THERAPEUTIC MODULATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY GAMMA SECRETASE INHIBITION

APPROVED BY SUPERVISORY COMMITTEE

Todd Eagar, Ph.D.

David Farrar, Ph.D.

Amelia Eisch, Ph.D

Olaf Stüve, MD., Ph.D.

DEDICATION

First and foremost, I would like to thank my mentor, Todd Eagar, Ph.D. I am proud to have worked with him, and I wish him the best as he moves into the next stage of his own career.

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THERAPEUTIC MODULATION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY GAMMA SECRETASE INHIBITION

MATTHEW AARON CUMMINGS

DISSERTATION

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The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

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Publication No_____

Matthew Aaron Cummings, Ph.D.

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Todd Eagar, Ph.D.

The acquisition of pro-inflammatory phenotypes constitutes a checkpoint in the progression toward autoimmunity. Small molecule **inhibitors of γ-secretase (γSI)** have been shown to suppress T_H1 differentiation and reduce the severity of experimental autoimmune encephalomyelitis (EAE), presumably by affecting Notch signaling. To understand the mechanisms through which γSI alter autoimmune T cell responses, we utilized an adoptive transfer model of EAE. We found that γSI treatment *in vivo* reduced EAE severity after adoptive transfer of myelin-specific TCR-transgenic T cells. This was accompanied with a decrease in the production of IFNγ by T cells ex-vivo. To determine the mechanisms of reduced EAE, we tested the effects of γSI on T_H1 and T_H17 differentiation. Culture with γSI reduced the expression of IFNγ and IL-17 without altering T cell activation or proliferation. The expression of Tbet and RORγt was strongly reduced by γSI while the number of FoxP3-expressing T cells increased. Additionally the *in vitro* treatment of naïve CD4+ T cells with γSI during T_H17 induction prevented adoptive EAE. Taken together, these results suggested that γSI regulate effector T cell responses by

preventing T_H1 and T_H17 differentiation and promoting the generation of regulatory T cells.

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LIST OF DEFINITIONS

γSI – Gamma secretase inhibitor

μg – Microgram

μl – Microliter

Aβ – Abeta fragment of APP

AD – Alzheimer's disease

APC – Antigen presenting cell

APC – Allophycocyanin

APH-1 – Anterior pharynx 1

APP – Amyloid precursor protein

B10.PL – A mouse strain

BBB – Blood brain barrier

C57B6/L – A mouse strain

CCR2 – Chemokine receptor 2

CCR6 – Chemokine receptor 6

CD – Cluster of Differentiation

CD40L – CD40 ligand, also know as CD154

CFA – Complete Fruend's adjuvant

CFSE – 5,6-Carboxyfluorescein succinimidyl ester

CNS – Central nervous system

CPD-E – Compound E, a gamma secretase inhibitor

CSL – CBF1, Suppressor of Hairless, Lag-1; a transcription factor

DAPT – A gamma secretase inhibitor

DBZ – A gamma secretase inhibitor

DC – Dendritic Cell

DNA – Deoxyribonucleic acid

DLL1 – Delta-like 1DLL4 – Delta-like DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

EAE – Experimental autoimmune encepahlomyelitis

EBV – Epstein-Barr virus

EDSS – Expanded disability status scale

ELISA – Enzyme-linked immunosorbent assay

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

FITC – Fluorescein isothiocyanate

FoxP3 – Forkhead box P3

FDA – Federal drug administration

GA – Glatiramer acetate

GATA-3 – protein encoded by the *GATA3* gene

GI – Gastro-intestinal

GS – Gamma secretase

HEPES – (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HES-1 – Hairy and enhancer of split-1

Het – Heterozygote

HLA – Human leukocyte antigen, also known as MHC

IFN-β – Interferon beta

IgG – Immunoglobulin G

IFNγ – Interferon gamma

IFNγR – Interferon gamma receptor

IL-2 – Interleukin-2

IL-2Rα – Interleukin-2 receptor subunit alpha

IL-4 – Interleukin-4

IL-6 – Interleukin-6

IL-12 – Interleukin-12

IL-12Rβ2 – Interleukin-12 receptor subunit beta 2

IL-17 – Interleukin-17

IL-17A – Interleukin-17A, commonly called IL-17

IL-17F – Interleukin-17F

IL-21 – Interleukin-21

IL-22 – Interleukin-22

IL-23 – Interleukin-23

IL23-R – Interleukin-23 receptor

IL-CHO – A gamma secretase inhibitor

IPEX – Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

iT_{reg} – induced T_{reg}

IACUC – Institutional Animal Care and Use Committee

Jak – Janus Kinase

Ko – Knockout

LFA-3 – Lymphocyte function-associated antigen 3, also known as CD58

LNC – Lymph node cells

LPS – Lipopolysaccharide

LY-411,575 – A gamma secretase inhibitor

MACS – Magnetic activated cell sorting

MBP – Myelin basic protein

MHCII – Major histocompatibility complex class II

mg – Milligram

miRNA – MicroRNA

ml – Milliliter

MOG – Myelin oligodendrocyte glycoprotein

MRI – Magnetic resonance imaging

mRNA – Messenger RNA

MS – Multiple sclerosis

MW167 – A gamma secretase inhibitor

NICD – Notch intracellular domain

ng – nanogram

$Notch1^{IC}$ – Notch1 intracellular domain

nT_{reg} – natural T_{reg}

PBS – Phosphate-buffered saline

PE – Phycoerythein

PE-Cy7 – Phycoerythein-cyanine7

PEN-2 – Presenilin enhancer 2

PFA – Paraformaldehyde

PLP – Proteolipid protein

PMA – Phorbol 12-myristate 13-acetate

PML – Progressive multifocal leukoencephalopathy

PPMS – Primary progressive multiple sclerosis

PSEN1 – Presenilin 1

PSEN2 – Presenilin 2

PTx – Pertussis toxin

RBPJk – Recombining binding protein suppressor of hairless kappa

RORα – Retinoic acid receptor-related orphan receptor alpha

RORγt – Retinoic acid receptor-related orphan receptor gamma t

RRMS – Relapsing remitting multiple sclerosis

S1P – Sphingosine-1 phosphate

siRNA – Small interfering RNA

SJL – A mouse strain

SPC – Splenocytes

SPMS – Secondary progressive multiple sclerosis

STAT1 – Signal transducer and activator of transcription 1

STAT3 – Signal transducer and activator of transcription 3

STAT4 – Signal transducer and activator of transcription 4

T2-B cell – Transitional type 2 B cell

Tbet – T box transcription factor

Tbx21 – The gene encoding Tbet

TCR – T cell receptor

TGFβ – Transforming growth factor beta

T_H1 – T helper cell type 1

T_H17 – T helper cell type 2

T_{reg} – T regulatory cell

Tyk – Tyrosine kinase 2

Wt – Wild type

XLAAD – X-linked autoimmunity-allergic disregulation

CHAPTER ONE

Introduction

MULTIPLE SCLEROSIS

Demographics

Multiple Sclerosis (MS) is a disorder of the central nervous system (CNS) characterized by inflammation, demyelination, gliosis, and neuronal loss. The age of onset is typically between 20 and 40 years and women are approximately three times more likely than men to acquire MS. Approximately 350,000 individuals in United States and 2.5 million individuals worldwide are afflicted with MS making it second only to trauma as the leading cause of neurologic disability beginning in early to middle adulthood [1].

Symptoms and Diagnostics

The initial clinical symptoms of MS vary depending on the particular nerve fibers affected but often present as sensory loss, optic neuritis, limb weakness, paresthesias (tingling sensation) or ataxia [2]. There are three main classes of MS. Relapsing remitting MS (RRMS) accounts for ~85% of initial MS diagnoses and is characterized by defined intervals of recovery between exacerbations. Secondary progressive MS (SPMS) occurs in up to 90% of those initially diagnosed with RRMS who eventually exhibit progressive neurologic decline between acute attacks without definite periods of remission. Primary progressive MS (PPMS) occurs in ~10% of patients and is characterized by progression of disability from onset without remissions or improvements.

