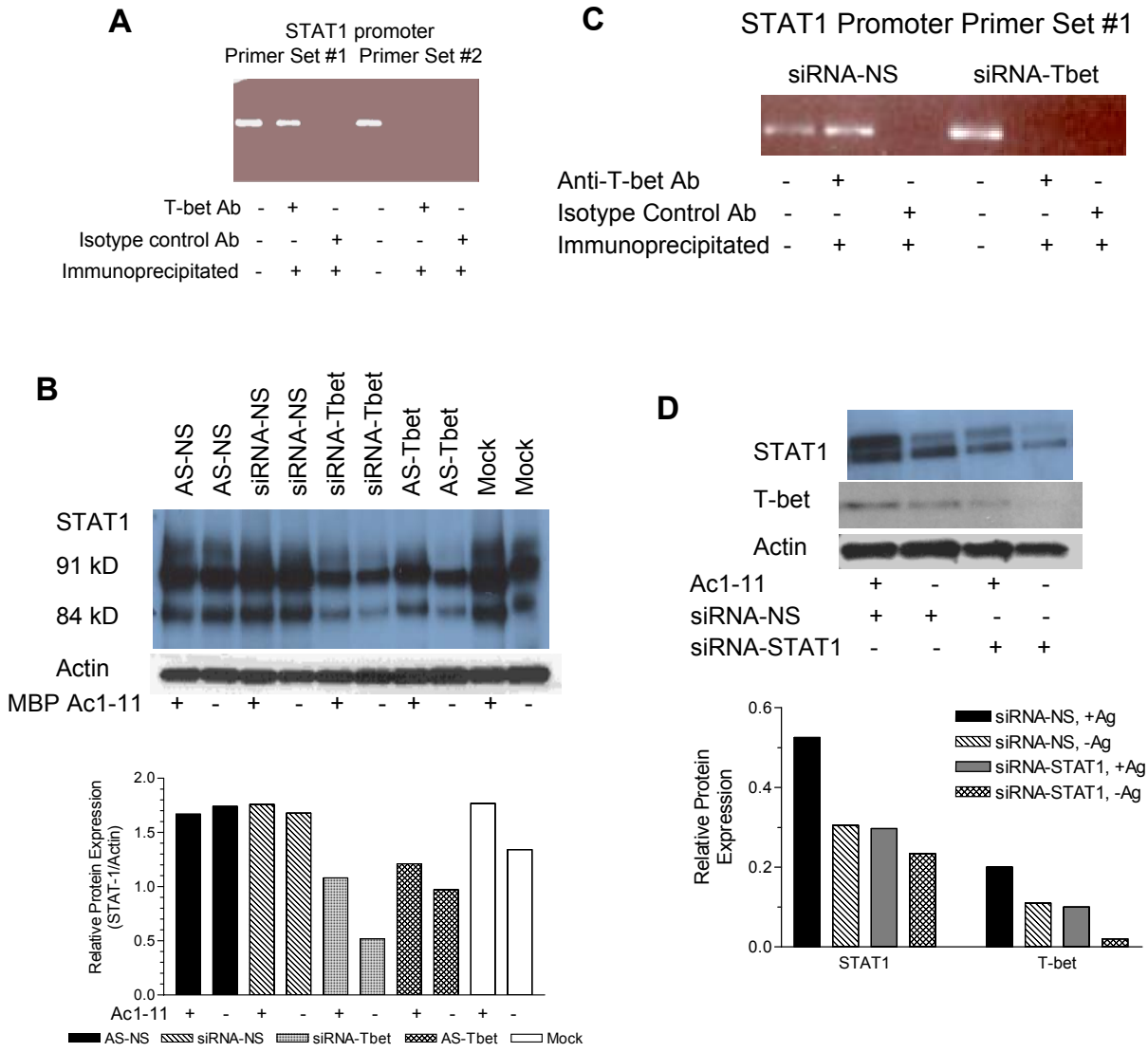


Figure 15. T-bet regulates STAT1 in a positive feedback loop. (A) ChIP assay was performed and DNA immunoprecipitated with anti-T-bet antibody from Ac1-11 activated cells was used as the template in PCR reactions using 3 primer sets for the STAT1 promoter region. One of the 2 primer sets (#1) shown specifically amplified the STAT1 gene. (B) Naïve Vβ8.2 transgenic splenocytes were transfected with AS NS, siRNA NS, siRNA T-bet, AS T-bet or mock-transfected 24 hr prior to activation with Ac1-11. Nuclear extracts were prepared after a 24 hr activation, 30 µg protein was loaded per lane on a 4-20% gradient SDS/PAGE gel, and a western blot was performed using a STAT1 antibody, demonstrating reduced STAT1 expression when T-bet was silenced during activation. Densitometry was used to normalize STAT1 to actin. (C) Vβ8.2 transgenic splenocytes were transfected with siRNA NS or siRNA T-bet prior to activation. ChIP assay was performed using an antibody specific for T-bet antibody and the STAT1 promoter was PCR amplified using primer set #1. (D) Vβ8.2 transgenic splenocytes were transfected with siRNA NS or siRNA-STAT1, activated with MBP Ac1-11 for 48 hr and nuclear extracts were prepared. Western blots were performed for STAT1 and T-bet. Densitometry was used to normalize STAT1 and T-bet to actin.

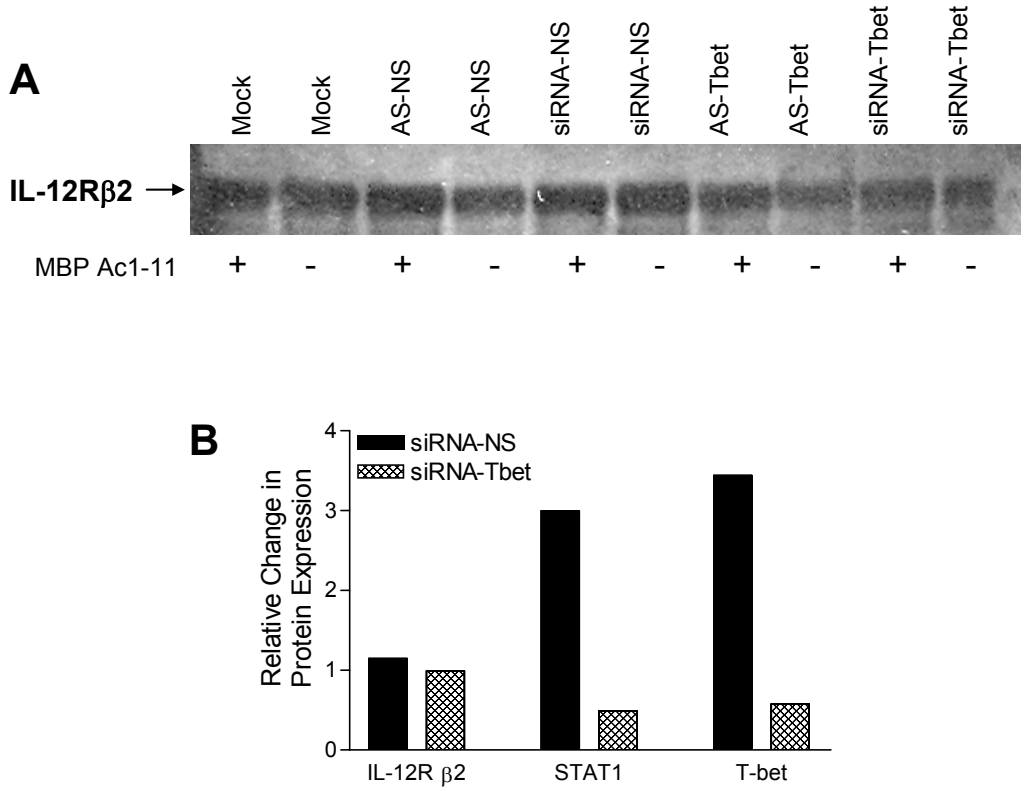


Figure 16. Silencing T-bet does not affect IL-12Rβ2 expression. (A) Vα2.3/Vβ8.2 TCR transgenic splenocytes were transfected *in vitro* with AS NS, AS T-bet, siRNA NS, siRNA T-bet, or mirus transfection reagent and activated with MBP Ac1-11 for 24 hr. Whole cell lysates were made, 20 μg of protein per lane was loaded on a 4-20% gradient SDS/PAGE gel, and a western blot was performed for IL-12Rβ2. Very little change in IL-12Rβ2 expression was observed when T-bet was silenced. (B) The same whole cell lysates as described in panel (A) were also used as the target antigen in an ELISA. Anti-IL-12R β2, anti-STAT1 and anti-T-bet were the primary antibodies. Relative change in protein expression was determined by subtracting the background and then determining the ratio of the OD of the antigen-activated cells to the OD of the no antigen cells.

Chapter IV. T-bet Regulates the Fate of Th1 and Th17 Lymphocytes in Autoimmunity

Introduction

EAE is an inflammatory demyelinating disease mediated by myelin-specific CD4+ Th1 lymphocytes. The disease can be induced in mice by immunization with various myelin proteins or peptides emulsified in CFA, or by the transfer of activated myelin-specific CD4+ Th1 lymphocytes into naïve recipients [69]. Both clinically and histopathologically, EAE is similar to Multiple Sclerosis (MS), yet the etiology of MS remains unknown. In order to develop therapies that target encephalitogenic T cells, we have been utilizing small interfering RNA (siRNA) as a mechanism to suppress particular proteins essential to CD4+ Th1 cells. One protein that we have targeted is the transcription factor, T-bet, which has been found to be a key regulator of the pro-inflammatory immune response [83]. T-bet is a member of the T-box family of transcription factors and binds to a core DNA sequence in promoter regions of a diverse set of genes. T-bet has been found to be expressed in Th1 cells, but not Th2 cells, and ectopic expression of T-bet in Th2 lymphocytes results in IFN-γ production and suppression of Th2 cytokines [83, 150, 151]. We found previously that silencing T-bet could prevent the development of EAE. Similarly, T-bet-deficient mice are resistant to the disease [99].

Recently, IL-23, which is produced by macrophages and dendritic cells, was also shown to be essential for the development of EAE [48]. IL-23, a member of the IL-12 family, is a heterodimeric cytokine comprised of the p40 subunit of IL-12 and a specific p19 subunit. In addition, IL-23 has been shown to play an important role in the maintenance of a unique population of T cells known as Th17 cells [50, 171]. This population of T cells, which appears to develop in the presence of TGF-β and IL-6, depends on IL-23 for growth and survival [172-174]. Th17 cells are believed to be pathogenic in EAE, but the transcription factors necessary for their development are still undefined. Although it is clear that T-bet plays an essential role in the differentiation of CD4+ Th1 lymphocytes [83, 85, 175], the potential role of T-bet in the IL-23/IL-17 pathway is not well understood. Recently, it was shown that Th17 cells can develop in the absence of T-bet and STAT1 *in vitro* [52]. However, it was suggested that T-bet is important for continued IL-17 production in the presence of IL-23 [52]. To investigate additional mechanisms by which suppression of T-bet ameliorates EAE, and to examine the ability of T-bet to regulate the IL-23/IL-17 pathway, T-bet-specific siRNA was administered to mice with established EAE induced by adoptive transfer of activated Th1 lymphocytes. EAE was ameliorated in these mice and this therapeutic benefit was mediated in part by suppression of IL-17 expression in the CNS, and by limiting development and effector functions of autoreactive Th1 lymphocytes.

Results

Amelioration of EAE by silencing T-bet

To determine if suppression of T-bet following disease manifestation may be clinically effective in EAE, siRNA specific for T-bet (siRNA T-bet) was administered i.v. to mice with clinically established disease. Naïve Vα2.3/Vβ8.2 TCR transgenic splenocytes, which recognize myelin basic protein (MBP) peptide Ac1-11, were differentiated *in vitro* with MBP Ac1-11 plus IL-12 and transferred into naïve B10.PL mice. Lymphocytes differentiated in this manner have a Th1 phenotype, producing significant amounts of IFN-γ, but no detectable IL-4 or IL-17 (Fig. 17A). Two days after the onset of clinical signs of EAE, siRNA T-bet or siRNA NS (nonsense) was administered i.v. The disease appeared to stabilize in the siRNA T-bet treated group, but progressed in the siRNA NS treated group (Fig. 17B). A second dose of siRNA was administered two weeks later because the effect was not sustained in mice treated with a single dose (data not shown). By day 40, the siRNA T-bet treated mice showed a remarkable improvement in their clinical scores, while the control mice continued to deteriorate ($p<0.001$), demonstrating that suppression of T-bet was beneficial in the treatment of EAE.

Clinical improvement correlates with reduced T-bet and IFN-γ

To verify that T-bet was suppressed *in vivo*, the spleen was removed from siRNA treated mice at various time points, nuclear extracts were prepared and T-bet expression was evaluated by western blotting. After the first dose of siRNA T-bet (Day 18), T-bet expression was significantly reduced in siRNA T-bet treated mice compared to the siRNA NS mice (Fig. 17C). In contrast, GATA3, a Th2-specific transcription factor, was found to be upregulated in siRNA T-bet treated mice. T-bet remained suppressed in the siRNA T-bet treated mice three weeks after the second siRNA treatment (day 44). On day 37, GATA3 levels were increased, but by day 44, there was no significant difference in GATA3 levels between the two groups of mice (Fig. 17C).

Since T-bet is known to regulate IFN-γ expression in CD4+ T cells, antigen-induced IFN-γ expression was measured in the splenocytes of mice treated with siRNA T-bet or siRNA NS. IFN-γ levels were reduced at day 18 and as late as day 60, in the siRNA T-bet treated group compared to the controls (Fig. 18). Since GATA3, which is necessary for optimal IL-4 production, was increased in the siRNA T-bet treated mice, we evaluated whether there was an increase in IL-4 in response to MBP Ac1-11. However, there was no significant IL-4 expression in splenocytes from any of the mice regardless of treatment or time point (data not shown), suggesting that a shift to a Th2 phenotype in the MBP Ac1-11-specific T cells was not responsible for the clinical improvement.

Furthermore, the presence of IL-17 could not be detected in splenocytes from any of the experimental animals by western blot or ELISA (data not shown).

Regulation of the IL-23 receptor (IL-23R) by T-bet

Since we were unable to detect IL-17 in the experiments described above, we sought to examine whether T-bet could play a role in the IL-23/IL-17 pathway upstream of IL-17 induction. Therefore, the promoter region of the IL-23R, which is expressed on activated or memory T cells [176, 177], was examined and found to contain a T-box consensus sequence 3243 bases upstream of the ATG start codon, as well as four STAT binding sites(Fig. 19A), indicating that T-bet could potentially regulate the IL-23R. Since it has previously been shown that mice deficient in IL-23 were resistant to EAE [48], the IL-23R may be critical in EAE pathogenesis. To determine if T-bet binds directly to the IL-23R promoter, chromatin immunoprecipitation assays were performed. Five primer sets for the PCR reactions were designed around the T-bet and STAT binding sites in the IL-23R promoter (Fig. 19A). Only primer set #2 amplified a sequence within the IL-23R promoter (data not shown). To verify the specificity of this reaction, ChIP assays were performed in which the MBP Ac1-11-specific splenocytes were transfected with siRNA NS or siRNA T-bet prior to activation with MBP Ac1-11 (Fig. 19B). DNA that was specifically bound to T-bet in these cells was subsequently used as the template in PCR reactions using primer set #2. The IL-23R promoter sequence was amplified from the siRNA NS-transfected cells,

but not from the siRNA T-bet-transfected cells, verifying the direct binding of T-bet to the IL-23R promoter (Fig. 19C).

It has been suggested that detected binding of a transcription factor at a promoter site by ChIP assay does not necessarily result in an obligate functional role for that transcription factor in the regulation of the associated gene [178]. Therefore, it is necessary to analyze both the ability of a transcription factor to bind to a promoter region by ChIP assay and to evaluate the ability of this binding to result in a functional consequence. This can be done by examining target gene expression in response to overexpression or absence of the transcription factor [178]. To confirm that T-bet was regulating the expression of the IL-23R, T-bet was over-expressed in EL4 cells using the pCS2 expression vector resulting in a significant increase in IL-23R mRNA (Fig. 20). This data suggests that T-bet can directly transactivate the IL-23R gene.

To determine if silencing T-bet inhibited IL-23R protein expression, splenocytes from an EAE mouse were transfected *in vitro* with siRNA NS or siRNA T-bet and activated with antigen. Western blot analysis demonstrated that T-bet and IL-23R expression were upregulated with antigen stimulation in the siRNA NS splenocytes, and expression of both molecules was decreased in the cells transfected with siRNA T-bet (Fig. 21).

In vivo suppression of T-bet results in decreased IL-23R and IL-17

To determine if silencing T-bet *in vivo* was resulting in down-regulation of IL-23R, whole cell lysates were prepared directly *ex vivo* from the splenocytes of EAE-affected mice which had been treated with siRNA NS or siRNA T-bet. Western blot analysis demonstrated a 45% reduction in IL-23R at day 18 and IL-23R was undetectable by day 44 in the siRNA T-bet treated mice (Fig. 22). Since IL-23R engagement can drive IL-17 expression [50, 171], we analyzed IL-17 production by ELISA following MBP Ac1-11 activation *in vitro* from splenocytes of siRNA NS and siRNA T-bet treated mice at multiple time points and found no IL-17 production (data not shown). In addition, IL-17 expression was analyzed by western blot using the same whole cell lysates that were used to evaluate IL-23R expression ex vivo, but no IL-17 was observed (data not shown). Since IFN-γ was expressed by the antigen-specific splenocytes (Fig. 18), but IL-17 could not be detected, we wanted to determine if this observation was due to the differential expression of IL-12 and IL-23 in the spleen. IL-12 p70 expression was the same in the siRNA T-bet and siRNA NS treated mice (Fig. 22). However, there was no IL-23 p19 expression, suggesting that there was no IL-23 present in the spleen to drive IL-17 expression in mice which develop EAE by transfer of Th1 lymphocytes.

Since it had previously been demonstrated that IL-17 was expressed in the plaques of MS patients by DNA microarray analysis [179], and increased

levels of IL-17 expressing mononuclear cells were found in the cerebrospinal fluid of MS patients [180], IL-23R and IL-17 expression were analyzed in the brains of EAE-affected mice treated with siRNA NS or siRNA T-bet. IL-23R and IL-17 were observed in the brains of control mice, but expression was significantly reduced in the siRNA T-bet treated mice (Fig. 23), suggesting that T-bet is regulating IL-23R expression and the subsequent expression of IL-17 in the CNS. It had previously been shown that IL-23 is expressed in the CNS by activated microglia in mice with EAE [181]. Analysis of IL-12 p70 and IL-23 p19 expression revealed similar levels between the groups of mice (Fig. 23), indicating that the reduced IL-17 in the siRNA T-bet treated mice was not due to lack of IL-23 in the CNS.

Discussion

This study provides evidence that the IL-23R and the subsequent expansion of IL-17-producing T cells are regulated by T-bet and demonstrates the benefit of silencing T-bet for treatment of an inflammatory mediated autoimmune disease, such as MS. We show that EAE affected mice treated with siRNA specific for T-bet were able to recover significantly from disease. Clinical improvement correlated with reduced T-bet expression, reduced IFN-γ production, and a reciprocal increase in the Th2 transcription factor GATA3 in splenocytes. These data indicate that silencing T-bet, even after EAE is induced, can alter pathogenesis and reduce clinical signs of EAE.

Analysis of other genes that may be regulated by T-bet in encephalitogenic T cells identified the IL-23R as a potential candidate and, subsequently, shed light on the significant clinical improvement observed in EAE. It has been shown that mice deficient in IL-23 are resistant to disease, indicating a critical role for IL-23 in the pathogenesis of EAE [48]. Moreover, T-bet deficient mice have been shown to be resistant to EAE [99] and we recently demonstrated that silencing T-bet at the time of EAE induction reduced the incidence and severity of disease, indicating that T-bet is critical for the differentiation of autoreactive T cells [175]. Therefore, because T-bet can regulate the IL-23R gene and, in doing so, limit IL-23 engagement and affect the fate of pathogenic

Th17 cells, loss of T-bet can result in decreased encephalitogenicity, incidence and severity of disease.

Although we were unable to detect measurable levels of IL-17 expression in the spleen, we were able to detect IL-17 in the CNS of EAE affected mice. When T-bet was silenced, we observed a subsequent decrease in both IL-23R expression and IL-17 expression in the CNS. This leads us to believe that IL-23R engagement and subsequent IL-17 production may play an important role in the pathogenesis of EAE in the target organ. Recent studies have shown that pathogenic IL-23-driven IL-17 producing cells can invade the CNS and promote the development of chronic CNS inflammation associated with autoimmunity [51]. Indeed, microarray analysis demonstrated IL-17 expression in the plaques of MS brains [179] and increased IL-17-expressing mononuclear cells were present in the cerebrospinal fluid of MS patients [180]. Using bone marrow chimeric mice in which the p40 subunit common to IL-12 and IL-23 was absent in the CNS, it was shown that IL-23 produced by resident microglia and infiltrating macrophages in the CNS was critical to the clinical onset of EAE, but not for chronic CNS inflammation, suggesting that IL-23R engagement in the CNS was critical to the pathogenicity of infiltrating myelin-specific T cells [181]. In addition, IFN-γ was found to enhance IL-23 p19 expression in primary microglia cultures, supporting the notion that the infiltrating Th1 cells in EAE may mediate IL-23 p19 production in the CNS [176].

An interesting possibility has been raised that IL-12 driven Th1 cells and IL-23 driven Th17 cells are related and arise from the same T-bet expressing precursor [182]. In this situation, T-bet would play a central role in regulating both IL-12-driven IFN-γ production and IL-23-driven IL-17 production, both of which have been shown to be important in EAE. However, two recent studies have concluded that Th17 cells are derived via a lineage distinct from Th1 cells [53, 183]. Initial studies also suggested that Th17 cells are generated most effectively *in vitro* when IL-23 is present, and IFN-γ and IL-4 neutralized [53, 183]. However, we found IL-17 expression in the CNS of EAE-affected mice, in which disease was induced by transfer of IFN-γ producing autoreactive T cells. This is in accordance with a recent study in which both T-bet+/+ and T-bet-/- pathogenic CD4+ T cell lines produced elevated levels of IFN-γ or IL-4, respectively, but also secreted significant and comparable amounts of IL-17 [184]. It was also demonstrated that T-bet deficient mice are more susceptible to experimental autoimmune myocarditis (EAM) due to an increase in IL-17 production [184]. This disease exacerbation is believed to result from a decrease in CD8-specific IFN-γ production [184]. These results correlate with findings that T-bet deficient mice, which produce little IFN-γ, and T cells from WT mice cultured with anti-IFN-γ, produce increased IL-17 [51-53, 183]. This is in contrast to our findings that IL-17 expression in the CNS decreases when T-bet is silenced. There may be a number of reasons for this discrepancy. The aforementioned studies were performed in or using cells from T-bet deficient mice. These mice develop with an impaired ability to produce IFN-γ. IFN-γ,

similar to IL-17, plays an important role in maintaining cell-mediated immune responses [185]. Therefore, T-bet deficient mice may produce increased amounts of IL-17 to compensate for a lack in IFN-γ production and protect them from infection. In our system, T-bet is knocked down using RNAi, but is not completely absent, as is the case with knockouts. Therefore, IFN-γ production is decreased, but still present, and is limited to specific cell populations, such as CD8+ T cells and NK cells, which rely less on T-bet for optimal IFN-γ production [85]. Non CD4+ T cell IFN-γ production in our system may be playing a regulatory role and limiting the expansion of pathogenic IL-17 producing cells. In addition, unlike the EAM model, in EAE, T-bet-/- mice are protected from disease [99]. Therefore, IL-17 may be playing different pathogenic roles and may be regulated slightly differently in these two disease models. For example, there is evidence that T-bet functions differently in various models of rheumatoid arthritis [95, 96]. In addition, it was speculated that IFN-γ-deficient mice develop severe EAE, because of an enhanced myelin-specific Th17 cell population [53]. However, if IL-17 producing T cells are the primary encephalitogenic cells and their production is enhanced in T-bet-deficient mice, this contradicts the observation that T-bet-deficient mice, which express little IFN-γ, are resistant to EAE induction.

Several recent papers indicate that TGFβ in the presence of IL-6 is critical for the differentiation of Th17 cells, and that IL-23 is important for the growth and function of these cells [172-174]. In our *in vivo* model, we believe that both IFN-

γ-producing Th1 cells and Th17 cells are present in the CNS. Resident CNS cells have the capacity to produce IL-23, IL-6 and TGFβ [48, 186, 187], which could contribute to the differentiation and expansion of a unique Th17 cell population in the CNS.

One concern about the suppression of T-bet and the upregulation of GATA3 that we observed is the potential to predispose to the development of Th2 mediated diseases such as asthma, since T-bet-deficient mice develop both physiological and inflammatory features characteristic of asthma [90]. In addition, *in vivo* suppression of GATA3 with an antisense oligonucleotide protects mice from the development of asthma [155]. To address this concern, the lungs of mice treated with siRNA specific for T-bet were evaluated by immunohistochemistry (data not shown) and there was no evidence of inflammation, suggesting that the level of T-bet suppression and the transient increase in GATA3 were not sufficient to predispose the mice to Th2-mediated disease.

In the present study, EAE was induced by adoptive transfer of classical MBP-specific Th1 lymphocytes, and Th17 cells could not be found in the periphery of these mice. However, IL-17 was observed in the CNS of these mice, suggesting that Th17 cells may be present in the target organ. Thus, it appears that classical Th1 cells can at least initiate the inflammatory phase of EAE and that IL-17 producing T cells may participate in target organ damage and

disease progression. The recent observation that T-bet is required for optimal IL-17 production in the presence of IL-23 [52], taken together with the mechanistic data shown in this report, supports the conclusion that one mechanism by which T-bet modulates IL-23/IL-17 mediated inflammation is by directly regulating the IL-23R gene. Moreover, the orphan nuclear receptor RORγt was shown to be required for the differentiation of T_H17 cells and RORγt deficient mice had reduced EAE severity [188]. Therefore, both RORγt and T-bet appear to be required for EAE. This study demonstrates that T-bet is an important regulator of the pro-inflammatory immune response and explains why silencing T-bet can be both protective and therapeutic in autoimmunity.

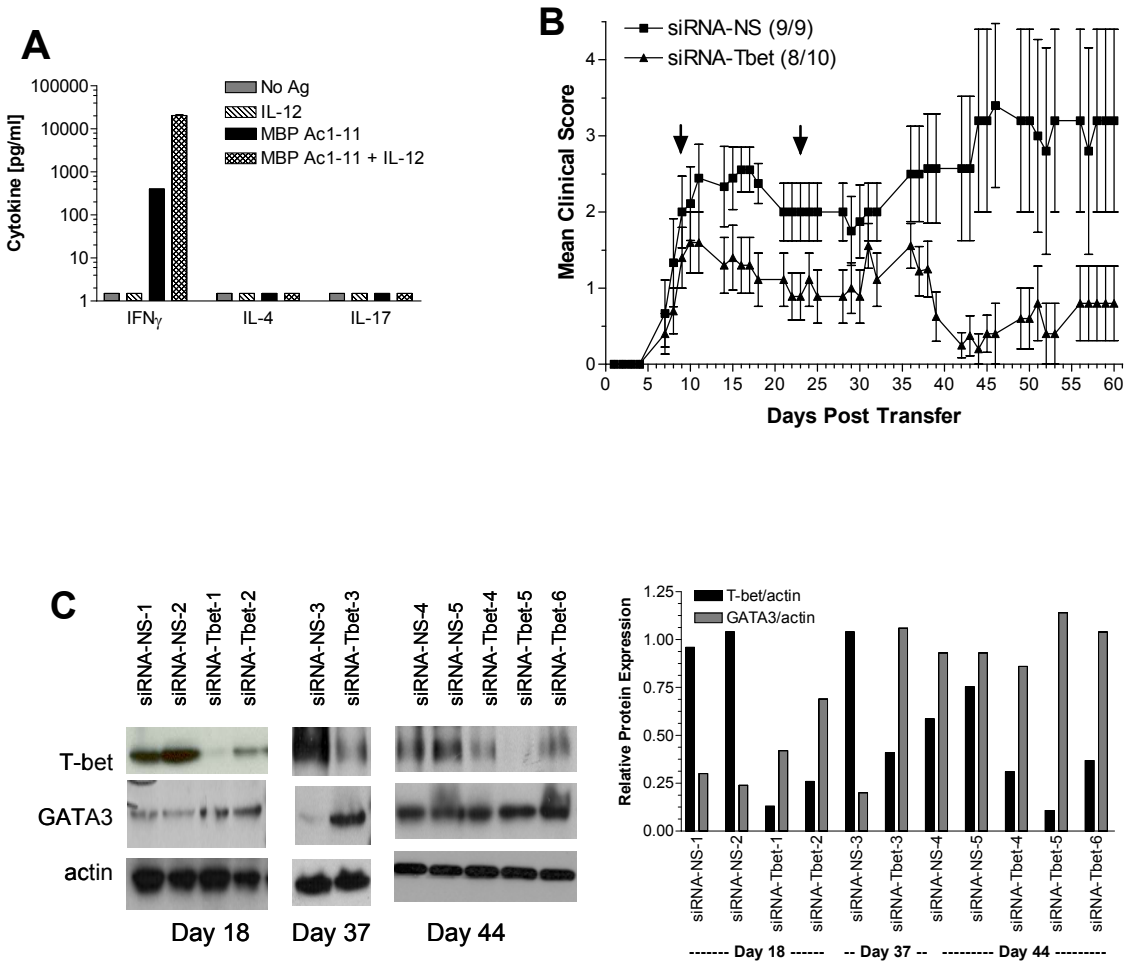


Figure 17. Therapeutic administration of siRNA T-bet ameliorates clinically established EAE. (A) Vα2.3/Vβ8.2 TCR transgenic splenocytes were differentiated *in vitro* into Th1 cells with MBP Ac1-11 and 1ng/ml IL-12. Splenocytes were also cultured with no antigen, IL-12 alone, or MBP Ac1-11 alone as a control. Cytokines in the supernatants were measured by ELISA. (B) EAE was induced via adoptive transfer of Th1 Vα2.3/Vβ8.2 TCR transgenic splenocytes into B10.PL mice. Nonsense (siRNA NS) or T-bet-specific siRNA was injected into mice (n=9 for siRNA NS and n=10 for siRNA T-bet) via the tail vein when 70% of the mice had developed clinical EAE (day 9). A second dose of siRNA was injected two weeks later. Mice were monitored for clinical signs of EAE. (C).Splenocytes were isolated from EAE affected B10.PL mice treated with siRNA 18, 37, and 44 days post-transfer. T-bet, GATA3 and actin expression were analyzed in the nuclear extracts of splenocytes by western blotting. Each lane is representative of a different mouse. Densitometry was performed and relative T-bet and GATA3 expression was determined by normalizing to actin. Results shown are representative of a minimum of 2 independent experiments.

*Technical assistance from Rehana Hussain and Lihong Ben for panels (A) and (B)

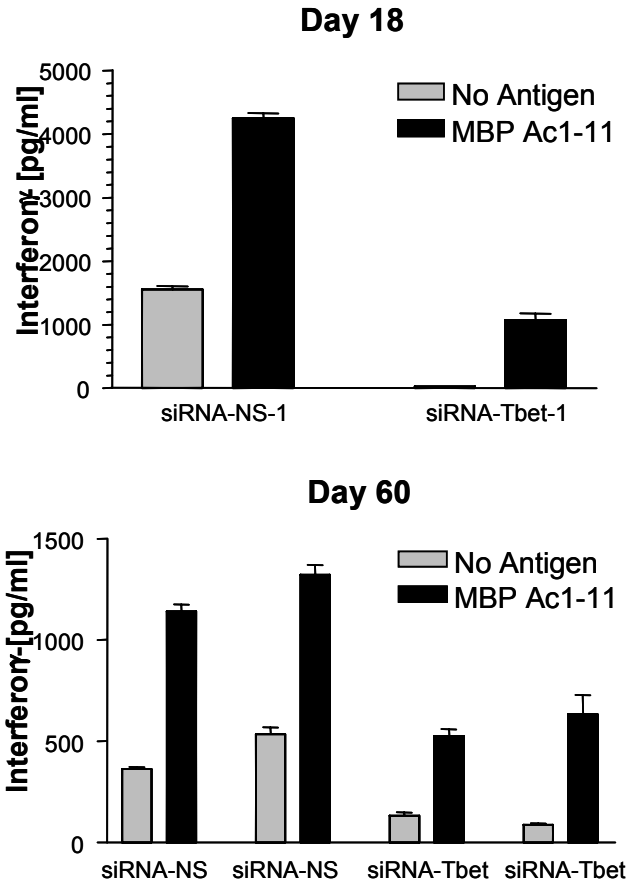


Figure 18. Antigen-induced IFN-γ secretion is decreased in siRNA T-bet treated mice. Splenocytes were isolated from siRNA T-bet or siRNA NS treated mice and restimulated *in vitro* with MBP-Ac1-11 for 48 hours. IFN-γ, IL-4 and IL-17 secretion were measured in the supernatants by ELISA at days 18 and 60 post-transfer. No IL-4 or IL-17 was detected (data not shown) and IFN-γ production was decreased in siRNA T-bet treated mice compared to controls. Results shown are representative of a minimum of 2 independent experiments.