In order to obtain a diagnosis of MS an individual must have experienced symptoms that last for greater than 24 hours in two distinct episodes separated by a month or more along with two or more signs that reflect pathology such as MRI evidence of new focal white matter lesions [3].

Pathology

The major cause of progressive and irreversible neuro-disability in MS is believed to result from the accumulation of inflammation, slowing of nerve conduction, and subsequent axonal loss. Classical RRMS pathology is characterized by plaques located in the subcortical or periventricular white matter, optic nerve sheath, brainstem and spinal cord. New demyelinating lesions form in the periventricular space due to disruption of the blood-brain barrier (BBB) as evidenced by gadolinum infiltration on MRI scan. White matter plaques are regions where the myelin sheath produced by the oligodentrocyte has been stripped away from the nerve cell axon it surrounds. The inflammatory infiltrate of the MS lesion consists largely of clonally expanded CD8+ T cells, CD4+ T cells [4,5], γδ T cells[6], monocytes, and large numbers of macrophages containing myelin debris[7]. Additionally, small numbers of B cells also infiltrate the CNS with evidence of immunoglobulin and complement deposition observed [8]. Axon survival is typical in MS with newly differentiated or surviving oligodendrocytes able to partially remyelinate denuded axons, visible as shadow plaques on MRI images. Additionally ectopic follicles resembling secondary

lymphoid structures of T and B cells appear in perivascular and meningeal regions over time.

The progressive phase of MS is characterized by a slow and gradual expansion of preexisting plaques at their margins with highly activated microglia and sparse inflammatory infiltrates composed largely of T cells in perivascular regions. Also characteristic of progressive MS is extensive axonal injury associated with demyelinated lesions, containing little to no remyelination. Diffuse, abnormal inflammation of the normal appearing white matter is also observed.

Recently the demyelination of axons in the cortex and other grey matter regions of the brain has gained increasing attention and significance among researchers. Focal demyelination in the white matter cannot explain the full extent of clinical, particularly cognitive, deficits in patients with MS [9]. Cognitive deficits such as memory impairment, attention deficits, and reduced mental processing are present in 45–65% of patients with MS [10,11]. These deficits are better explained by demyelination and resultant damage to the neurons in the grey matter than in the white matter. Indeed, MS patients with cognitive decline have more cortical damage than patients who are cognitively preserved [12,13].

Causes

Both environmental and genetic factors have been shown to contribute to MS susceptibility. There is a clear genetic influence in MS as an identical twin of an MS patient has a 33% chance of also developing MS. The HLA-DRB1 locus of chromosome 6 is strongly associated with MS susceptibility. Individuals

heterozygous for the *DRB1*15:01* allele have three times the risk while those homozygous for *DRB1*15:01* have nine times the risk of acquiring MS[14]. HLA-DR along with HLA-DP and HLA-DQ are human class II major histocompatibility genes (MHCII). MHCII is responsible for displaying small peptides of endocytosed proteins on the surface of antigen presenting cells. MHCII interacts exclusively with CD4+ T helper cells to incite an immune response to combat extracellular pathogens. However self-protein derived peptides are also presented in MHCII, which under certain conditions could lead to autoimmune tissue destruction. The high association of MS with an MHCII allele provides evidence that MS is, at least in part, an immune-mediated disease.

Association studies have also identified many other genes each of which slightly contributes to MS susceptibility. Most MS-associated genetic variants have known roles in the immune system such as IL-7R, IL-2R, and LFA-3[15-17].

Due to the low disease concordance between genetically identical twins, environmental factors must also play a role in MS. Individuals sero-positive for Epstein-Barr Virus (EBV) have a greater risk of acquiring MS (reviewed in[18]) and antibodies to the EBV and cytomegalovirus have been associated with brain atrophy in MS [19-21]. Smoking has been shown to increase MS risk[22]. MS prevalence also has a geographic component with prevalence generally increasing with distance from the equator. At high latitudes average vitamin D biosynthesis is lower due to decreased sunlight intensity, and vitamin D deficiency has been shown to increase MS risk. An early environmental effect is evident as children who relocate prior to adolescence

assume the risk of the area they moved to, whereas individuals retain the MS risk of the region where they were raised if they move after adolescence. There also appears to be a pre-natal component as a non-identical twin of an MS patient carries twice the risk of also developing MS over that of a non-twin sibling. An additional pre-natal factor is likely responsible for association of birth month with MS risk. May births in the Northern hemisphere and November births in the southern hemisphere have an increased risk of developing MS.

TREATMENT OF AUTOIMMUNE DEMYELINATION

No cure exists for MS and current treatment is limited to the management of acute attacks and slowing disease progression. When new demyelinating attacks or acute relapses of RRMS occur, intravenous glucocorticoids are used to reduce the severity and shorten the duration of attacks. The efficacy of glucocorticoid treatment is likely due to both anti-inflammatory and immunosuppressive mechanisms. However, immunomodulatory therapies are used for disease maintenance due to the side effects of long-term glucocorticoid use and the questionable efficacy of long-term corticosteroid treatment.

Immunomodulatory Therapy

There are currently eight FDA-approved drugs that reduce disease activity and progression in MS patients: the interferons (Avonex, Rebif, Betaseron and Extavia), mitoxantrone (Novantrone), natalizumab (Tysabri), fingolimod (Gilenya), and

glatiramer acetate (Copaxone) all of which act upon the immune system to suppress relapses [23].

Interferon-ß

Four interferon beta drugs have been approved for the treatment of MS; Betaseron, Extavia, Avonex, and Rebif. All of these injectable medications have similar efficacies and reduce the attack rate as well as improve disease severity measures such as Expanded Disablility Status Scale progression and MRI-documented disease burden. Efficacy in MS probably results from immunomodulatory properties, including downregulating expression of MHC molecules on antigen-presenting cells, inhibiting proinflammatory and increasing regulatory cytokine levels, inhibiting T cell proliferation, and limiting the trafficking of inflammatory cells in the CNS. The main treatment-limiting side effect of these drugs was a flu-like syndrome associated with the injections. However, less than half of patients respond to IFNβ treatment and some responders eventually develop neutralizing antibodies against INFβ, necessitating the development of alternative MS therapies.

Glatiramer Acetate

Glatiramer Acetate (GA, Copaxone) is a synthetic, random polypeptide composed of four amino acids (alanine, lysine, glutamate and tyrosine) in a ~ 4.2:3.4:2.1:1 ratio. GA was developed as a cheap synthetic mimic to myelin basic protein peptide. However, instead of inducing autoimmune demyelination in animal models, GA treatment protected mice from disease. The mechanism of action of GA is unclear but may include the induction of antigen-specific suppressor T cells, the binding of

GA to MHC molecules and displacing bound MBP, or the alteration of the balance between proinflammatory and regulatory cytokines. GA is given as a daily subcutaneous injection. Its main clinical advantage is its lack of flu-like symptoms in comparison to IFN-β treatment, but it does cause similar local skin injection reactions. Comparison clinical trials suggest that glatiramer acetate has nearly equal efficacy to high dose IFN-β treatment.

Natalizumab

Natalizumab (Tysabri) is a humanized monoclonal antibody directed against the α4 subunit of α4β1 integrin, a cellular adhesion molecule expressed on the surface of lymphocytes. It blocks binding to adhesion molecules necessary for transmigration of lymphocytes across the blood–brain barrier. Natalizumab demonstrated a significantly better relapse reduction over IFN-β. However incidences of progressive multifocal leukoencephalopathy caused by reactivation of latent JC virus were found to increase after 2 years on the medication, and in patients who had previous exposure to immune-suppressive medications. Currently only 12-18 month periods of Natalizumab treatment are recommended.