*Technical assistance from Lihong Ben

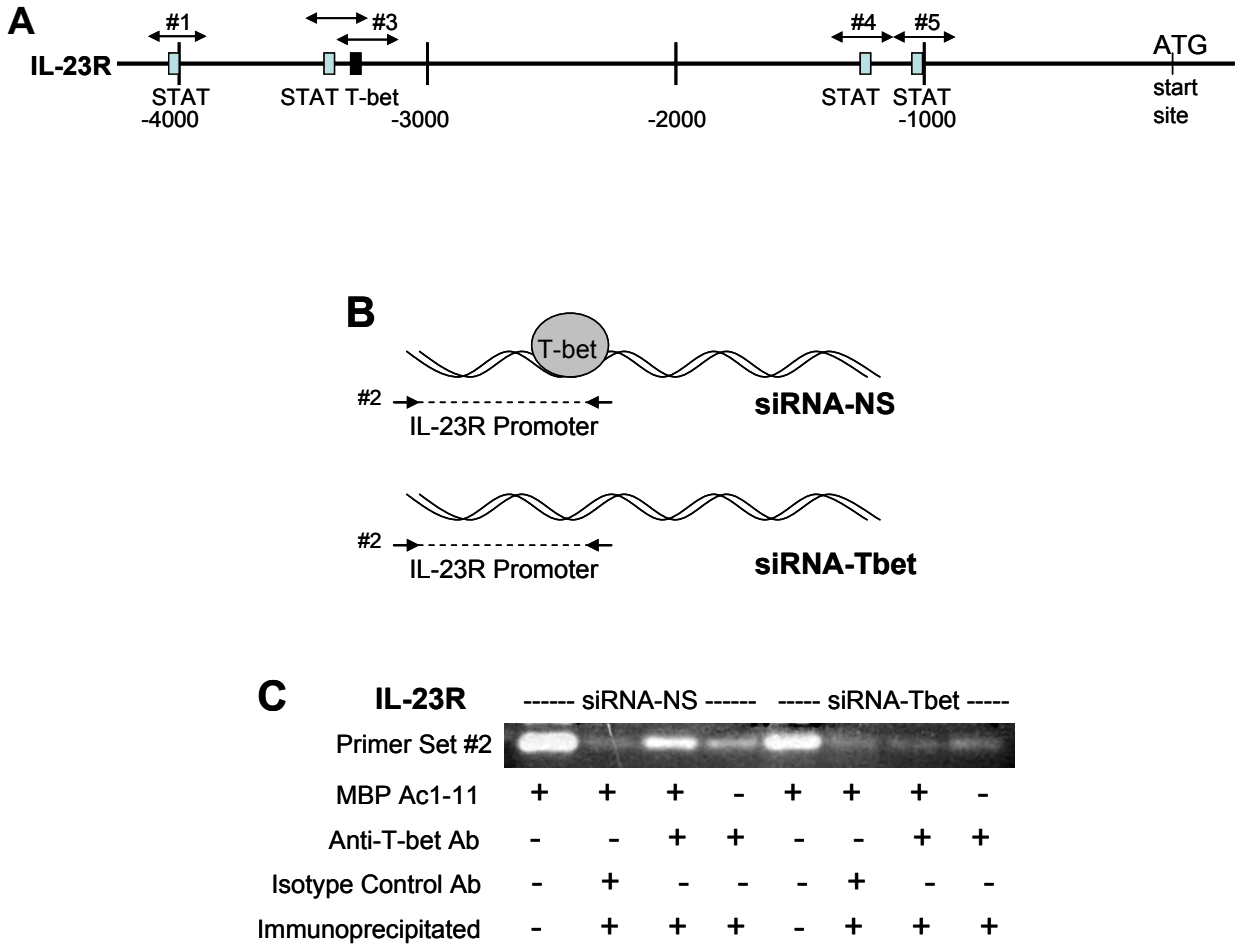


Figure 19. T-bet regulates transcription of the IL-23R. (A) Promoter region of the IL-23R gene is shown, illustrating the T-bet and STAT consensus sequence sites and location of the primer sets #1-5 for the IL-23R promoter used in the chromatin immunoprecipitation assays. (B) Splenocytes from a Vβ8.2 TCR transgenic mouse were transfected with siRNA T-bet or siRNA NS. A ChIP assay using IL-23R primer set #2, which had been the only primer set that amplified the IL-23R promoter in non-transfected MBP Ac1-11-specific T cells in a prior ChIP assay, was used to amplify the region containing the T-box consensus sequence. (C) Immunoprecipitation was performed with an antibody specific for T-bet. DNA bound to T-bet was purified and used as a template for PCR reactions. Primer set #2 specifically amplified the IL-23R gene in cells transfected with siRNA NS, but this binding was abolished in cells transfected with siRNA T-bet (lanes 3 and 7 in panel C). Lanes 1 and 5 represent input DNA from each sample as a positive control. An isotype control antibody was used in Lanes 2 and 6, while lanes 4 and 8 represent non-activated TCR splenocytes. Results shown are representative of 3 independent experiments.

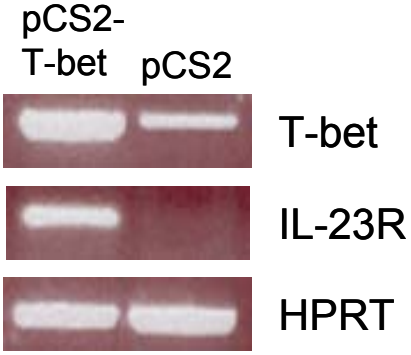


Figure 20. Overexpression of T-bet enhances IL-23R mRNA expression. Transient transfection of EL4 cells was performed using the AMAXA nucleofection system. 2 million cells per transfection were resuspended in nucleofection solution L. Setting C-009 was used for nucleofection with the standard protocol. Cells were transfected with a pCS2-T-bet expression construct or an empty vector control. 24 hours post-transfection, RT-PCR was performed to examine the effect of T-bet overexpression on mRNA expression levels for the endogenous target gene IL-23R. Results shown are representative of 2 independent experiments.

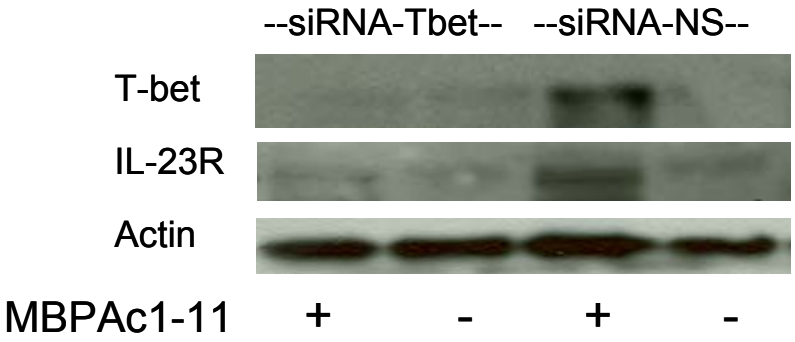


Figure 21. Silencing T-bet *in vitro* reduces IL-23R expression. Splenocytes from a B10.PL EAE-affected mouse were transfected *in vitro* with siRNA T-bet or siRNA NS and stimulated with MBP Ac1-11 for 48 hours. Whole cell lysates were made, protein was quantitated, and western blot analysis was performed to determine the level of T-bet and IL-23R expression. When T-bet was silenced, there was a concordant decrease in IL-23R expression. Results shown are representative of 3 independent experiments.

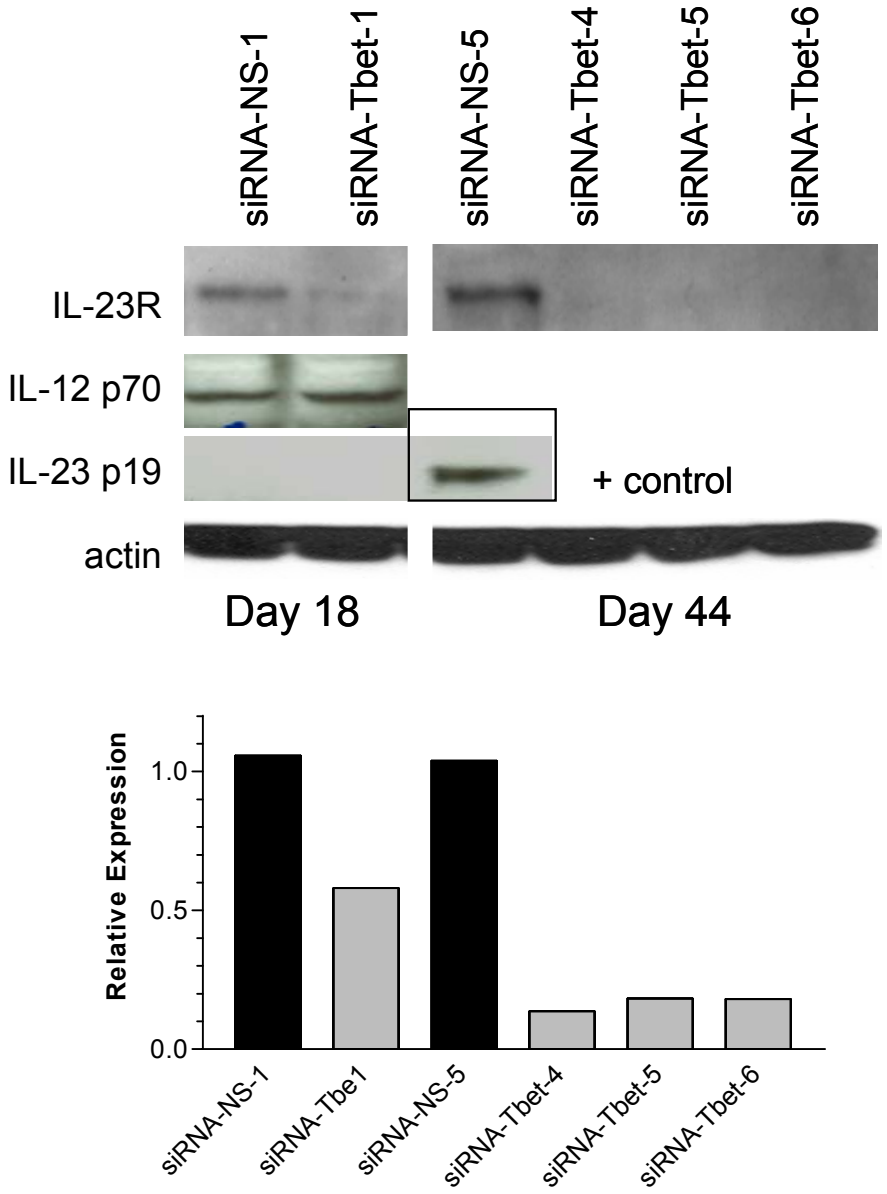


Figure 22. Silencing T-bet *in vivo* results in decreased IL-23R expression. Splenocytes were isolated from EAE affected B10.PL mice treated with siRNA T-bet or siRNA NS at 18 and 44 days post-transfer. Western blot analysis was performed for IL-23R, IL-12 p70 and IL-23 p19 expression. Recombinant IL-23 p19 was run as a positive control and is shown in rectangle. IL-17 expression was also evaluated but was not detectable. Densitometry was performed and relative IL-23R expression was determined by normalizing to actin. Each lane is representative of a different mouse. Results shown are representative of 3 independent experiments.

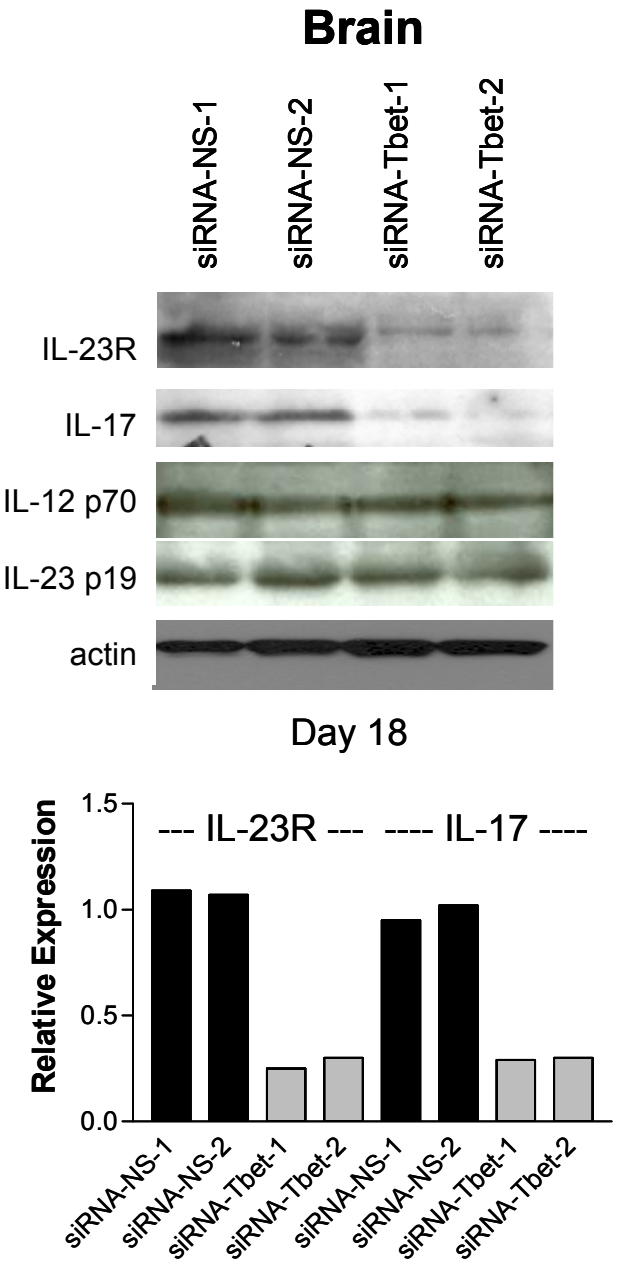


Figure 23. In vivo suppression of T-bet results in decreased IL-23R and IL-17 expression in the CNS. Brains were isolated from EAE affected B10.PL mice treated with either siRNA T-bet or siRNA NS at day 18. Lysates were prepared, protein was quantitated and western blotting was performed to analyze IL-23R, IL-17, IL-12 p70 and IL-23 p19 expression in the CNS. Densitometry was performed and relative IL-23R and IL-17 expression was determined by normalizing to actin. Each lane is representative of a different mouse. Insufficient protein was recovered from the spinal cords for evaluation. IL-23R and IL-17 expression were decreased in siRNA T-bet treated mice compared to control animals. Results shown are representative of 3 independent experiments.

Chapter V. Transcriptional Modulation of the Immune Response by PPARα Agonists Ameliorates Autoimmune Encephalomyelitis

Introduction

Experimental Autoimmune Encephalomyelitis (EAE) is an immune-mediated inflammatory disease that serves as a model for the human disease Multiple Sclerosis (MS). EAE can be induced in genetically susceptible strains of rodents by immunization with myelin or myelin peptides emulsified in complete Freund's adjuvant (CFA) or by adoptive transfer of myelin specific CD4+ Th1 lymphocytes into naïve recipient animals [69, 189]. It has been shown by our lab and others that shifting the phenotype of autoreactive CD4+ T cells from pro-inflammatory Th1 cells to IL-4 producing Th2 cells can be beneficial in EAE [69, 70, 73]. Several different reagents such as altered peptide ligands, retinoids, and peroxisome proliferator-activated receptor (PPAR) agonists have been shown to ameliorate disease in this manner [70, 148, 190, 191]. However, the mechanism by which these agents induce a Th2-like phenotype is not clearly defined.

PPARs are ligand activated transcription factors belonging to the nuclear hormone receptor superfamily which includes steroid, retinoic acid, and thyroid hormone receptors [114]. Three different isoforms of PPARs have been

identified to date, PPARα, PPARγ, and PPARβ/δ. These isoforms are encoded by separate genes and have varied tissue distribution and ligand specificity [122, 123]. Upon heterodimerizing with retinoid X receptors (RXR), PPARs can positively or negatively regulate gene expression by binding to PPAR response elements (PPREs) in the regulatory regions of target genes [117, 118, 130]. PPARα, the first PPAR to be cloned, is known to regulate lipid homeostasis and is a target of the class of drugs known as fibrates [121, 126, 129, 192]. Fibrates, such as gemfibrozil, have been used clinically for the treatment of hypertriglyceridemia for a number of years and are safe and well tolerated by patients. Recently, PPARα has been shown to be expressed in immune cells, including macrophages, dendritic cells, and B and T lymphocytes, and PPARα agonists are believed to play a role in regulating the inflammatory response [130, 141, 142, 145, 193]. More specifically, PPARα ligands have been shown to inhibit IL-2, TNF-α, and IFN-γ production by activated CD4+ T cells, and induce IL-4 production in splenocytes, suggesting the ability of these agonists to induce a Th2-like phenotype [137, 148, 194]. In addition, PPARα agonists may partially regulate inflammation by sustaining expression of the negative regulator IκBα, thereby preventing nuclear translocation and activation of NF-κB, a major transcription factor involved in initiating pro-inflammatory immune responses [130, 144]. Furthermore, the PPARα agonist WY 14,643 was shown to induce apoptosis of lymphocytes and to inhibit IgG responses in myelin oligodendrocyte glycoprotein (MOG) 35-55 immunized mice [145]. More recently, we have demonstrated that oral administration of PPARα agonists can prevent the

development of EAE. We also found that PPARα agonists can increase the production of the Th2 cytokine IL-4, reduce the production of the Th1 cytokine IFN-γ, suppress antigen-specific T cell proliferation, and reduce nitric oxide (NO) production by microglia [148].

In this study we characterized the mechanism by which the PPARα agonist gemfibrozil can induce a Th2-like response and protect mice from EAE. More specifically, we investigated the ability of gemfibrozil to interact with its receptor, PPARα, and modulate transcription of genes involved in the immune response that occurs during EAE. We examined the effects of gemfibrozil on the expression of two key transcription factors involved in T helper cell differentiation, T-bet and GATA3, and determined whether this agonist mediates its effects in EAE in an IL-4 and receptor-dependent manner using IL-4 deficient mice and a small interfering RNA (siRNA) specific for PPARα. This study also demonstrated that PPARα can positively regulate the Th2 cytokine genes IL-4 and IL-5 and illustrated specifically that PPARα binds the IL-4 promoter in the presence of the coactivator SRC-1, indicating its ability to transactivate this gene. Finally, we show that the PPARα agonists gemfibrozil and fenofibrate can effectively ameliorate established EAE, suggesting that these drugs could potentially be used therapeutically for MS.

Results

Gemfibrozil increases GATA3 and decreases T-bet expression in splenocytes

It has been shown previously that PPARα agonists can increase IL-4 production and decrease IFN-γ production in immune cells [137, 145, 147, 148]. We wanted to determine if the agonists were mediating these effects by modulating the transcription factors GATA3 and T-bet, which are known to regulate IL-4 and IFN-γ production, respectively [83, 108]. Splenocytes from naïve Vβ8.2 TCR transgenic mice were cultured with the myelin basic protein (MBP) peptide Ac1-11 in the presence or absence of 100μM gemfibrozil, nuclear extracts were prepared, and western blots were performed to measure GATA3 and T-bet expression. GATA3 expression increased and T-bet expression decreased when cells were cultured with gemfibrozil, suggesting that this PPARα agonist can not only alter IL-4 and IFN-γ production, as shown previously [148], but can also modulate the transcription factors that regulate expression of these cytokines. (Fig. 24A).

In addition to increasing IL-4 production and decreasing IFN-γ production, PPARα agonists have been shown to ameliorate EAE [148]. Furthermore, splenocytes taken from gemfibrozil treated EAE mice have decreased inflammatory cytokine production and increased IL-4 production when cultured *in vitro* [148]. To determine whether these changes in disease state and cytokine

secretion correlate with altered expression of key transcription factors involved in T helper cell differentiation, T-bet and GATA3 expression, as well as PPARα expression, were examined in splenocytes directly *ex-vivo* from EAE affected mice that were given a diet supplemented with gemfibrozil. Directly *ex-vivo*, similar to what was observed *in vitro*, GATA3 expression was increased and T-bet expression was decreased in gemfibrozil fed mice compared to vehicle fed controls at days 15 and 30 post-immunization and feeding. These changes correlated with an increase in nuclear PPARα expression in the gemfibrozil fed mice (Fig. 24B). In addition, because we have demonstrated that T-bet can directly regulate the IL-23R and shown that this regulation contributes to the pathogenicity of EAE (Chapter IV), we wanted to determine if IL-23R expression, similar to T-bet expression, was decreased directly *ex-vivo* in EAE mice that were fed gemfibrozil. We found that IL-23R expression was decreased in splenocytes of EAE affected mice that were given gemfibrozil at 30 days post-immunization/feeding compared to vehicle fed controls at this same time point, correlating gemfibrozil treatment with decreased disease severity (Fig. 24B).

Gemfibrozil partially mediates its protective effects in EAE in an IL-4-dependent manner

Prior work suggests that gemfibrozil can decrease disease severity in EAE and that this decreased severity correlates with an increase in the cytokine IL-4. In addition, an increase in the transcription factor GATA3 was observed in the

presence of gemfibrozil (Fig. 24A & B) and GATA3 is known to enhance IL-4 production in an amplification loop. Therefore, we wanted to determine if the efficacy of gemfibrozil treatment in EAE was dependent on production of IL-4. IL-4 deficient or WT B10.PL mice were started on a diet continuously supplemented with 0.25% w/w gemfibrozil 1 day prior to immunization with MBP Ac1-11. Gemfibrozil fed mice that were deficient in IL-4 were not protected from disease to the same extent as the WT gemfibrozil fed mice, although there was a decreased incidence of disease in IL-4-/- gemfibrozil fed mice (3 out of 5 mice developed EAE) as compared to vehicle fed controls (5/5 mice developed EAE) (Fig. 25A). The differences between EAE groups were found to be statistically significant (IL-4-/- Gem vs. WT Gem, $p< 0.01$, WT Gem vs. WT ETOH, $p< 0.01$). This data indicates that deficiency in IL-4 reduces the efficacy of gemfibrozil treatment in EAE, suggesting that one mechanism by which the drug exerts its protective effect is dependent on IL-4. However, because the gemfibrozil fed IL-4 deficient mice demonstrated a decreased incidence of disease, it is possible that there is more than one mechanism by which PPARα agonists work to ameliorate disease. This experiment was repeated in B10.PL transgenic mice expressing a TCR specific for the myelin basic protein peptide, MBP Ac1-11, that were WT or deficient in the IL-4 gene and the results were similar to the data described above. IL-4 deficient mice that were fed gemfibrozil were not protected from EAE, when compared to WT gemfibrozil mice, which had reduced disease severity (Fig. 25B). It is important to note that the WT gemfibrozil fed mice were not protected from disease to the extent seen previously [148]

because these mice expressed the Vβ8.2 TCR transgene and develop a more severe form of EAE [71, 156].

We also wanted to determine whether deficiency in IL-4 affected PPARα expression, and if changes in PPARα correlated with disease severity. To do this, PPARα expression was evaluated directly *ex-vivo* by western blot in splenocytes from mice in the previous experiment. IL-4 deficient mice were found to have decreased nuclear PPARα expression compared to WT controls even in the presence of gemfibrozil (Fig. 25C). This decreased expression correlated with increased disease severity.

The PPARα agonist gemfibrozil modulates the immune response in a receptor-dependent manner

It has been suggested previously that PPARα agonists mediate their anti-inflammatory effects in a PPARα independent manner [137]. To determine if this was true in our system, naïve splenocytes from a Vβ8.2 TCR transgenic mouse were transfected with an siRNA specific for PPARα *in vitro*. Cells were then stimulated in the presence of 100μM gemfibrozil and PPARα expression was measured by western blot. We found that we were able to successfully knock-down PPARα expression using siRNA *in vitro*. Furthermore, when PPARα expression was knocked down there was no longer an increase in GATA3

expression in the presence of gemfibrozil (Fig. 26). This data suggests that the increase in GATA3 expression that occurs in the presence of gemfibrozil (Fig. 24A & B) occurs in a receptor-dependent manner.

To investigate whether the PPARα agonist gemfibrozil was mediating its protective effects in EAE in a receptor-dependent manner *in vivo*, siRNA specific for PPARα or an siRNA NS control was administered via the tail vein to B10.PL mice. In addition, the diet of these mice was supplemented with 0.25% w/w gemfibrozil or vehicle control (ETOH) beginning 1 day prior to immunization with MBP Ac1-11. Silencing PPARα *in vivo* decreased the efficacy of gemfibrozil treatment in EAE (siRNA NS vs siRNA PPARα, $p < 0.001$) (Fig. 27A). In the gemfibrozil fed groups, only 1 mouse given siRNA NS developed EAE and this occurred late in the disease course compared to 3 out of 4 mice developing EAE in the siRNA PPARα treated group. Overall, disease was most severe in mice that received siRNA PPARα (gemfibrozil and ETOH fed), suggesting an important role for this receptor, and possibly its endogenous ligands, in protection from EAE. To confirm that PPARα expression was successfully silenced *in vivo*, splenocytes were isolated from mice 15 days post-immunization and PPARα expression was measured by western blot. PPARα expression was decreased in mice given siRNA specific for PPARα compared to siRNA NS controls, confirming that our knock down was successful *in vivo* (Fig. 27B).

To evaluate the ability of gemfibrozil to induce a shift to a Th2-like phenotype *ex vivo* in the absence of PPARα, splenocytes were isolated from siRNA PPARα or siRNA NS EAE mice 15 days post-immunization and restimulated *in vitro* with MBP Ac1-11. Supernatants were collected and IL-4 and IL-5 production were measured by ELISA. Interestingly, splenocytes taken from siRNA PPARα treated mice were unable to secrete increased IL-4 and IL-5 even if the mice were fed gemfibrozil. Mice given siRNA NS and fed gemfibrozil were able to produce increased IL-4 and IL-5 compared to ETOH fed controls (Fig. 28). This data suggests that the ability of the PPARα agonist gemfibrozil to induce a Th2-like phenotype, as determined by increased Th2 cytokine secretion, is receptor-dependent.

In addition, because it has been demonstrated recently that IL-17 production contributes to the pathogenesis of EAE and because we have found that T-bet, via direct regulation of the IL-23R, can influence the fate of pathogenic Th17 cells (Chapter IV), we considered the possibility that gemfibrozil was ameliorating EAE not only by increasing Th2 cytokine production, but by inhibiting Th17 cytokine production as well. Therefore, we evaluated the ability of gemfibrozil to impair IL-17 production *ex-vivo* in mice that were given siRNA PPARα compared to siRNA NS controls. We found that splenocytes taken from siRNA PPARα treated mice produced increased amounts of IL-17 even if the mice were fed gemfibrozil *in vivo* compared to ETOH fed controls (Fig. 28). Contrary to what was found for IL-4 and IL-5, siRNA NS treated mice that were

fed gemfibrozil were impaired in their ability to secrete IL-17 compared to ETOH fed controls. Therefore, this suggests that gemfibrozil, via interaction with its receptor PPARα, ameliorates EAE by increasing Th2 cytokine production and decreasing pathogenic IL-17 production.

PPARα regulates IL-4 and IL-5

Because we no longer observed an increase in the Th2 cytokines IL-4 and IL-5 in the presence of gemfibrozil when PPARα is silenced, we wanted to ascertain whether PPARα could directly regulate these genes. To determine if PPARα binds directly to the IL-4 and/or IL-5 promoter regions, chromatin immunoprecipitation (ChIP) assays were performed. PPARα specific DNA was amplified using primers specific for the IL-4 and IL-5 promoter regions. In the presence of gemfibrozil, following stimulation with antigen, PPARα bound the IL-4 and IL-5 promoters (Fig. 29A). This binding was not observed in the absence of ligand or when we immunoprecipitated with an isotype control antibody. In addition, the IL-4 and IL-5 promoters contain a PPAR response element (PPRE) located approximately 317 and 1738 base pairs upstream from the transcription start site respectively, and this is the region of each promoter that was amplified. To determine if PPARα could also regulate IL-4 and IL-5 production indirectly via regulation of the transcription factors GATA3 and c-Maf, which are known to regulate Th2 cytokine genes, the PPARα specific DNA was also amplified using

primer sets specific for the GATA3 and c-Maf promoters. We were unable to detect binding to either of these regulatory regions by PPARα (Fig. 29B) suggesting that regulation of IL-4 and IL-5 production is not occurring indirectly through regulation of GATA3 or c-Maf. Rather, it is possible that PPARα dependent Th2 cytokine production, specifically IL-4 production, can lead to increased GATA3 expression that is seen in the presence of gemfibrozil (Fig. 24A & B).

To verify the specificity of this binding, another ChIP assay was performed in which the splenocytes were transfected with siRNA PPARα or siRNA NS prior to activation. When PPARα expression is inhibited using RNAi, we no longer observe binding to the IL-4 or IL-5 promoters in the presence of gemfibrozil compared to the siRNA NS transfected controls(Fig. 29C).

It has been suggested that binding to regulatory regions of a gene by a transcription factor does not always result in functional transactivation. In an unliganded state, PPARα is bound to a corepressor complex. Following ligand binding, this corepressor complex is displaced and targeted for degradation [130]. The PPARα/RXR heterodimer is then bound by a co-activator complex containing SRC-1. This complex can bind to PPREs in promoter or enhancer regions of target genes and transactivate these genes [130]. To prove that PPARα is transactivating the IL-4 gene, and therefore indicate functional binding, we examined whether PPARα is bound to the coactivator SRC-1 when it is

binding to the IL-4 promoter. To accomplish this, a ChIP re-ChIP assay was performed. Splenocytes were stimulated in the presence or absence of gemfibrozil and ChIP was performed using an antibody specific for PPARα. Following immunoprecipitation with PPARα another immunoprecipitation was performed using an antibody specific for the coactivator SRC-1. DNA that was specifically bound to PPARα when SRC-1 was bound was then used as a template in a PCR reaction. We found that a primer set which spans a PPRE, amplified a sequence within the IL-4 promoter when the cells were cultured in the presence of gemfibrozil (Fig 30). This data, in combination with the inability of gemfibrozil to increase IL-4 production when PPARα is silenced (Fig. 28), suggests that PPARα can transactivate the IL-4 promoter and that the binding is functional.

Treatment of EAE mice with the PPARα agonists gemfibrozil and fenofibrate ameliorates disease course

PPARα agonists have been shown to protect mice from the development of EAE. To determine if treatment with the PPARα agonists gemfibrozil and fenofibrate could ameliorate EAE once disease has already been established, C57BL/6 mice were immunized with MOG 35-55 to induce EAE. Once all mice developed disease, the mice were fed gemfibrozil, fenofibrate, or vehicle controls by gavage for 5 days. After 5 days, the mice were given a diet supplemented with 0.25% w/w gemfibrozil, fenofibrate or vehicle control for the duration of the

experiment. Treating mice with gemfibrozil and fenofibrate after disease was established significantly ameliorated their disease course (GEM vs. ETOH, $p < 0.001$, FEN vs. DMSO, $p < 0.001$) (Fig. 31). This data suggests that these drugs could be useful for therapeutic purposes in inflammatory immune mediated diseases.

Discussion

The current study investigates the mechanism by which the PPARα agonist gemfibrozil induces an anti-inflammatory phenotype and protects from EAE. Gemfibrozil has been shown to affect cytokine production, but was also found in this study to alter the expression of the transcription factors T-bet and GATA3, which are essential for the differentiation of Th cells. Changes in expression of these transcription factors was observed following culture *in vitro* as well as directly *ex-vivo* when taken from mice that had been given a diet supplemented with gemfibrozil. In addition, it has been shown by us and others that decreased T-bet expression correlates with protection from EAE. T-bet knockout mice and mice that have had T-bet silenced using RNAi are protected from disease and have been shown to have a correlative increase in GATA3 expression [99, 175](and Chapter IV). Furthermore, we have demonstrated that T-bet, via direct regulation of the IL-23R, can influence the fate of pathogenic IL-17 producing cells (Chapter IV). Although Th17 cells have been shown to differentiate independently of T-bet, they appear to rely on T-bet for optimal IL-23 responsiveness and, therefore, survival. We have found that T-bet can directly regulate the IL-23R and when T-bet is silenced using RNAi, IL-23R expression and IL-17 expression are decreased, resulting in protection from EAE (Chapter IV).The current study suggests that at least one mechanism by which PPARα agonists exert their protective effects in EAE is through down-regulation of T-bet and up-regulation of GATA3. This altered transcription factor expression results

in a Th2-like phenotype and may lead to decreased proliferation/expansion of encephalitogenic Th1 or Th17 cells. Furthermore, it has been shown that T-bet and GATA3 can regulate one another and in doing so may affect downstream signaling [195, 196]. Therefore, if PPARα, through interaction with its ligand, can regulate either of these transcription factors, directly or indirectly, this could result in the regulation of the other transcription factor.