Fingolimod

Fingolimod (Gilenya) is a sphingosine- 1-phosphate (S1P) inhibitor and it prevents the egress of lymphocytes from the secondary lymphoid organs such as the lymph nodes and spleen. Its mechanism of action is due to the sequestration of lymphocytes in the periphery and the prevention of proliferation and egress into the peripheral blood stream thereby preventing auto-reactive T and B cells from

reaching the brain. A greater than 50% reduction in relapse rate was seen with Fingolimod compared to placebo. A large head-to-head phase III randomized study demonstrated the clear superiority of fingolimod over low dose (weekly) IFN-β treatment. Fingolimod is given as a once-a-day oral tablet resulting in greater patient compliance than the injectable drugs. However, potentially serious side effects such as bradycardia, hypertension, macular edema have limited the use of Fingolimod. Due to these side effects most physicians prescribe Fingolimod as a second-line agent for patients who either do not obtain good disease control with the self-injection agents or are unable to tolerate them.

Mitoxantrone

Mitoxantrone is the only medication approved for progressive MS. Mitoxantrone inhibits the proliferation of rapidly dividing cells by intercalating into DNA producing both strand breaks and inter-strand cross-links, interfering with RNA synthesis, and inhibiting topoisomerase II involved in DNA repair. Mitoxantrone likely acts by inhibiting the proliferation of T cells, B cells, and macrophages thought to be responsible for most of the neuroinflammation in MS. Numerous studies showed decreased disability in both relapsing–remitting and progressive disease courses after treatment with Mitoxantrone. Unfortunately the main side effect of this medication is dose dependent cardiotoxicity. This limits the duration of use for this medication and limits its long-term benefits.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis is the experimental model of multiple sclerosis wherein T lymphocytes activated by peptides derived from myelin proteins in the periphery differentiate into inflammatory helper T cells. These cells are able to pass the blood-brain barrier where they recognize their cognate target antigen and initiate an inflammatory cascade leading to tissue damage. T cell infiltration is largely localized to the spinal cord in classic EAE with ascending paralysis beginning in the tail and progressing to the forelimbs. The extent of paralysis is used as a measure of disease severity.

EAE can be induced in susceptible animals by active immunization with myelin antigens mixed with adjuvant, or by passive transfer of myelin reactive $CD4^+$ T helper cells. EAE disease course and severity varies with mouse strain and encephalitogen used. For instance, SJL mice experience a relapsing-remitting disease course of severe or moderate severity depending upon whether EAE is induced with PLP or MBP respectively, whereas C57B6/L mice experience a monophasic or chronic disease course of mild to moderate severity induced by MBP or MOG respectively. Additionally passive transfer models of EAE have been developed. These models use CD4+ T cells from previously immunized mice, or myelin-specific CD4+ TCR-transgenic T cells from naïve mice to induce disease in unimmunized recipients. In the transgenic models, EAE can be induced by the passive transfer of activated $MBP_{AC1\text{-}11}$ specific CD4+ T cells in the B10.PL mouse

causing monophasic disease or moderate severity, or by and transfer of MOG_{35-55} specific CD4+ T cells into irradiated C57B6/L mice [24]. At peak EAE severity both T_H1 and T_H17 proinflammatory CD4+ subsets have been detected in the CNS with the immunization model of EAE, and both subsets are able to transfer disease to naïve mice individually using the adoptive model of EAE [25,26].

T Helper Cells

T cells are a type of white blood cell that plays a central role in cell-mediated immunity. They are called 'T' cells because they mature in the thymus. Each T cell expresses T cell receptors that all specifically recognize the same unique peptide:MHC complex, as a result of somatic recombination of the genes encoding the individual subunits of the TCR. T cells can be further divided into two groups; cytotoxic T cells which express CD8 and respond to peptide:MHCI complexes, and helper T cells (T_H) which express CD4 and respond to peptide:MHCII complexes.[27] T_H cells were given the name 'helper' because they provide 'help' to B cells[28] in the form of cytokines that direct immunoglobulin class switching (reviewed in [29]). Helper T cells have been further divided into subtypes based upon the transcription factors that regulate their differentiation and the cytokines they produce[30-33], however the process of defining T_H subsets is ongoing[34]. Of particular interest are the proinflammatory T_H1 and T_H17 subsets, which have been implicated in the pathogenesis of EAE and MS.

T_H1 cells

T_H1 cells are defined as cells that produce large quantities of interferon gamma (IFNγ). T_H1 cells are predominantly involved in the clearance of intracellular pathogens through the activation of macrophages and induction of immunoglobulin class switching to complement-fixing antibodies [30]. IFNγ-producing T_H1 cells have also been associated with the pathogenesis of many organ-specific autoimmune diseases [35,36]. Early EAE studies identified IFNγ at the site of tissue inflammation during the peak of disease and T_H1 cells were able to transfer disease in mice [37-39].

T_H1 Differentiation

The differentiation of T_H1 cells is initiated by activation of T cells in the presence of IFNγ. This leads to the activation of STAT1 and the T_H1 specific transcription factor Tbet. Tbet induces IFNγ production and allows responsiveness to IL-12 via the expression of the IL-12Rb2 chain. Engagement of the IL-12 receptor by IL-12 induces the phosphorylation of STAT4, which further cooperates with Tbet to transactivate the IFNγ gene. A positive feedback loop then ensues wherein the increased IFNγ further up-regulates Tbet and thus strengthens T_H1 commitment (Figure 1). Multiple overlapping mechanisms likely contribute to stable T_H1 differentiation including epigenetic modifications, post-transcriptional regulation by miRNAs, changes in metabolic activity, and activation of transcription factors (reviewed in [40]). The T_H1 phenotype becomes more stable with increasing numbers of cell divisions or repeated rounds of stimulation in vitro suggesting heritable

epigenetic modifications are responsible of the terminal differentiation of effector phenotypes [41-43].

Additionally, mice deficient for the transcription factors of T_H1 differentiation such as Tbet and STAT4 are resistant to the development of EAE supporting the influence of T_H1 cells and T_H1-associated cytokines in organ-specific autoimmunity [44,45].