This study also demonstrates that the protective effects of gemfibrozil treatment in EAE are partially dependent on IL-4. The data indicates that there may be more than one mechanism by which gemfibrozil exerts it protective effects in EAE, but suggests that IL-4 does play an important role in this protection. An additional mechanism for protection may be mediated via antigen presenting cells, such as microglia which have been implicated in the pathology of EAE and MS [197]. PPARα and RXR agonists have been shown to inhibit microglial and astrocyte production of nitric oxide, IL-1β, TNF-α, IL-6 and MCP-1, all of which contribute to pathogenesis in EAE [187, 198], and the PPARα agonist fenofibrate has been demonstrated to suppress LPS induction of IL-12, IL-23, and IL-27p28 by microglia (personal communication with Dr. Paul Drew). In addition, PPARα agonists may mediate protection from EAE in part by repression of transcription factors, such as NF-κB and T-bet, that regulate Th1 inflammatory genes, or by negatively regulating the production of pro-inflammatory cytokines, such as IFN-γ, as has been demonstrated for PPARγ [199-201].

Increased nuclear expression of PPARα in WT gemfibrozil fed mice was also observed (Fig. 24B). However, this expression was decreased in IL-4-/- gemfibrozil fed mice to levels seen in vehicle controls, suggesting an important role for IL-4 in the regulation of PPARα. There is evidence of cross-talk between PPAR signaling pathways and STAT and GATA transcription factors [135]. It is possible that PPARα agonists induce increased IL-4 expression which then drives Th2 differentiation via STAT6 and GATA3. This differentiation can then result in increased IL-4 production which could lead to an increase in PPARα expression in a positive feedback loop. Additional studies are required to further investigate the role of IL-4 in induction of PPARα expression and to elucidate other mechanisms of protection by gemfibrozil in IL-4 deficient mice.

It has previously been suggested that fibrates increase IL-4 production via a PPARα independent mechanism. In a study by Cunard et al., the PPARα agonist WY14,643 was found to induce modest IL-4 production in splenocytes from PPARα knockout mice [137]. Contrary to these results, we found that gemfibrozil increases GATA3 expression and ex-vivo IL-4 and IL-5 production in a PPARα dependent manner. Furthermore, gemfibrozil was shown to exert its protective effects in EAE in a receptor-dependent manner when PPARα was silenced *in vivo* using siRNA. There may be a number of reasons for this discrepancy. Rather than using PPARα knockout mice, we silenced PPARα expression using RNA interference, thereby avoiding the redundancy or compensation that may occur when a gene is not expressed during development.

In addition, this study employed gemfibrozil rather than WY14,643 and B10.PL mice were used which have a different genetic background than PPARα deficient mice [202]. Moreover, there may be additional mechanisms by which gemfibrozil mediates protection in EAE, other than increasing IL-4 production, such as inhibiting production of the pro-inflammatory cytokines IL-17 or IL-23, that may be dependent on receptor-ligand interactions. Importantly, this study demonstrates that PPARα directly regulates IL-4 and IL-5 by binding to PPREs in the regulatory regions of these genes as shown by chromatin immunoprecipitation assay. This data, in combination with the inability of siRNA PPARα treated mice to produce increased IL-4 and IL-5, suggests that regulation of these cytokine genes is one way in which gemfibrozil induces immune deviation to a Th2-like phenotype and protects mice from EAE. Moreover, the finding that PPARα binds the IL-4 promoter when the coactivator SRC-1 is bound is further support that PPARα, following ligand binding, can directly transactivate the IL-4 gene. In an unliganded state, PPARα is bound to a corepressor complex. In the presence of ligand, this corepressor complex dissociates and is targeted for degradation. The PPARα/RXR heterodimer can then associate with a co-activator complex and bind to PPREs in promoters and enhancers of target genes. It is this coactivator complex that induces chromosomal modifications such as chromatin acetylation and remodeling and allows transcriptional machinery to gain access to the regulatory regions of genes that are regulated by PPARα [203]. Association of PPARα with the coactivator complex on the IL-4 promoter is

strong evidence of transactivation and receptor-dependent regulation of IL-4 in the presence of gemfibrozil.

Finally, in this study established EAE was ameliorated with the PPARα agonists gemfibrozil and fenofibrate. This is important because it suggests that PPARα agonists could be used clinically for the treatment of immune mediated inflammatory diseases such as MS. In diseases such as MS, patient treatment begins after the onset of clinical signs and symptoms of disease occur. Therefore, it is necessary to show that a drug can be effective in ameliorating established disease in an animal model before considering the drug for use in clinical trials. Gemfibrozil, and fenofibrate, have been used for many years for the treatment of hypertriglyceridemia and have been shown to be safe and effective in a large number of patients. For this reason, treating MS patients with these drugs would pose little threat of adverse reaction and may be more beneficial, if taken alone or in combination with current FDA approved drugs, than presently available MS drugs alone. In addition, fibrates are taken orally which differs from the method of administration of current MS therapies. Therefore, the use of these drugs could improve the quality of life of the MS patient if proven to be effective in reducing disease severity.

Overall, this study provides new insight into the anti-inflammatory mechanism of the PPARα agonist gemfibrozil and further delineates how this drug ameliorates EAE. It suggests for the first time that PPARα agonists mediate

their protective effects in a receptor dependent manner via transcriptional activation of the Th2 cytokine genes IL-4 and IL-5. This regulation of IL-4 and IL-5 results in immune deviation to a Th2-like phenotype via altered expression of the transcripton factors T-bet and GATA3. The model we propose for the protective mechanism of gemfibrozil in EAE is as follows (Fig. 32). In the presence of PPARα agonists, PPARα heterodimerizes with RXR, dissociates from its nuclear corepressor complex, associates with a coactivator complex and binds to PPREs in the promoter region of IL-4 and/or IL-5. The transactivation of IL-4/IL-5 leads to increased expression of GATA3 which in turn results in decreased T-bet expression and downregulation of the Th1 inflammatory response. This shift in the immune response to a Th2-like phenotype results in amelioration of EAE. This study suggests that fibrates could provide an effective therapy for MS and insight gained from dissecting the mechanism of action of fibrates in EAE could be used for the development of new drugs with fewer side-effects to be used for the treatment of immune-mediated inflammatory diseases.

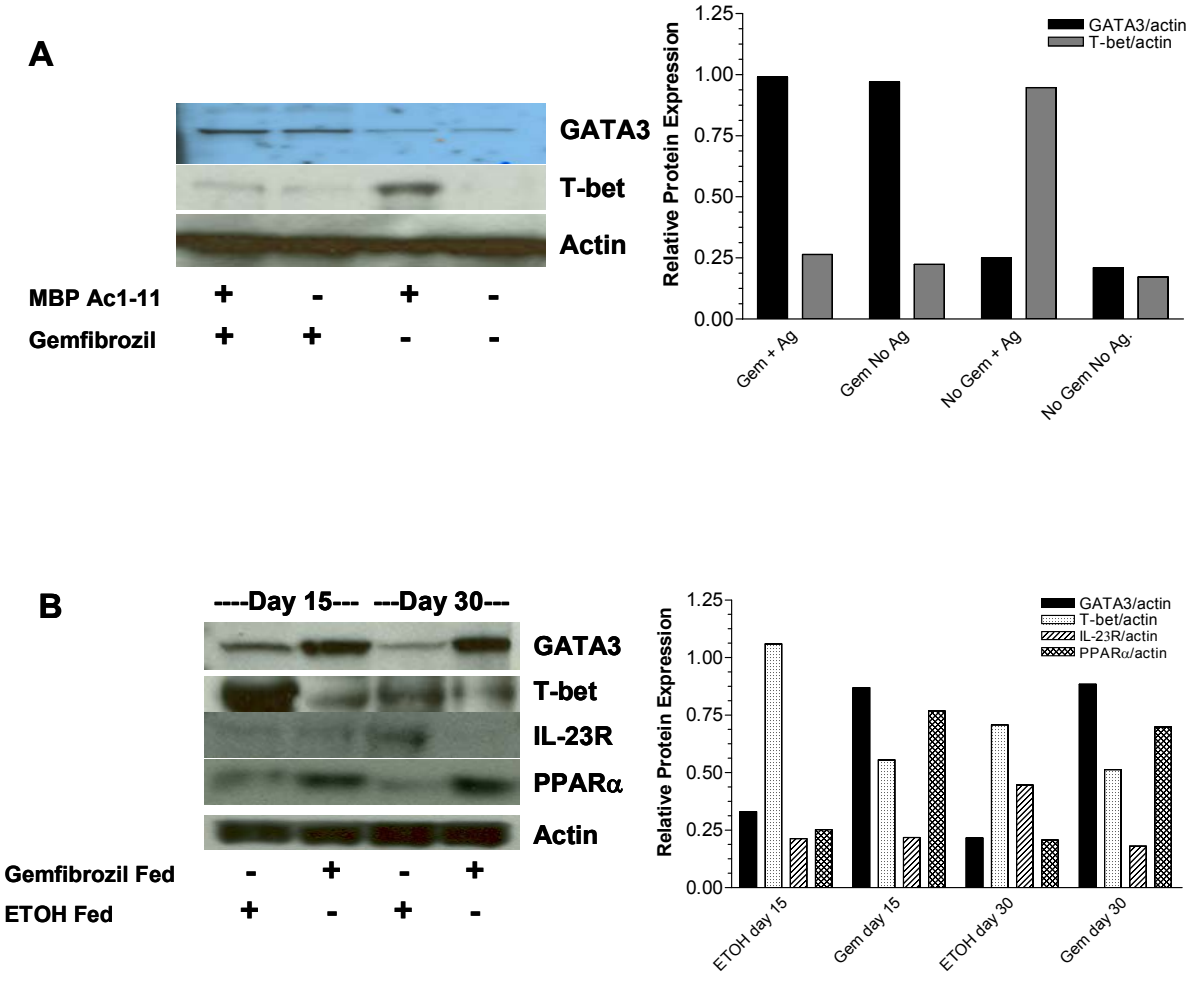


Figure 24. Gemfibrozil increases GATA3 and decreases T-bet expression *in vitro* and *ex-vivo* in mouse splenocytes. (A) The PPARα agonist gemfibrozil (100μM) or vehicle control (ETOH) was added to Vβ8.2 TCR transgenic splenocytes from a B10.PL mouse and cells were stimulated with MBP Ac1-11 for 48 hours. Nuclear extracts were made and GATA3 and T-bet expression were measured by western blot. (B) B10.PL mice were given a diet supplemented with 0.25% wt/wt gemfibrozil or vehicle control (ETOH) beginning 1 day prior to immunization with MBP Ac1-11. At days 15 and 30 post-immunization, splenocytes were isolated and GATA3, T-bet, IL-23R and PPARα expression were measured *ex-vivo* by western blot. Densitometry was performed on western blots and relative GATA3, T-bet,IL-23R,and PPARα expression was determined by normalizing to actin. Results shown are representative of a minimum of 3 experiments.

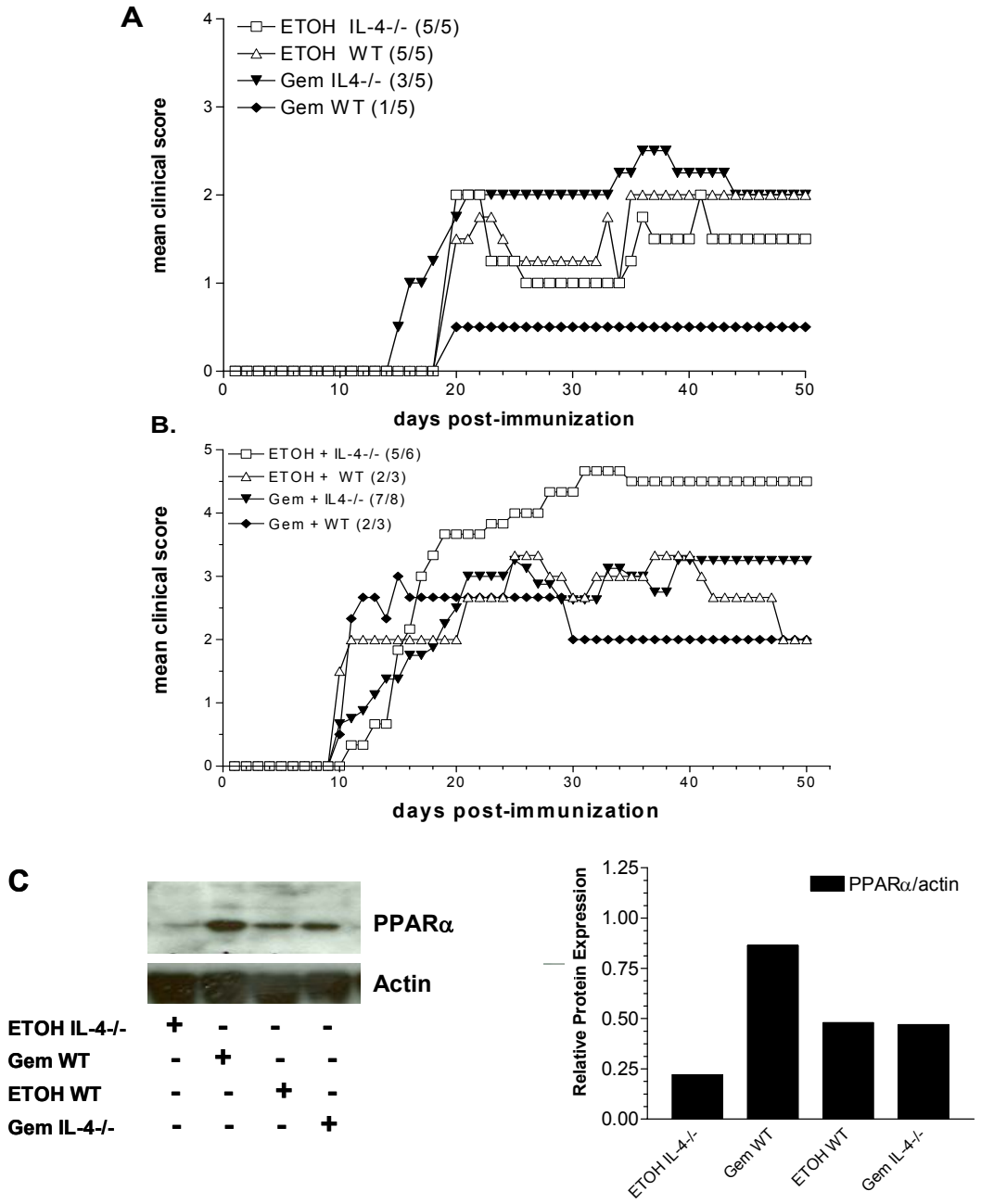


Figure 25. Gemfibrozil partially mediates its protective effects in EAE in an IL-4 dependent manner. (A) B10.PL mice deficient in IL-4 or WT for the IL-4 gene were given a diet supplemented with 0.25% wt/wt gemfibrozil or vehicle control (ETOH) beginning 1 day prior to immunization with MBP Ac1-11 to induce EAE. Mice were monitored for clinical signs of disease. Disease incidence is indicated in parentheses. A representative experiment is shown. IL-4-/- Gem vs. WT Gem, $p < 0.01$, WT Gem vs. WT ETOH, $p < 0.01$. (B) Vβ8.2 TCR transgenic B10.PL mice deficient in IL-4 or WT for the IL-4 gene were given a diet supplemented with 0.25% wt/wt gemfibrozil or vehicle control (ETOH) beginning 1 day prior to immunization with MBP Ac1-11 to induce EAE. Mice were monitored for clinical signs of disease. Disease incidence is indicated in parentheses and mortality occurred in all groups except for WT Gem group. IL-4-/- Gem vs. WT Gem, $p < 0.01$, WT Gem vs. WT ETOH, $p < 0.05$. (C) 30 days post-immunization and feeding splenocytes were isolated from representative mice in panel (A) and nuclear extracts were made. PPARα expression was measured directly *ex-vivo* by western blot. Densitometry was performed and relative PPARα expression was determined by normalizing to actin. Results shown are representative of a minimum of 2 experiments.