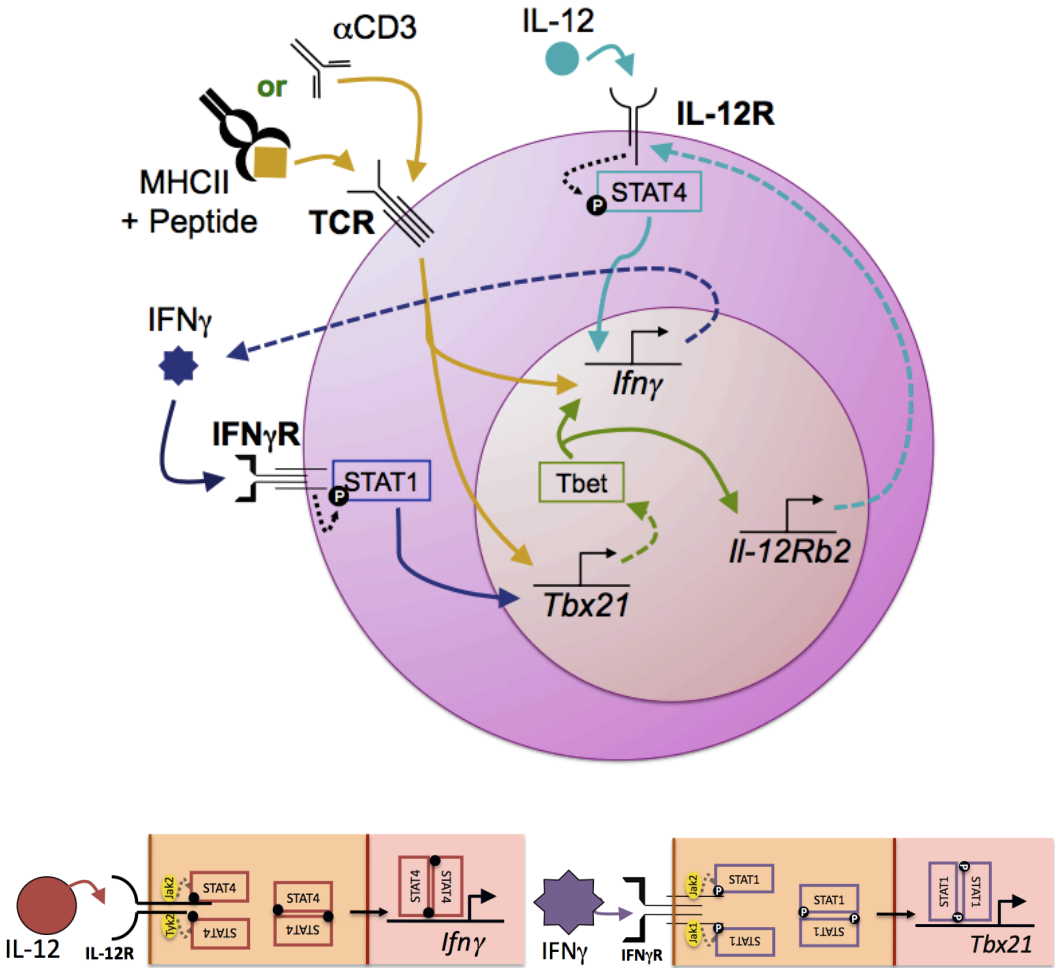

Figure 1: *Model of Th1 differentiation*. TCR engagement in conjunction with IFNγ signaling through STAT1 propagates a positive feedback loop of IFNγ production facilitating Th1 differentiation. IFNγ also induces IL-12 responsiveness to enhance IFNγ production.

T_H17 involvement in EAE

Despite the requirement of canonical T_H1 factors such as Tbet, STAT4, and the IL-12p40 subunit for EAE induction, IFNγ itself was found to be dispensable. In fact IFNγ deficient mice experienced more severe EAE than mice lacking IL-12p35 subunit or IL-12Rβ2 [46-50].

Despite initially being characterized solely as a T_H1 factor, the IL-12p40 subunit of IL-12 was later discovered to also pair with the p19 subunit of IL-23. IL-23p19 is also necessary for EAE induction [51]. IL-23 signals through a receptor composed of IL-12Rβ1 and IL-23R to drive the expansion of IL-17 producing T_H17 cells (Figure 2). Resident microglial cells and infiltrating macrophages have been observed to produce IL-23 during autoimmune inflammation of the CNS suggesting that IL-23 may function *in situ* to sustain a population of pathogenic T_H17 cells [51]. Despite the requirement for IL-23, IL-17 was also found to be dispensable for EAE induction, although EAE was less severe in IL-17 deficient mice [52,53]. However, $T_H17$cells are capable of inducing EAE upon adoptive transfer. T_H17 cells preferentially home to the brain and recruit a predominantly neutrophil CNS infiltrate, whereas T_H1 cells preferentially home to the spinal cord and induce infiltrates that are predominantly macrophages[25,54]. In regards to severity, T_H17 induced adoptive EAE was observed to be more severe than EAE induced by adoptive transfer of IL-12-driven T_H1 cells [55].

These studies clearly demonstrate the contribution of T_H17 cells to EAE. In mice the first line MS drug IFN-β reduces EAE severity in T_H1 mediated disease but

exacerbates EAE symptoms in T_H17 mediated disease. The heterogeneity in EAE induction is reflected in RRMS experienced by humans where IFNβ non-responders possess high IL-17F serum levels [56].

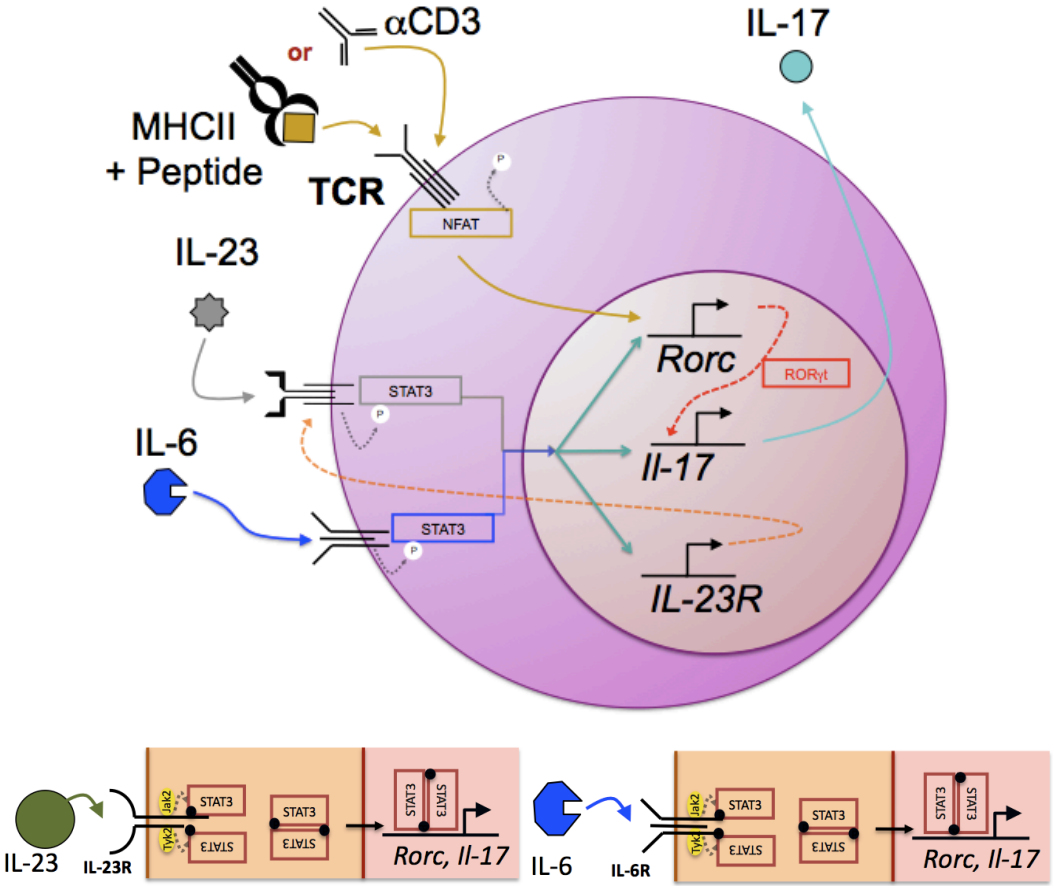

Figure 2: *Model of T_H17 Differentiation*. TCR engagement in conjunction with IL-6 signaling through STAT3 promotes T_H17 differentiation through the induction of RORγt and IL-23R expression. IL-23 signaling strengthens T_H17 differentiation in a synergistic manner and is required for stable IL-17 production.

REGULATORY T CELLS IN AUTOIMMUNE DISEASE

Regulatory T cells (T_{reg}) are a developmentally and functionally distinct T cell subpopulation that is engaged in sustaining immunological self-tolerance and homeostasis. T_{regs} are generated naturally as a product of thymic selection (nT_{reg}) or induced in the periphery (iT_{reg}) and both populations express high levels of CD25 (IL-2Rα) and the transcription factor FoxP3. Mutations in the gene encoding FoxP3 in humans causes two severe multi-organ autoimmune syndromes in humans, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and X-linked autoimmunity-allergic dysregulation syndrome (XLAAD) [57-59] and a fatal lymphoproliferative disorder in mice.[60] Additionally, T cell specific ablation of FoxP3 resulted in a lymphoproliferative autoimmune syndrome identical to that observed in Foxp3-deficient mice [33]. Defective FoxP3 protein leads to lethal immune dysregulation therefore FoxP3 is clearly essential for regulatory T-cell functions.