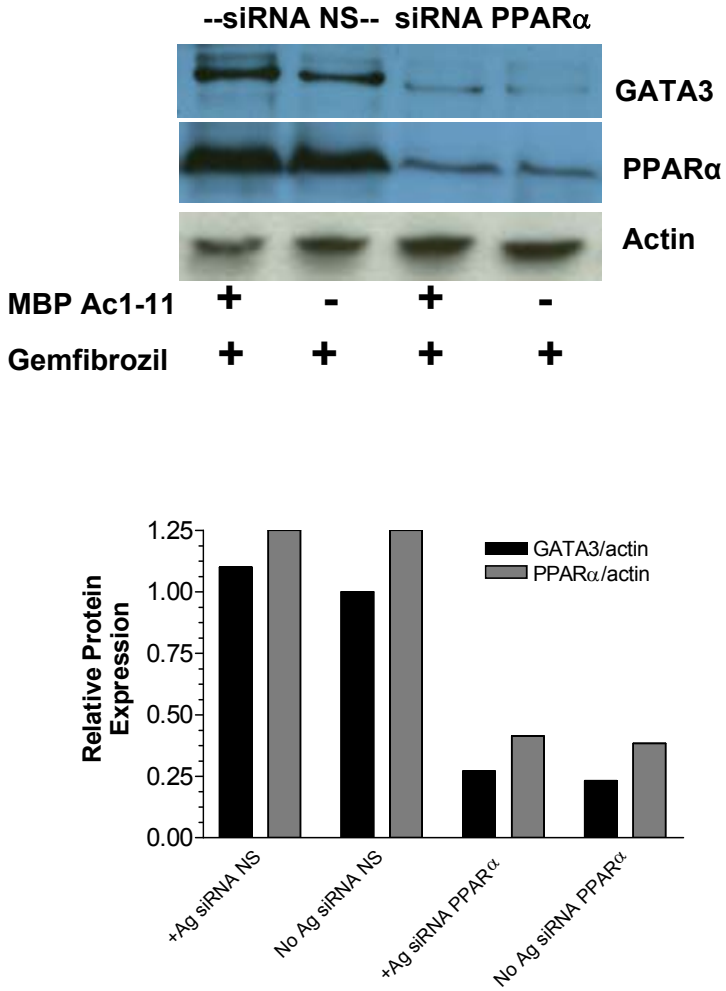


Figure 26. Gemfibrozil increases GATA3 expression in a PPARα-dependent manner. Splenocytes isolated from a Vβ8.2 TCR transgenic B10.PL mouse were transfected *in vitro* with an siRNA specific for PPARα or an siRNA NS control. Cells were cultured in the presence of 100μM gemfibrozil or vehicle control (ETOH) with or without the antigen MBP Ac1-11 for 48 hours. Whole cell lysates were prepared, protein was quantitated, and PPARα and GATA3 expression were measured by western blot. Densitometry was performed and relative PPARα and GATA3 expression were determined by normalizing to actin. Results shown are representative of a minimum of 2 experiments.

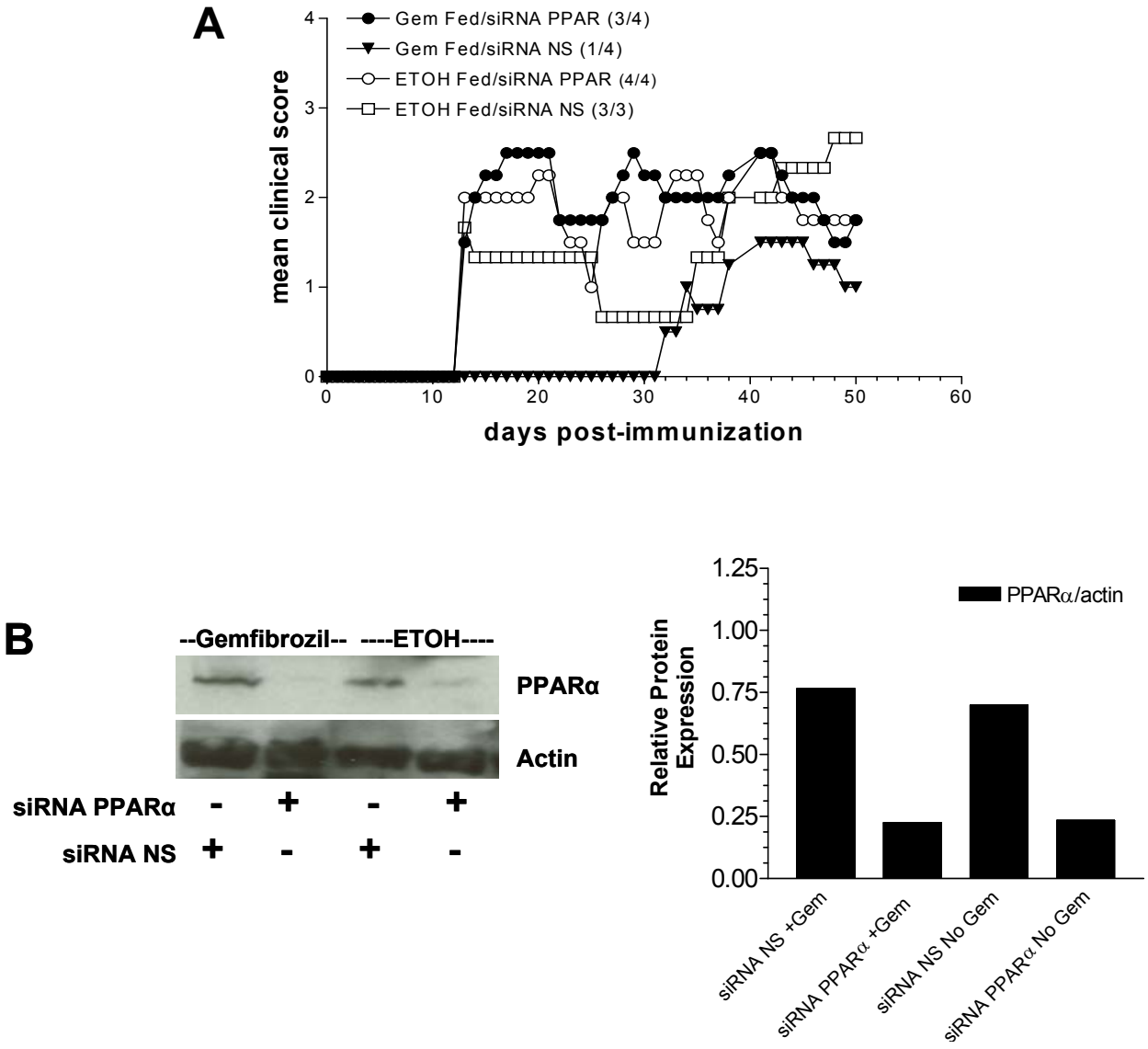


Figure 27. Gemfibrozil ameliorates EAE in a receptor dependent manner. (A) siRNA PPARα or siRNA NS were administered to B10.PL mice *in vivo* via tail vein and diet was supplemented with 0.25% wt/wt gemfibrozil or vehicle control (ETOH) 1 day prior to immunization with MBP Ac1-11. Mice were monitored for clinical signs of EAE. Disease incidence is indicated in parentheses. siRNA PPARα + Gem vs. siRNA NS + Gem, $p < 0.001$, siRNA NS + Gem vs. siRNA NS + ETOH, $p < 0.001$. (B) Splenocytes were isolated from mice receiving siRNA PPARα or siRNA NS 15 days post-immunization/feeding and PPARα expression was measured directly *ex-vivo* by western blot to verify gene silencing *in vivo*. Densitometry was performed and relative PPARα expression was determined by normalizing to actin. Results shown are representative of a minimum of 2 experiments.

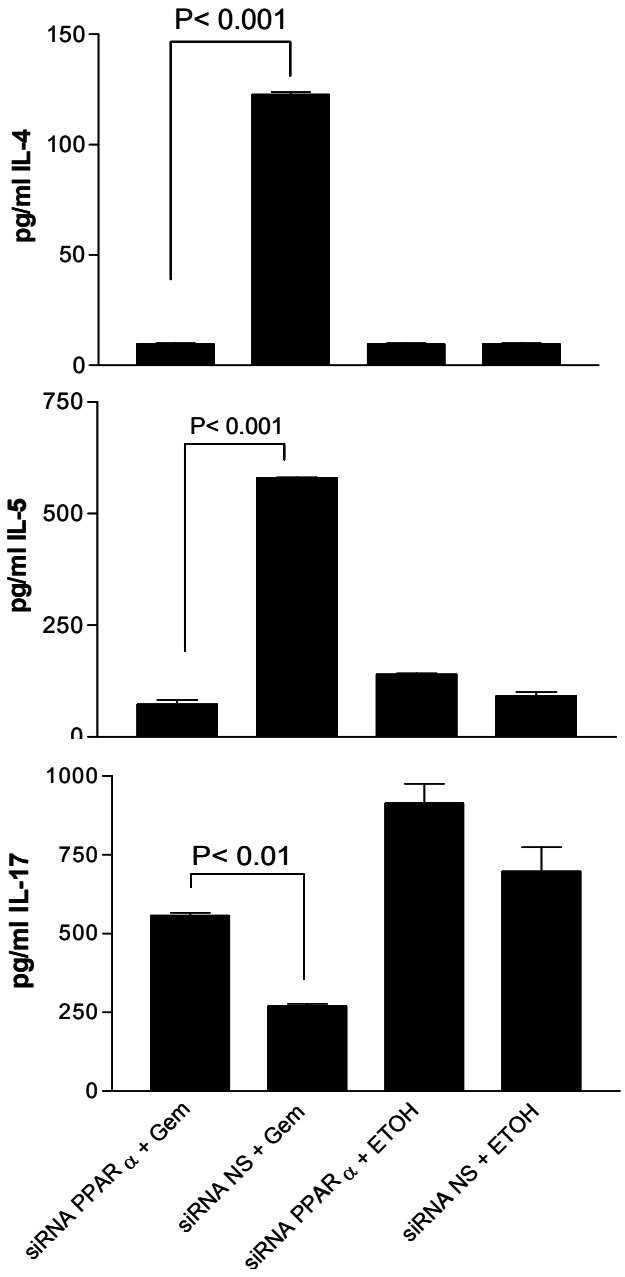


Figure 28. Gemfibrozil induces Th2 cytokine production in a receptor dependent manner. Splenocytes were isolated from representative EAE mice that had been administered siRNA PPARα or siRNA NS at the time of immunization and were fed gemfibrozil or ETOH and restimulated *in vitro* with MBP Ac1-11. At 72 hours supernatants were collected and IL-4, IL-5, and IL-17 secretion were measured by ELISA. siRNA PPARα + Gem vs. siRNA NS + Gem, p< 0.001 for IL-4 and IL-4. siRNA PPARα + Gem vs. siRNA NS + Gem, p<0.01 for IL-17. Results shown are representative of a minimum of 2 independent experiments.

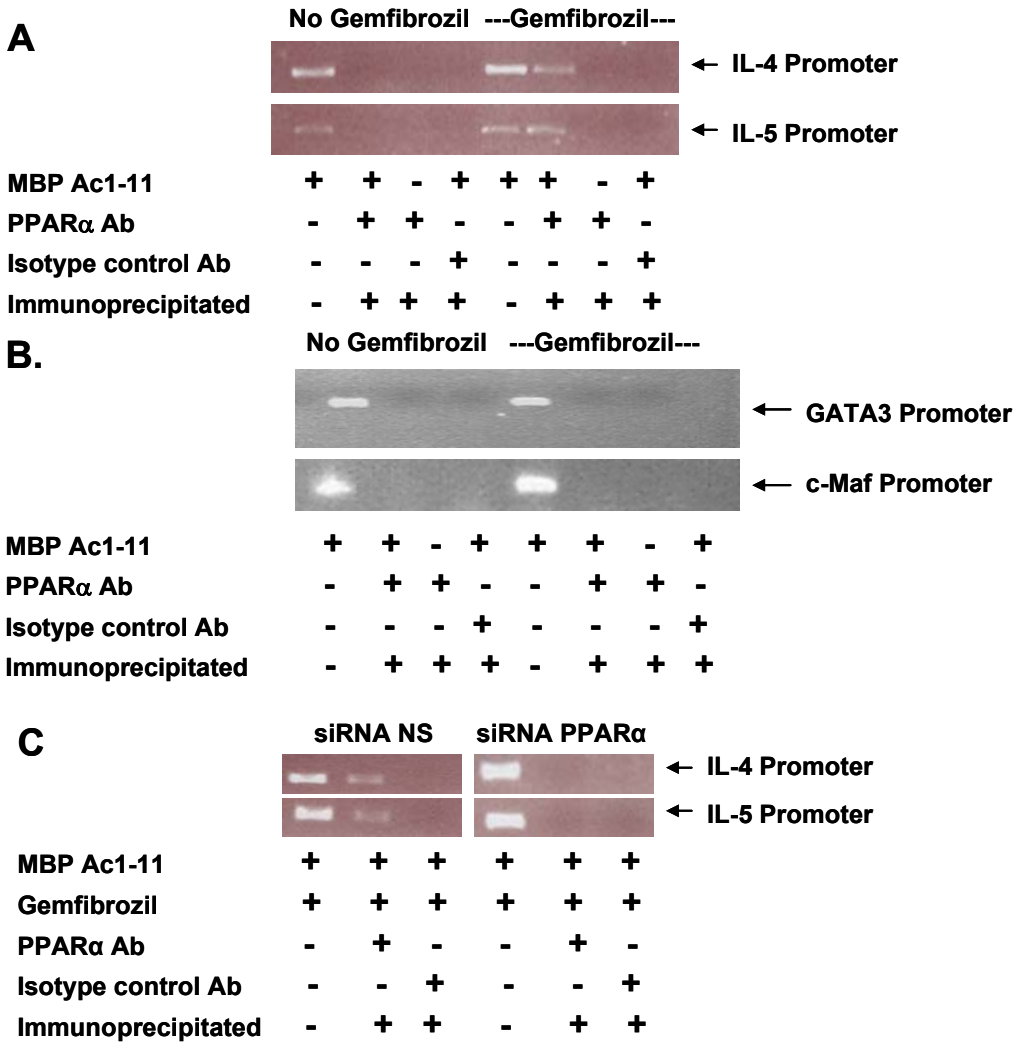


Figure 29. PPARα regulates transcription of Th2 cytokine genes in a ligand-dependent manner. A) Splenocytes were isolated from a Vβ8.2 TCR transgenic B10.PL mouse and cultured in the presence of 100μM gemfibrozil or vehicle control (ETOH). Cells were stimulated with or without MBP Ac1-11 for 48 hours and crosslinked for chromatin immunoprecipitation assays (ChIP). ChIP assays were performed using an antibody specific for PPARα and immunoprecipitated DNA was amplified using primers specific for the IL-4 and IL-5 promoter regions that contained PPREs (B) Splenocytes were isolated and cultured as described in panel (A) and ChIP assays were performed using an antibody specific for PPARα. Immunoprecipitated DNA was amplified using primers specific for the GATA3 and c-Maf promoter regions (C) Splenocytes were isolated as described in (A) and transfected *in vitro* with an siRNA specific for PPARα or an siRNA NS control. Cells were cultured in the presence of 100μM gemfibrozil or vehicle control (ETOH) with or without MBP-Ac1-11 for 48 hours and crosslinked for ChIP assays. ChIP assays were performed with a PPARα specific antibody and immunoprecipitated DNA was amplified with primers specific for the IL-4 and IL-5 promoter regions that contained PPREs. Results shown are representative of a minimum of 2 independent experiments.

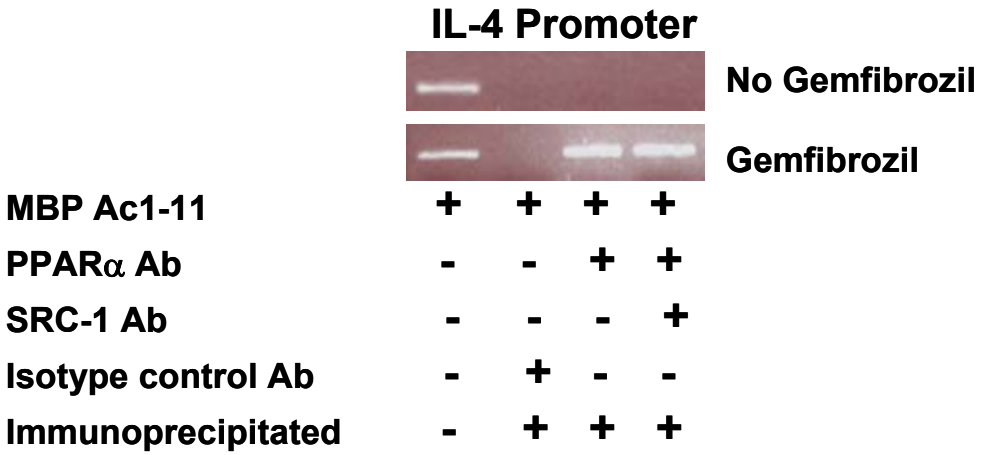


Figure 30. PPARα binds the IL-4 promoter region in the presence of the co-activator SRC-1. Splenocytes from a Vβ8.2 TCR transgenic mouse were isolated and cultured in the presence of 100μM gemfibrozil or vehicle control (ETOH). Cells were stimulated with or without MBP Ac1-11 for 48 hours and crosslinked for ChIP re-ChIP assay. ChIP re-ChIP was performed by immunoprecipitating first with a PPARα specific antibody. Eluted immune complexes were then immunoprecipitated again using an antibody specific for the steroid receptor co-activator (SRC-1). Immunoprecipitated DNA was amplified using primers specific for the IL-4 promoter region containing a PPRE. Results shown are representative of 3 independent experiments.