T_{reg} and T_H17 populations are believed to exist in a reciprocal balance because differentiation of both are promoted by TGFβ. While IL-6 pushes naïve CD4+ T cells toward a proinflammatory T_H17 state, IL-2 signaling induces naïve CD4+ T cell differentiation into a FoxP3+ regulatory population that suppresses inflammation (Figure 3) [61-63]. This balance between $T_H17$and T_{reg} differentiation is critical for both host immunity and the preservation of tolerance [64-67].

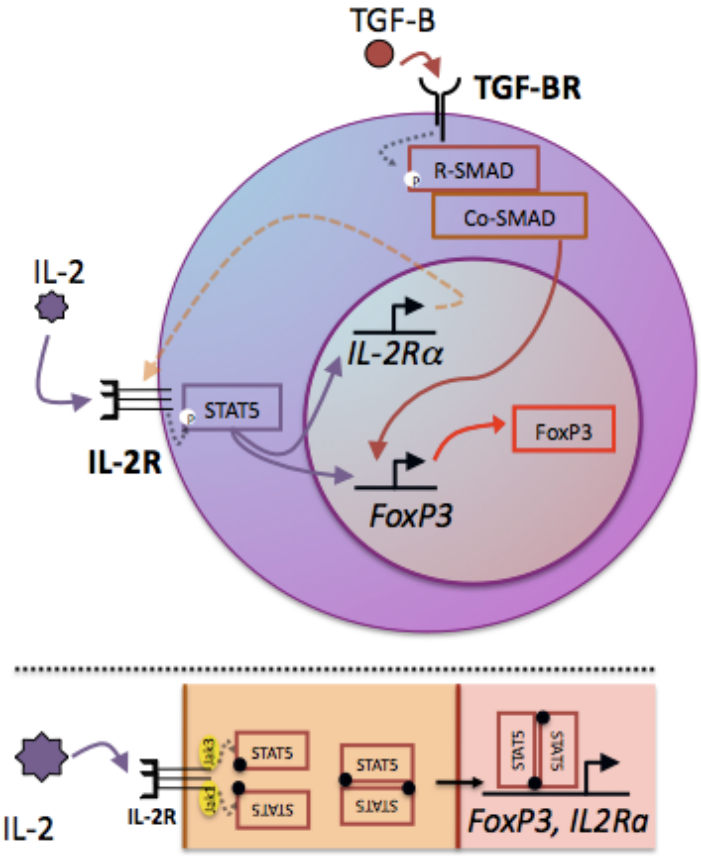

Figure 3: *Model of T_{reg} Differentiation*. TGF-β signaling in conjunction with IL-2 induces expression of the transcription factor FoxP3. T_{reg} cells do not produce IL-2 and therefore rely on exogenous IL-2 for their survival.

THE GAMMA SECRETASE COMPLEX

The gamma secretase complex belongs to a unique class of enzymes that cleave membrane-embedded portions of their substrates via hydrolytic proteolysis within the cell membrane. The gamma secretase complex is composed of four subunits, anterior pharynx-defective 1 (APH-1), presenilin enhancer 2 (PEN-2), nicastrin, and presenilin 1 (PSEN1) [68,69] in a 1:1:1:1 ratio [70,71]. The presenilin subunit is the catalytic core the gamma secretase complex[72]. Two versions of presenilin exist, PSEN1 and PSEN2, which are conserved among vertebrates[73]. Presenilin is a nine transmembrane proenzyme [71] which undergoes self endoproteolysis between transmembrane regions 6 and 7 [74] to form the active transmembrane aspartyl protease[75,76]. PSEN1 and PSEN2 mRNA are expressed ubiquitously and at comparable levels in most human and mouse tissues [77]. The non-enzymatic subunits of the gamma secretase complex stabilize the presenilin holoprotein in the complex (APH-1 and Nicastrin), promote endoproteolytic processing of presenilin (PEN-2)[78], or facilitate trafficking of the gamma secretase complex (Nicastrin)[79].

The most well characterized substrates of gamma secretase are the amyloid precursor protein and Notch. However, 55 type I transmembrane proteins are predicted to be substrates of gamma secretase. More than a dozen of the predicted gamma secretase substrates have been confirmed [80,81] including CD44, INFαR2, IL-1R2, and E-cadherin which are involved in immune cell trafficking, immune cell extravasation, antiviral responses, and cytokine signaling [82-85]

Origins and development of Gamma secretase inhibitors

APP proteolysis

The enzyme γ-secretase, along with β-secretase is responsible for amyloid precursor protein (APP) proteolysis. Gamma secretase (GS) imprecisely cleaves APP producing a collection of Aβ fragments of variable length[86]. Genetic mutations in presenilin1 and presenilin2 are linked to rare familial early-onset Alzheimer's disease [87-89] and favor the production of the amyloid plaque forming Aβ42 fragments. In an effort to retard amyloid plaque formation, and thereby halt Alzheimer's disease progression, a series of gamma secretase inhibitors were developed.

Inhibitor Development

Early gamma secretase inhibitors blocked substrate cleavage by binding to, and thereby blocking, the active site of enzymatic proteolysis. These first-generation inhibitors caused side effects in thymocyte development and in the GI tract, which were attributed to the blockage of Notch signaling [90-92]. However, some blockade of Notch processing by GS is tolerated, and the Bristol-Myers Squibb group determined that ~15-fold Notch sparing in a γSI is sufficient to avoid Notch-related gastrointestinal and T cell side effects [93]. Additionally the Wyeth group reported that an ~30% inhibition of Aβ synthesis was sufficient to reverse cognitive impairment in the contextual fear conditioning model of AD in Tg2567 mice [94]. Therefore it was hoped that a window of partial GS inhibition existed wherein a therapeutic reduction in Aβ could be achieved while retaining enough activity to

prevent Notch related side effects. This led pharmaceutical companies to search for compounds that bind alternative sites on GS. Several of these alternative binding site compounds entered into clinical trials.

Inhibitor Classification

γSIs can be separated into three general types based on where they bind to GS: (1) active-site-binding γSIs that mimic the transition state of APP cleavage by GS, (2) substrate docking-site-binding γSIs, and (3) alternative binding site γSIs that can be further subdivided into carboxamide- and arylsulfonamide-containing γSI s.[95] Of the inhibitors referenced in this work, IL-CHO and MW167 are class I inhibitors, whereas all other inhibitors belong to class III. The chemical structures of γSIs that have been released to the public are depicted in APPENDIX 2.

Alzheimer's Clinical Trials

Thus far all gamma secretase inhibitors that have entered clinical trials eventually failed. SPI-1865 failed pre-clinical trials due to adrenal toxicity. Begacestat and ELND007 were discontinued during phase I for undisclosed reasons. ELND006 failed phase I due to liver toxicity. Avagacestat (BMS-708163) was discontinued after phase II due to worsening of cognition. Semagacestat (LY-450,139) was terminated during phase III due to increased weight loss, incidence of skin cancer and infection, and worsening of dementia over placebo[96]. Lastly, Flurizan (R-flurbiprofen) failed phase III trials based on lack of efficacy.

Notch Signaling

In addition to cleavage of APP, gamma secretase is required for notch signaling[72]. The notch receptor is an evolutionarily conserved type I transmembrane protein which, in conjunction with its cognate ligands, facilitates the communication between cells that are physically contacting one another. In mammals notch signaling is accomplished via a binary combination of one of four notch receptors (Notch 1-4) with one of five notch ligands (Jagged 1, Jagged 2, Delta-like 1,2,4) [97]. After ligand binding, the extracellular domain of Notch is cleaved by TACE, an ADAM family metalloproteinase. Subsequently the remaining portion of the Notch protein is processed by gamma secretase, releasing the notch intracellular domain (NICD), which translocates to the nucleus and interacts with CSL to activate transcription of target genes[98]. Notch signals can promote or suppress cell proliferation, cell death, acquisition of specific cell fates, or activation of differentiation programs in a context dependent manner. Notch signaling plays a critical role in a wide range of tissues throughout development of an organism and the maintenance of self-renewing adult tissues including the adaptive immune system[99].

Role of Notch in Adaptive Immune Differentiation

Notch signaling is required for the differentiation of distinct subsets of B and T cells of the adaptive immune system from bone marrow-derived common lymphoid progenitor cells. In the spleen Delta-like 1 mediated Notch 2 signaling is critical in immature T2-B cells for differentiation into marginal zone B cells or follicular B cells[100-102]. Similarly notch signaling in the thymus is required for T cell linage

commitment [103-106] up to the double negative 3 stage of T cell differentiation. Notch signal strength is also involved in αβ versus γδ T cell determination[107,108]

Notch Regulation of Peripheral Immune responses

Naïve T cells express Notch1 and Notch2 [109] and upregulate expression of Notch 1-4 when activated [110], although expression levels may vary with antigen or mouse strain [111]. Notch signaling has been observed to regulate *in vitro* T helper differentiation into T_H1 or T_H2 depending on whether the notch ligand was delta-like 1, (T_H1) or jagged1 respectively (T_H2)[109]. While it has been clearly demonstrated that the NICD interacts with the IL-4 and GATA-3 promoters there have been conflicting results of NICD association with the promoter region of *TBX21* which encodes Tbet, the hallmark T_H1 transcription factor[112,113]. During canonical Notch signaling, the NICD translocates to the nucleus and binds the transcriptional repressor, RBPjk, converting it into an activator and inducing the expression of downstream target genes [97]. In human cells, RBPjk (also known as CSL) has also been shown to bind the IL-17 and RORgt promoter regions. One study reported that IL-17A production increased when $Notch1^{IC}$ was overexpressed while siRNA inhibition of Notch1 decreased IL-17A and IL-17F levels [114].

Notch Signaling modulates EAE

Modulation of notch and its ligands has been reported in the context of EAE. Specifically, Delta-like 1 (DLL1) expression was found to be upregulated on dendritic cells and B cells after priming, while Jagged1 was upregulated only on

dendritic cells.[115]. Additionally, CNS-infiltrating myeloid dendritic cells, macrophages, and resident microglia were all observed to express Delta-like ligand 4 (DLL4) after EAE induction [116].

Interestingly treatment with DLL1 was found to worsen EAE with increased T_H1 infiltration into the CNS whereas Jagged1 treatment promoted IL-10 and ameliorated disease in vivo [115]. Additionally, DLL4 blockade has been shown to decrease EAE by inhibiting T_H1/T_H17 while promoting T_H2/T_{reg} immune responses [117] or by altering T cell migration through repression of chemokine receptors CCR2 and CCR6 [116].

SPECIFIC AIMS

These findings form the basis of my dissertation work surrounding the hypothesis that γ-secretase inhibition represses inflammatory CD4+ T-cell differentiation, functioning as a mechanism of EAE suppression. To explore this idea, I have decided to focus my work into four specific aims, representing the four subsections of the Results chapter:

1) Investigate the impact of *in vivo* γSI treatment in mouse models that experience acute, monophasic EAE.
2) Investigate the impact of γSI on autoreactive T cells *in vivo*.
3) Investigate the impact of γSI on T_H1 differentiation *in vitro*.
4) Investigate the impact of γSI on the balance of T_H17/T_{reg} differentiation.

CHAPTER TWO

Methodology

Mice

Female B10.PL mice and B6 CD4-Cre mice aged 6-8 weeks were purchased from Jackson Laboratories. Female C57BL/6 mice were purchased from the UT Southwestern Mouse Breeding Core Facility. B10.PL MBP_{AC1-11} TCR transgenic mice[118] were obtained from Juan Lafaille at New York University. $PSEN1^{flox/flox}$ (B6:129P-$Psen1^{tm1Vln}$/J) were purchased from Jackson Laboratories and backcrossed onto a pure B6 background for ten generations. All mice were housed and bred in the UT Southwestern Medical Center Animal Resource Center in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC).

Active EAE Induction

Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice by subcutaneous immunization with 200 μg of MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK, GeneMed Synthesis Inc.), 200 μg recombinant MOG protein (provided by Nancy Monson lab and E. Sally Ward lab) or 200 μg $PLP_{178-191}$ (NTWTTCQSIAFPSK, GeneMed Synthesis Inc.) in emulsified CFA (Difco Laboratories) supplemented with 4 mg/ml M. tuberculosis strain H37Ra (Difco) followed by intraperitoneal injection of 250 ng of Bordetella pertussis toxin (Difco) in phosphate-buffered saline (PBS) at the time of and 2 days after immunization.

Clinical disease severity was monitored daily and scored according to the following scale: 0 – no clinical disease, 1 – limp tail, 2 – hind limb weakness, 3 – severe hind limb weakness and/or partial hind limb paralysis, 4 – complete hind limb paralysis, 5 –moribund/death [119].

Adoptive EAE Induction

T_H1 EAE: Splenocytes and lymph node cells were isolated from MBP_{AC1-11} TCR-transgenic mice and plated at $5x10^6$ / ml with IL-12 (10 ng/ml), plate bound anti-CD3 (1 μg/ml) and plate bound anti-CD28 (1 μg/ml). After 3 days of activation cells were washed in PBS and $10x10^6$ T cell blasts ($5x10^7$/ml) were injected intraperitonially into 8-10 week old B10.PL mice. Control and experimental groups consisted of equal numbers of male and female mice.

T_H17 EAE: Splenocytes and lymph node cells were isolated from MBP_{AC1-11} TCR-transgenic mice and depleted of CD25+ cells (Miltenyi) using an AutoMACS (Miltenyi). Cells were plated at $5x10^6$ in 24-well flat bottom plates. After a 30 minute incubation at 37°C 5% CO_2 with DBZ or DMSO cells were activated with MBP_{AC1-11} (48 ug/ml) (Ac-ASQKRPQRHG, GeneMed Synthesis Inc.), TGF-β1 (2 ng/ml), IL-6 (30 ng/ml) anti-IFNγ (10 μg/ml clone XMG1.2), and anti-IL-4 (1 μg/ml clone 11B11). Cells were treated with DBZ or DMSO every 24 hours except on days on which cells were split. Cells were split as needed (~48 hours and 6 days post activation) with media containing IL-23 (10 ng/ml). On day 7 cells were counted and replated at $2x10^6$/well in 24 well plates coated with anti-CD3 (2 μg/ml) and

anti-CD28 (2 μg/ml) overnight at 4°C. After 3 days of restimulation cells were washed with PBS and $3x10^6$ or $1x10^7$ T cell blasts were transferred into naïve 8-10 week old F1 B6xB10.PL mice. Control and experimental groups consisted of equal numbers of male and female mice.

In vitro Cell Culture

Cell Culture Media

Unless otherwise noted, all in vitro cell culture used DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products), HEPES (5 mM) (Cellgro), MEM nonessential amino acids (Cellgro), GlutaMAX-I (Gibco), sodium pyruvate (1mM) (Cellgro), penicillin/streptomycin solution (Gibco), and 2-mercaptoethanol (50 μM) (Sigma).