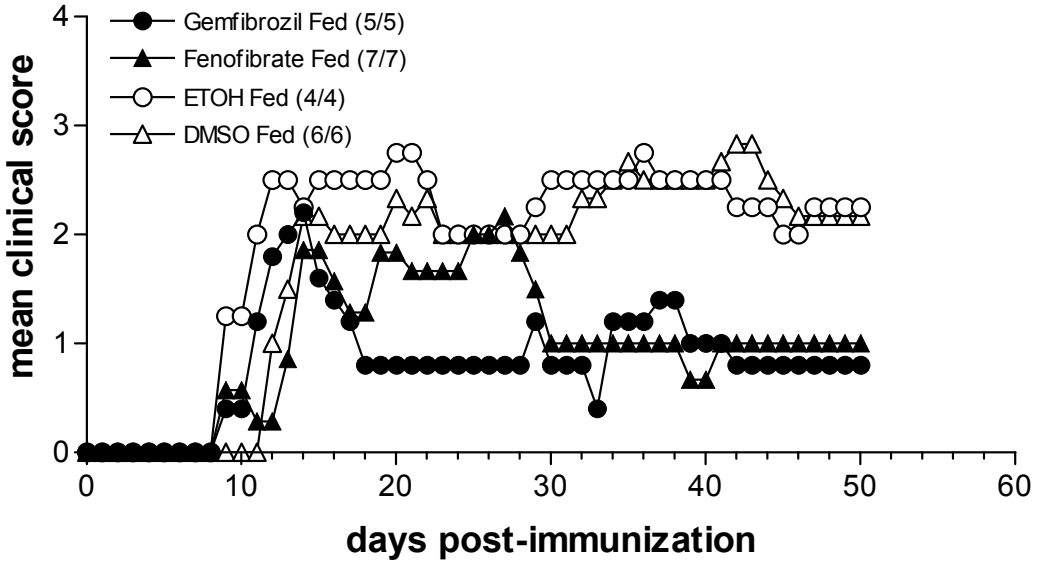


Figure 31. Treatment of mice after onset of EAE with gemfibrozil and fenofibrate ameliorates disease. C57/BL6 mice were immunized with MOG 35-55 to induce EAE. Treatment with PPARα agonists began at day 15 when all mice had clinically definite EAE. Mice were given 500μg of gemfibrozil or fenofibrate by gavage for 5 days followed by a diet supplemented with 0.25% wt/wt gemfibrozil, fenofibrate, or vehicle control (ETOH or DMSO) for the duration of the experiment. Mice were evaluated daily for clinical signs of EAE. Disease incidence is indicated in parentheses. Treatment with gemfibrozil and fenofibrate ameliorated disease compared to control treated groups. GEM vs. ETOH, $p< 0.001$, FEN vs. DMSO, $p< 0.001$. Results shown are representative of 2 independent experiments.

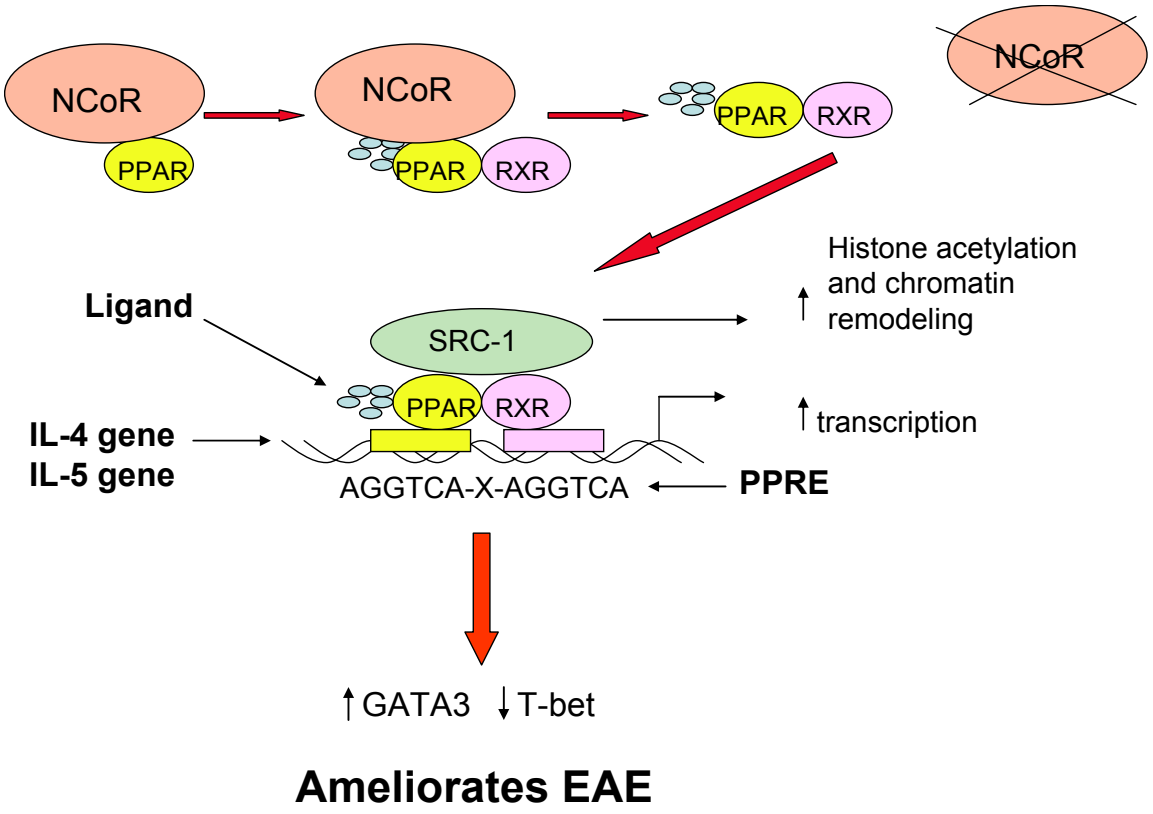


Figure 32. Model for a protective mechanism of gemfibrozil in EAE In the presence of PPARα agonists, such as gemfibrozil, PPARα heterodimerizes with RXR, dissociates from its nuclear corepressor complex, and associates with a coactivator complex containing SRC-1. SRC-1 promotes transactivation of target genes by increasing histone acetylation and chromatin remodeling. Following association with the coactivator complex, the PPAR/RXR heterodimer can bind to PPREs in the promoter region of IL-4 and/or IL-5. The transactivaiton of IL4/IL-5 leads to increased expression of GATA3 which in turn results in decreased T-bet expression and downregulation of the inflammatory immune response. This shift in the immune response to a Th2-like phenotype results in protection from EAE.

Chapter VI. Discussion

This work sought to examine the importance of transcriptional regulation of T helper cells in EAE. The experimental results presented in chapters III – V clearly illustrate that modulation of specific transcription factors can influence the clinical outcome of this disease. Moreover, the data suggest that targeting T-bet and/or PPARα by gene silencing and administration of specific agonists could provide a novel therapy for Th1 mediated autoimmune diseases such as MS. However, further understanding of the molecular mechanisms of action of these transcription factors in the immune system is necessary to generate the most effective form of treatment. This dissertation only begins to answer some of the important questions related to transcriptional regulation of T helper cells in autoimmune disease, but does contribute to the foundation of this ever growing research topic and can be expanded upon in future studies.

Targeting T-bet in EAE and MS: Is this a good strategy?

It was reported previously that mice deficient in T-bet are resistant to the induction of EAE [99]. This was confirmed in our studies utilizing a different approach. Instead of using mice that had the T-bet gene deleted, we silenced T-bet in normal mice using a synthetic small interfering RNA (siRNA) or an antisense oligonucleotide. This technique provided an advantage over the use of knockout mice because it allowed us to investigate the effects of targeting T-bet

while avoiding the redundancy or compensation that may occur when a gene is not expressed during development. Moreover, when considering T-bet as a target for therapeutic intervention in MS, gene knockouts are not an option. Humans with MS express T-bet during development, thereby making gene silencing a more biologically relevant technique to study the role of T-bet in this disease. There are potential problems associated with the use of siRNA in humans, including non-specific activation of the immune system and the induction of a type I interferon response [204]. However, type I interferons are a current therapy for MS and the effects of administering siRNA to MS patients, whether deleterious or beneficial, have not been determined.

The results presented herein demonstrate that T-bet can be successfully silenced *in vitro* resulting in decreased protein expression and reduced IFN-γ production compared to controls. Furthermore, splenocytes in which T-bet was silenced were less capable of inducing EAE in a passive transfer model of disease. This was supported by the finding that *in vivo* administration of siRNA or AS T-bet resulted in decreased incidence and severity of actively induced EAE. T-bet expression was decreased *ex vivo* in lymph nodes and splenocytes of mice that received siRNA or AS T-bet, and gamma interferon production was reduced compared to controls when the cells were restimulated with antigen *in vitro*. Importantly, these studies also examined the role of T-bet in the Th1 differentiation pathway and illustrated through the use of chromatin immunoprecipiation assays and western blotting that T-bet and STAT1 can

regulate one another in a positive feed back/amplification loop. This observation was a bit surprising given the finding that T-bet expression was absent in STAT1 deficient cells [151]. Furthermore, silencing T-bet was shown to be ineffective in the prevention of EAE if exogenous IL-12 was added to the splenocyte cultures prior to adoptive transfer, indicating that T-bet is acting upstream or independently of the IL-12/STAT4 signalling pathway of Th1 differentiation. Taken together this data suggests that silencing T-bet can favorably alter the differentiation of encephalitogenic Th1 cells and implicates T-bet as a target for therapy in EAE. However, the question remains, is targeting T-bet a good strategy for the treatment of MS?

Previous studies have demonstrated a pathogenic role for T-bet in Th1 mediated autoimmune disease. For example, increased T-bet activity and/or expression has been demonstrated in Celiac and Crohn's diseases, and in type I diabetes mellitus, two T-bet polymorphisms have been shown to be associated with disease [92, 93, 98]. Studies performed in the EAE model obviously implicate T-bet in MS pathogenesis, but what could the side effects be? T-bet is believed to be the master regulator of Th1 mediated immunity and plays an essential role in the amplification of the pro-inflammatory immune response by transactivating the IFN-γ gene. IFN-γ is the principal macrophage activating cytokine and is critical for the elimination of intracellular microbes and infections. Therefore, targeting T-bet could lead to decreased IFN-γ production and eventually result in increased susceptibility to infection. However, in a situation

where too much IFN-γ is being produced or IFN-γ production is detrimental, targeting T-bet could be beneficial. Whether this is truly the case in MS has not yet been determined. Furthermore, it is now believed that IFN-γ producing cells are not as important as IL-17 producing cells in disease pathogenesis. Therefore, the question of the moment becomes can T-bet regulate the development of IL-17 producing cells in MS?

Th17 vs. Th1 cells in EAE: T-bet can regulate both populations

Recently, a unique population of Th cells was identified and named Th17 cells because of their ability to produce the cytokine IL-17. This population of cells is believed to be derived via a lineage distinct from Th1 cells and has been implicated in the pathogenesis of EAE and possibly MS [50, 51, 53, 179, 183]. Initial studies suggested that Th17 cells are generated most effectively *in vitro* in the presence of exogenous IL-23 when IFN-γ and IL-4 are neutralized [51, 53]. However, more recent studies have demonstrated that these cells develop in the context of inflammation in the presence of IL-6, IL-1, and TGF-β1, and IL-23 is believed to be essential for their growth and survival [172-174]. The first studies connecting Th17 cells with EAE came secondary to studies implicating IL-23 rather than IL-12 as the critical cytokine in EAE [48]. A study by Langrish et al. demonstrated that IL-17 producing cells generated in the presence of exogenous IL-23 could induce EAE by adoptive transfer [51]. This led to the belief that IL-17 producing cells rather than IFN-γ producing cells are the primary

encephalitogenic cells in EAE and helped to explain why mice deficient in IFN-γ and the IFN-γ receptor displayed an exacerbated disease course. Furthermore, it has been shown that cells from T-bet deficient mice produce increased amounts of IL-17 [52]. This data supports the concept that Th17 cells are generated most effectively when IFN-γ is suppressed (i.e. in the absence of T-bet). However, if Th17 producing cells are the pathogenic cells in EAE and they are increased in the absence of T-bet, this contradicts the observation that T-bet deficient mice are protected from EAE. The experimental results contained in chapter IV help to explain this paradox.

We found that therapeutic administration of siRNA T-bet significantly ameliorated clinically established EAE. This decrease in disease severity correlated with a decrease in T-bet protein expression, reduced IFN-γ production, and a reciprocal increase in GATA3 protein expression. Because there was an increase in GATA3 expression, we expected to see an increase in Th2 cytokine production. However, there was no detectable IL-4 or IL-10 produced by siRNA T-bet treated mice. It has been demonstrated recently that T-bet and GATA3 can regulate one another. More specifically, T-bet is believed to repress Th2 lineage commitment via tyrosine kinase mediated interactions that interfere with the binding of GATA3 to its target DNA [195]. In addition, a study by Usui et al. demonstrated that retroviral expression of GATA3 in developing Th1 cells can suppress Th1 development through downregulation of STAT4. GATA3 expression did not block Th1 differentiation in cells coexpressing STAT4, but did

inhibit Th1 development in cells coexpressing T-bet [196]. These studies may explain why we see an increase in GATA3 when T-bet is silenced without any correlative increase in Th2 cytokine production.

We also observed a decrease in IL-23R expression both ex-vivo in splenocytes from siRNA T-bet treated mice and when cells were transfected with siRNA T-bet and subsequently activated *in vitro*. This observation was correlated by the finding that T-bet binds to a T-box consensus site located in the IL-23R promoter region. Furthermore, when T-bet was overexpressed in EL4 cells there was a concordant increase in IL-23R mRNA expression. This result taken together with the ChIP data suggests that T-bet can directly regulate/transactivate the IL-23R. Therefore, because T-bet can regulate the IL-23R gene and, in doing so, limit IL-23 engagement and affect the fate of pathogenic Th17 cells, loss of T-bet can result in decreased encephalitogenicity, incidence and severity of EAE.

Although we were unable to detect measurable levels of IL-17 expression in the spleen, we were able to detect IL-17 in the CNS of EAE affected mice. When T-bet was silenced, we observed a subsequent decrease in both IL-23R expression and IL-17 expression in the CNS. This leads us to believe that IL-23R engagement and subsequent IL-17 production may play an important role in the pathogenesis of EAE in the target organ. Using bone marrow chimeric mice in which the p40 subunit common to IL-12 and IL-23 was absent in the CNS, it

was shown that IL-23 produced by resident microglia and infiltrating macrophages in the CNS was critical to the clinical onset of EAE, but not for chronic CNS inflammation, suggesting that IL-23R engagement in the CNS was critical to the pathogenicity of infiltrating myelin-specific T cells [181]. In addition, IFN-γ was found to enhance IL-23 p19 expression in primary microglia cultures, supporting the notion that the infiltrating Th1 cells in EAE may mediate IL-23 p19 production in the CNS [176].

It has been suggested that Th1 cells and Th17 cells may arise from the same T-bet expressing precursor cell. In the presence of IL-23 and possibly STAT3 or STAT4 signaling, Th17 cells develop that express IL-17, IL-6, TNF-α, and IL-23R. If the cells develop in the presence of IL-12 and STAT4 signaling, they become classic Th1 cells that express IFN-γ, perforin, granzymes, and IL-12Rβ2 [205]. Our data also favors the hypothesis that these two distinct cell types could be derived from the same progenitor, or at the least, both depend on T-bet for optimal expansion and survival. In a Th1 adoptive transfer model, we observed a decrease in both IFN-γ production and IL-17/IL-23R expression when T-bet was silenced. This suggests that T-bet can regulate both IFN-γ producing Th1 cells and Th17 cells via regulation of the IFN-γ and IL-23R genes respectively, and leads to the speculation that classical Th1 cells can initiate the inflammatory phase of EAE and IL-17 producing T cells may participate in target organ damage and disease progression. More importantly, this data provides

one possible explanation as to why T-bet deficient mice are protected from EAE when IFN-γ deficient mice develop an exacerbated disease course.

PPARα Agonists as a Therapy for MS

Chapter V investigates the mechanism of action of the PPARα agonist gemfibrozil in EAE. PPARα agonists have been shown previously to be protective in EAE and to induce a phenotypic switch from Th1 to Th2 as evidenced by altered cytokine production [145, 148, 199]. However, little work has been performed to investigate the manner in which these agonists induce this immune deviation. We found that splenocytes stimulated in the presence of gemfibrozil *in vitro* upregulated GATA3 expression and downregulated T-bet expression. Furthermore, ex vivo analysis of splenocytes from EAE mice given a diet supplemented with gemfibrozil also exhibited increased GATA3 and decreased T-bet expression. These changes correlated with a decrease in disease severity as would be expected based on previous findings that mice that have had T-bet knocked out or silenced are protected from EAE [99, 175]. This altered transcription factor expression resulted in a Th2-like phenotype and may lead to decreased proliferation/expansion of encephalitogenic Th1 or Th17 cells. In addition, as mentioned previously, it has been shown that T-bet and GATA3 can regulate one another and in doing so may affect downstream signaling [195, 196]. Therefore, if PPARα, through interaction with its ligand, can regulate either

of these transcription factors, directly or indirectly, this could result in the regulation of the other transcription factor.

Importantly, we also found that the protective effects of gemfibrozil in EAE are at least partially dependent on IL-4 and appear to be receptor dependent as well. IL-4 deficient mice were not protected from EAE to the same extent as wildtype controls in the presence of gemfibrozil, and when PPARα was silenced using RNAi, disease was no longer ameliorated with gemfibrozil treatment. Moreover, PPARα was demonstrated to regulate the IL-4 and IL-5 genes, suggesting that one mechanism by which gemfibrozil ameliorates EAE is via transactivation of Th2 cytokine genes, resulting in an upregulation of GATA3, and a downregulation of T-bet and the pro-inflammatory immune response. Finally, we found that therapeutic administration of the PPARα agonists gemfibrozil and fenofibrate significantly ameliorated clinically established EAE. Overall, these results suggest that fibrates, via induction of immune deviation, could provide an effective therapy for MS and insight gained from dissecting the mechanism of action of fibrates in EAE could be applied to developing treatments for immune-mediated inflammatory diseases. However, is altering the phenotype of encephalitogenic cells from a Th1/Th17 to a Th2 phenotype sufficient to treat inflammatory, immune-mediated diseases such as MS?

Is Immune Deviation a Valid Therapy for MS?

Studies performed in EAE mice suggest that altering the phenotype of encephalitogenic cells ameliorates the disease. IL-4 has been associated with the remission stage of EAE when expressed in the CNS [68] and most IL-4 producing Th2 cells are nonencephalitogenic [69]. In addition, IL-10 expression in the CNS during EAE was found to correspond to the onset of the recovery phase of disease [73], and *in vivo* delivery of IL-10 inhibited EAE [74]. However, EAE is merely a model for MS and in humans there has been little evidence for immune deviation being sufficient to treat disease. Current MS therapies include Avonex, which is interferon beta 1a, and Copaxone, which is a random copolymer originally designed to mimic myelin basic protein. Why these drugs are effective in MS is not completely understood. It is believed that copaxone may induce a Th2 bias or may stimulate CD8+ cells to kill myelin reactive CD4+ cells [190, 206-208]. However, these drugs are only mildly effective in treating MS. Moreover, clinical trials in which immune deviation was the strategy, including treatment of relapsing remitting and secondary progressive MS patients with recombinant IL-4, IL-10, TGF-β2 or neutralizing antibodies to TNF-α, were terminated or failed because of insufficient efficacy or induction of exacerbations [209]. In addition, attempts at remyelination with the use of immunoglobulins in MS patients did not prove to be successful [209]. This is probably due to the nature of the disease. MS is an inflammatory demyelinating disease of the central nervous system. Initial inflammation is followed by demyelination,

oligodendrocyte cell death, and destruction of axons, eventually resulting in impaired nerve conduction (Fig. 33). For this reason, autoreactive T cells may not play a major role in the later stages of disease. Therefore, strategies that target the immune system, such as immune deviation may be effective very early in disease, but may not prove beneficial at later stages. It is important to consider this when developing new therapies. It may be more advantageous to develop therapies that target both the immune-mediated aspect and the demyelinating/ axonal damage aspect of MS. For example, studies in our lab are also focusing on targeting NOGO-A. NOGO-A is a strong inhibitor of neurite outgrowth and may play a role in negatively regulating regeneration in the CNS [210, 211]. We are attempting to silence this inhibitor using siRNA, thereby allowing axonal sprouting and repair of damaged axons to occur. This strategy coupled with one that targets the immune system, such as those presented herein, may be the most effective way to treat this debilitating disease. Furthermore, by dissecting the molecular pathways that lead to immune deviation, it may be possible to specifically target one mechanism, thereby resulting in new therapies with fewer side effects.

Advantages of PPAR Agonists over Current MS Therapies

The PPARα agonists gemfibrozil and fenofibrate have been used for many years for the treatment of hypertriglyceridemia and have been shown to be safe and effective in a large number of patients. For this reason, treating MS patients

with these drugs would pose little threat of adverse reaction and may be more beneficial, if taken alone or in combination with current FDA approved drugs, than presently available MS drugs alone. In addition, fibrates are taken orally which differs from the method of administration of current MS therapies, which are delivered by subcutaneous or intramuscular injection daily or weekly. Therefore, the use of PPARα agonists, while they may not alleviate all signs of disease, could improve the quality of life of the MS patient if proven to be effective in reducing disease severity. Moreover, gemfibrozil is given to patients at a dose of 1200mg/day which is equivalent to the dose that was given to the EAE mice in the studies presented herein, suggesting that the current recommended dose may be sufficient for use in MS patients. This differs from other FDA approved drugs, such as statins, which have to be given at the highest recommended dose or higher to be effective and may result in undesirable side-effects. Therefore, it may be worthwhile to test the safety and efficacy of PPAR agonists in MS as well as other inflammatory immune mediated diseases.

Future Directions

Future studies will investigate the role of T-bet in the IL-23/IL-17 pathway to provide insight for the development of new MS therapies. While we believe that T-bet influences the fate of Th1 cells and pathogenic Th17 cells via regulation of the IL-23 receptor, it is possible that T-bet affects this pathway in multiple ways that have yet to be explored. More specifically, the effects of silencing T-bet as well as silencing IL-23 and IL-17 in actively induced EAE and

in EAE that has been induced by the adoptive transfer of Th17 cells will be investigated. In this manner experiments will be performed to examine the ability of T-bet to influence the differentiation of IL-17 producing cells in the CNS of immunized and treated mice. It will be determined if the effects are time and dose dependent. Furthermore, because it has been shown that TGF-β, IL-6, and IL-1 contribute to the differentiation of Th17 cells, we will investigate the relationship between T-bet and these cytokines. Experiments will be performed to examine whether silencing T-bet influences the availability of these cytokines, thereby inhibiting the differentiation of Th17 cells. Moreover, additional transcription factors that may be directly regulating IL-17 or IL-23 will be examined, and it will be determined if these transcription factors are influenced by or somehow related to T-bet. By definitively determining the role of T-bet in the IL-23/IL-17 pathway we hope to elucidate specific molecules or pathways that could be targeted for therapeutic purposes.

In addition to studying the role of T-bet in the IL-23/IL-17 pathway in EAE, the effects of silencing T-bet in human lymphocytes will be explored as well. It is possible and likely that T-bet functions differently in humans than in mice. Therefore, studies that have been performed in mice will need to be repeated using human lymphocytes. Futhermore, experiments will be performed to examine whether T-bet expression varies in MS patients compared to healthy individuals and/or among MS patients with different forms and duration of disease. Moreover, it has been shown that the role of IFN-γ differs in EAE compared to MS, as demonstrated by a clinical trial in which MS patients were

treated with gamma interferon resulting in relapse and disease exacerbation. This result differed from mouse experiments in which IFN-γ and IFN-γ receptor deficient mice displayed an exacerbated disease course. Therefore, the role of Th17 cells in MS patients will be explored as well. T-bet may affect this cell population differently in humans than has been demonstrated in mice.

Additional experiments will combine the use of siRNA T-bet with other approaches, such as the use of siRNA specific for NOGO-A, in order to target the initial inflammatory phase of disease and the later neurodegenerative phase. These studies are currently being performed in the EAE model and will eventually be tested in human cells as well.

Future studies for the PPARα portion of this project will include further investigation of the mechanism of action of PPARs in EAE. More specifically, experiments will be performed to investigate whether fibrates can induce PPARα to negatively regulate pro-inflammatory genes such as IFN-γ, IL-23, IL-17, iNOS, or other transcription factors such as T-bet and NFkB. This will be studied using chromatin immunoprecipiation assays and ChIP re-ChIP assays to examine whether PPARα alone or in response to agonist can remain bound by nuclear corepressors thereby resulting in transrepression of target genes. In addition we would like to explore in detail the effects of PPARα and PPARγ agonists on antigen presenting cells in EAE. For example, experiments will be performed to determine if PPARs regulate cytokine genes expressed by APCs that influence the fate of T cell differentiation. The effects of different PPARα agonists will be

investigated by isolating antigen presenting cells and T cells. The CNS of EAE animals treated with PPAR agonists will also be examined to evaluate differences in APC function.

Additional experiments will explore the synergistic or additive effects of PPAR agonists and RXR agonists. Previous studies have shown that treating mice with PPAR agonists in combination with RXR agonists results in an additive or synergistic effect in EAE [187, 198, 212]. The mechanism by which this occurs will be explored using techniques that were employed for the PPARα studies shown within.

Experiments will also be performed to investigate whether different PPARα agonists, such as gemfibrozil and fenofibrate, function in different capacities to ameliorate EAE. For example, maybe gemfibrozil induces PPARα to transactivate Th2 cytokine genes where as fenofibrate negatively regulates inflammation via effects on antigen presenting cells. This could aid in explaining discrepancies in results that occur when different agonists are used.

Finally, experiments performed to study the mechanism of these drugs in EAE mice will be repeated to examine the effects of PPAR agonists on human lymphocytes. Furthermore, it may be useful to perform a retrospective analysis of MS patients that have taken fibrates for hypertriglyceridemia to determine whether there were any advantageous effects. Eventually, the safety and efficacy of fibrates in MS patients should be tested in a clinical trial to ultimately

determine whether these drugs could be used as a therapy for this inflammatory immune mediated disease.

Conclusions

The studies presented in this body of work investigated the role of the transcription factors T-bet and PPARα in the differentiation and effector function of T helper cells in the EAE model. More specifically, we investigated the effects of silencing T-bet on the differentiation of encephalitogenic T cells and demonstrated that this transcription factor plays a critical role not only in the development of these cells, but also in the induction of EAE. The experimental results add to the current knowledge of T-bet's role in the Th1 differentiation pathway by demonstrating the ability of T-bet and STAT1 to regulate one another in a positive feedback loop. Moreover, we explored the role of T-bet in the IL-23/IL-17 pathway as well. The data provides the first evidence that T-bet can regulate the IL-23R and is the only work to date that offers an explanation as to why T-bet deficient mice are protected from EAE when IFN-γ deficient mice display an exacerbated disease course. The importance of this paradox has often been overlooked in recent studies with no plausible explanation. The results also show that T-bet can regulate both IFN-γ and IL-17 expression. With the recent surplus of data implicating IL-17 producing cells as the pathogenic cells in EAE, it has been ignored that EAE can be induced with the injection of classical Th1 cells that do not produce IL-17. This would suggest that both Th1 and Th17 cells play a role in EAE, as is likely the case in MS. Our data was

generated in such a way as to explore the function of both of these cell populations and supports the notion that IFN-γ has both regulatory and pathogenic roles in EAE.

In contrast to T-bet, PPARα plays a beneficial role in EAE pathogenesis. We found that administration of the PPARα agonists gemfibrozil and fenofibrate treated and protected mice from the development of EAE. Furthermore, the data shows for the first time that this protection is mediated in an IL-4 and receptor-dependent manner. The evidence that PPARα can regulate the Th2 cytokine genes IL-4 and IL-5 supports previous data that demonstrate an increase in Th2 cytokine production in the presence of PPARα agonists. Moreover, we developed a model to explain the protective mechanism of PPARα in EAE. Upon ligand binding, PPARα transactivates Th2 cytokine genes which lead to upregulation of the Th2 transcription factor GATA3 and down regulation of the inflammatory immune response. This leads to immune deviation to a Th2-like phenotype and protection from EAE. The data reported herein are the first to identify a mechanism of action for PPARα in EAE. The understanding gained from our study is essential for future studies that may seek to use PPARα agonists as a therapy for MS.

Overall, the studies performed for this dissertation provide an increase in the understanding of the transcriptional regulation of T helper cells in EAE. Although the role of transcription factors in EAE and MS has become increasingly more defined, additional studies are required to dissect specific

mechanisms of transcriptional activation that occur during disease pathogenesis. It is this increased understanding of transcriptional activation of T helper cells in EAE/MS that will ultimately result in the discovery of novel and more specific targets for therapeutic intervention.

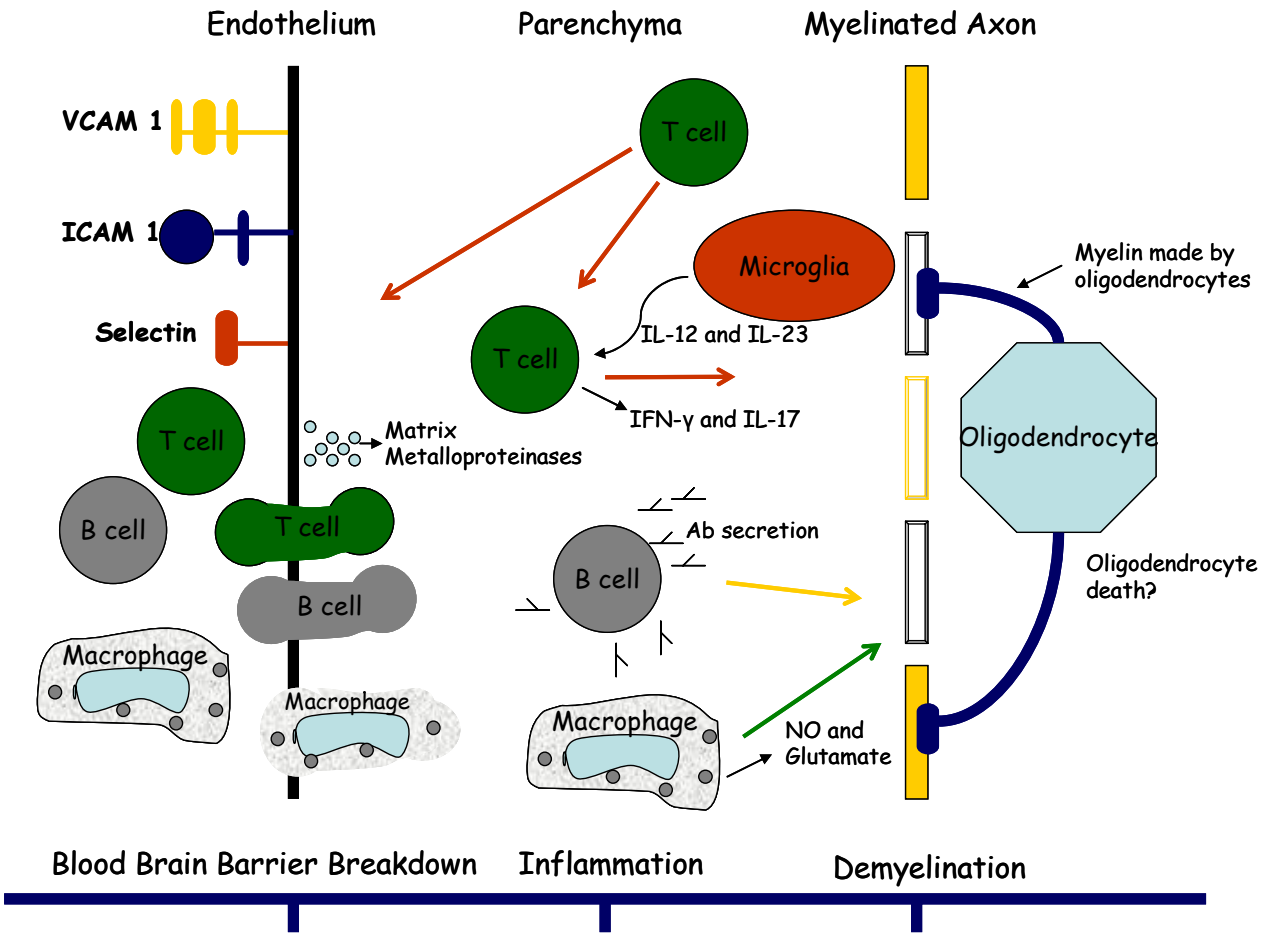


Figure 33. MS Pathogenesis
Adhesion molecules interact with T cells, B cells, and macrophages at the endothelial surface and facilitate migration of these cells into the central nervous system. Infiltrating B cells secrete antibodies that can mediate axonal damage. Infiltrating T cells secrete the inflammatory cytokines IFN-γ and IL-17 which promote further damage. Macrophages and resident microglia release nitric oxide (NO) and secrete IL-12 and IL-23 which induce the myelin reactive T cells to secrete increased IFN-γ and IL-17. These inflammatory events culminate in a series of detrimental processes resulting in axonal damage, and eventually axonal transection and neurodegeneration.

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VITAE

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