In vitro T Helper Differentiation

Spleens and cervical, brachial, and axillary lymph nodes were harvested from mice for in vitro differentiation. In vitro differentiation was using either magnetically isolated naïve CD4+ T cells or unsorted cells. Naïve T cells were isolated using a two-step magnetic positive selection kit for CD4+ CD62L+ cells from Miltenyi on an AutoMACS magnetic cell sorter. In order to activate naïve sorted TCR-transgenic CD4+ T cells with their cognate peptide, antigen presenting cells were obtained by depleting CD4+ T cells from bulk splenocytes with a CD4+ selection kit from BD Biosciences and the BD IMagnet™. Unless otherwise stated, unsorted cells were plated unto flat-bottomed 24-well plates at a density of $5x10^6$ per well whereas

sorted cells were plated at a density of $2x10^6$ per well with an equal number of CD4+-depleted APCs. Cells were incubated with γSI at 37°C for 30 minutes prior to activation. Cells were then activated with 12 μg/ml MBP_{AC1-11} for 3 days, rested for 2 days in fresh media, then restimulated with 12 μg/ml MBP_{AC1-11} for an additional 3 days under the following culture conditions: **T_H1** IL-12 (10 ng/ml) stimulation, IL-2 (2 ng/ml) rest, IL-12 (2 ng/ml) IL-2 (10 ng/ml) restimulation. **T_H17** anti-IFNγ (10 ug/ml) anti-IL-4 (1 ug/ml) TGFβ1 (2 ng/ml) IL-6 (20 ng/ml) stimulation, IL-7 (2 ng/ml) rest, IL-23 (20 ng/ml) restimulation. **T_H1 non-skewing** only received 2 ng/ml IL-2 during the resting phase. All blocking antibodies were purchased from Biolegend and all cytokines were purchased from Peprotech.

Gamma secretase inhibition

In vivo gamma secretase inhibition was accomplished by intraperitonial injection of 100 μg (~5 μg per gram of body weight) of either DAPT or DBZ solublized in DMSO at 100 mM and delivered in a total volume of 100 μl utilizing vegetable oil as a vehicle. γSI treatment was initiated one day prior to active immunization or one day post induction of adoptive EAE. Mice were treated with γSI or DMSO control every 48 hours unless otherwise specified.

Gamma secretase inhibition of *in vitro* cultures was accomplished by a 30 minute pre-incubation at 37°C 5% CO_2, then a daily treatment with DAPT, DBZ, or Compound E to the final concentration of 200 nM, 8.5 nM, or 0.3 nM respectively. All inhibitors were delivered in 1 ul DMSO per 1 ml culture medium. All gamma

secretase inhibitors were purchased from Axon Medchem.

Flow Cytometry and Analysis

Flow cytometry was performed on BD LSR II or Accuri C6 flow cytometer. All fluorescent antibodies were purchased from eBioscience unless otherwise stated. Flow cytometry data were analyzed using FlowJo and Cytobank software.

Intracellular flow cytometry

To assess intracellular expression of transcription factors and cytokines were fixed using the eBioscience FoxP3 Staining Buffer Set with a slight modification that cell surface markers were stained post fixation instead of prior. Additionally, for intracellular cytokine staining cells were incubated with PMA (50 ng/ml), Ionomycin (250 ng/ml), and Brefeldin A (5 μg/ml) for 4-6 hours prior to fixation. Transcription factor flow panel included anti-mouse CD4-Pacific Blue (Biolegend) and the following anti-mouse antibodies all purchased from eBioscience: CD25-FITC, Tbet-PE, RORγt-APC, and FoxP3-Alexa700. The intracellular cytokine panel consisted of anti-mouse CD4-Pacific Blue (Biolegend) and the following anti-mouse antibodies all purchased from eBioscience: CD25-APC, IL-17A-FITC, IFNγ-PE-Cy7, and either GM-CSF-PE orIL-4-PE.

Phosphorylated STAT

After T_H1 or T_H17 culture cells were washed 2x with fresh culture media and rested for 30 minutes at 37°C before stimulation with IFNγ (400 ng/ml), IL-12 (80 ng/ml), IL-23 (80 ng/ml) or IL-6 (60 ng/ml) to induce phosphorylation of STAT1, STAT4, STAT3, and STAT3 respectively or with PBS as a negative control. Cells were quickly spun down, aspirated and resuspend in 50 μl PBS. 50 μl of 4% PFA was immediately added, briefly and gently vortexed and incubated for 10 minutes at 37°C to fix the cells. Cells were chilled on ice for 1 minute then 900 μl of ice cold methanol was slowly added to the cells. Tubes were manually shaken for 30 seconds to remove the cellular membranes then incubated an additional 30 minutes on ice. Cells were then spun down and MeOH was aspirated completely flowed by 2 washes in FACS buffer then staining for 60 minutes at room temperature in the dark with the following anti-mouse antibodies: CD4-Pacific Blue (Biolegend), CD25-PE-Cy7 (eBioscience), and one of the following: pSTAT1-Alexa 647 (BD Biosciences), pSTAT4-PE (BD Biosciences), or pSTAT3-Alexa 647 (BD Biosciences).

CFSE-Based Proliferation Assay

Carboxyfluorescein succinimidyl ester (CFSE) dilution assays were performed as previously described [120]. Briefly, cells were suspended at 1 x 10^6 cells/ml in PBS and incubated at 37°C for 7 minutes with 0.25 μM carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE). Cells were then washed 3X with 10% fetal bovine serum (FBS, Gemini Bio-Products) in PBS. Cells were then resuspended in complete media

(DMEM supplemented with 10% FBS, Lglutamine (2 mM), penicillin/streptomycin (100 IU/ml / 100 µg/ml), HEPES (5 µM), sodium pyruvate (1 mM), non-essential amino acids (all from Mediatech), and β-mercaptoethanol (50 µM) (Sigma)) and incubated for 120 hours at 37°C and 5% CO_2. Cells were washed with FACS buffer (PBS with 1% bovine serum albumin (BSA) and 0.1% sodium azide) and stained with fluorescent antibodies. Flow cytometric data were acquired on a BD LSR II running FACSDiva software. Data were analyzed by FlowJo (TreeStar).

Statistical Analyses

Statistical analyses were performed using Prism 6 software from Graphpad. The Kolmogorov-Smirnov test used to determine significance in EAE severity over time between treatment and control groups. The unpaired t-test was for all other analyses.

CHAPTER THREE

Results

SECTION 1: IMPACT OF γSI ON EAE

Previous studies have shown that γSI treatment was able to reduce the severity of very mild EAE in the SJL/J strain of mouse immunized with $PLP_{139-151}$ when the inhibitor is administered intraperitonially [112] or supplemented into the mouse chow [114]. Additional studies demonstrated that intraventricular injection of the Sigma γSI, MW167, at the peak of disease resulted in modestly better recovery from acute EAE in $PLP_{139-151}$. Histology of γSI treated mice revealed milder CNS pathology and increased remyelination compared to controls [121,122].

To further investigate the effects of gamma secretase inhibition on demyelinating disease and evaluate the potential of γSIs as a possible MS therapeutic, we induced acute monophasic EAE and assessed the impact of *in vivo* γSI treatment. Herein, we utilized both the active immunization model of disease with MOG in C57BL/6 mice and the adoptive transfer model of EAE using MBP_{AC1-11}-specific TCR-transgenic T cells in B10.PL mice in an effort to dissect the mechanism of γSI-mediated EAE suppression.

γSI Treatment Modulates Active EAE

Previous data from Eagar Lab revealed that γSI treatment suppressed active EAE in C57BL/6 mice immunized mice with MOG of human origin. Mice that received DMSO control experienced paralysis associated with severe EAE whereas the mice treated with DAPT a much less severe disease course (Figure 4). While γSI treatment protected mice immunized with full length MOG protein, γSI failed to protect mice immunized with the immunodominant 35-55 epitope of MOG in the C57BL/6 strain. (Figure 5). γSI treatment also failed to reduce disease severity in C57BL/6 mice immunized with $PLP_{178-191}$ (Figure 6). In both cases DAPT treatment of peptide-immunized EAE resulted in a trend toward increased EAE severity without being statistically significant.

Additionally EAE disease course was not significantly altered in mice in which PSEN1 expression is decreased in all T cells (Figure 7) using cre-recombinase under expression of the CD4 promoter in mice heterozygous for a floxed exon 7 of the PSEN1 gene [123]. *In vitro* measurements of HES-1, a transcription factor used to assess notch signaling, was reduced in PSEN1(flox+/-) activated T cells to a level similar to the resting state of wild type control. Therefore these heterozygous mice should be useful in investigating the role of presenilin and gamma secretase in T cells. However no reduction in disease severity and was observed in the mice with a CD4 conditional reduction of PSEN1. PSEN2 is capable of compensating for the loss of PSEN1 in forming a functional gamma secretase complex, which may explain the

lack of impact on EAE severity. Alternately, the therapeutic effect of γSI treatment on EAE severity *in vivo* may be due to inhibition of GS in a non-T population.

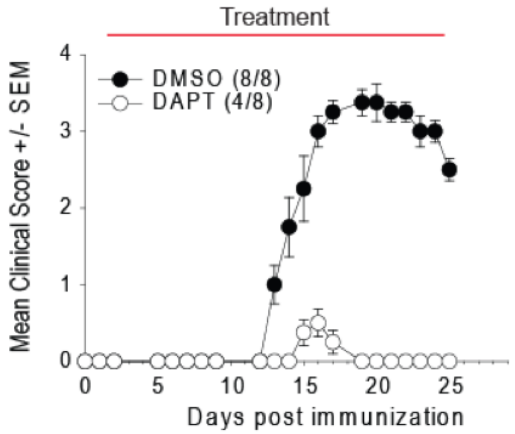

Figure 4 *γSI treatment protects mice from MOG$_{1-125}$ induced EAE*. C57BL/6 mice immunized with MOG1-125 and treated with γSI beginning one day post immunization and every other day thereafter were protected from EAE. EAE incidence in γSI treated mice was half that of the control. An extremely mild disease course was observed in the γSI treated mice that did experience EAE.

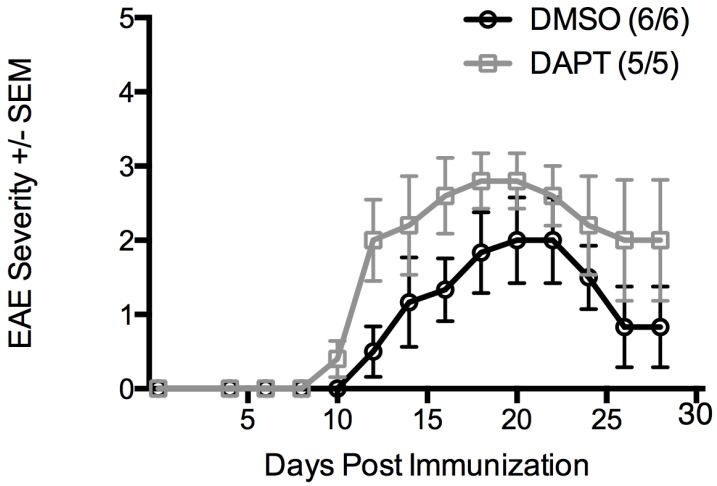

Figure 5 *MOG$_{35-55}$ EAE severity not reduced by in vivo γSI treatment.* C57BL/6 mice were treated with γSI intraperitoneally one day prior to immunization and every other day thereafter. No reduction was observed in EAE severity. Disease incidence was 100% in both groups with 6/6 and 5/5 mice experiencing EAE in the DMSO and DAPT treated groups respectively.

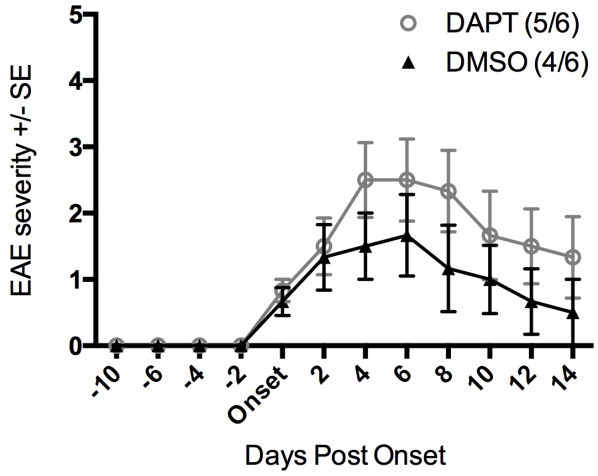

Figure 6 *$PLP_{178-191}$ EAE severity not reduced by in vivo γSI treatment*. C57BL/6 mice were treated with γSI intraperitoneally one day prior to immunization and every other day thereafter. No difference was observed in EAE severity. N=6 in each group with a disease incidence of 5/6 in DAPT and 4/6 in DMSO treated mice.

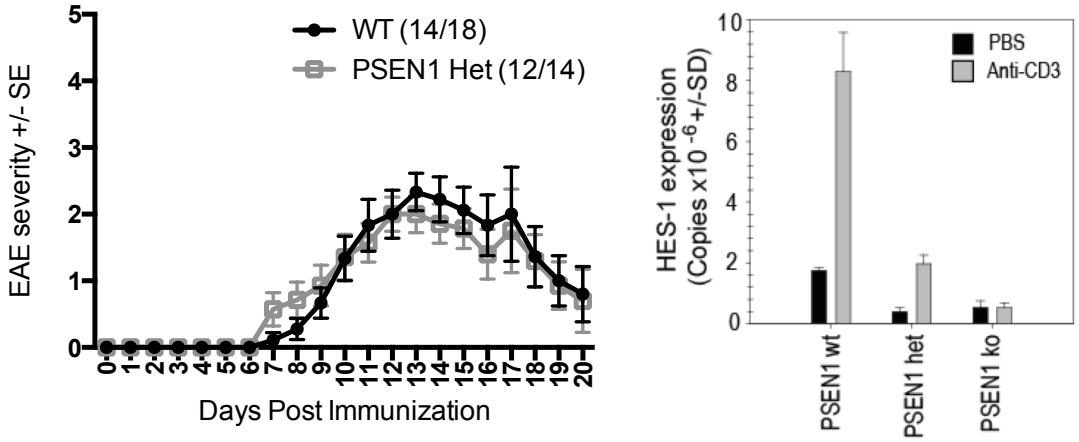

Figure 7 *EAE severity unaffected by genetic PSEN1 deficiency*. CD4-Cre mediated ablation of a single copy of PSEN1 in C57BL/6 mice did not reduce MOG_{35-55} induced EAE severity. Disease course was overlapping in the pooled results of two experiments (left panel). Disease incidence was 14 of 18 mice in the wild type condition and 12 of 14 in PSEN1 heterozygous mice. Deletion of a single copy of PSEN1 severely reduces Notch signaling as measured by expression of the downstream factor HES-1 (right panel).

γSI treatment Suppresses Adoptive EAE

In addition to disease induction by active immunization with myelin proteins, EAE can be induced through the adoptive transfer of activated and polarized myelin-specific CD4+ T cells. This model of EAE is useful in determining specific CD4+ factors necessary for disease induction as well as assessing the impact of treatment post-transfer on established immune responses.

To determine if γSI treatment could suppress adoptive EAE, T_H1 cells differentiated *in vitro* were transferred into naïve mice and then treated with γSI every other day. When IL-12 pushed T_H1 cells specific for MBP_{ac1-11} were transferred to naïve mice, γSI treatment reduced EAE severity (Figure 8).

Summary

Here we have demonstrated gamma secretase inhibition is a viable method of suppressing the severity of autoimmune disease in two models of EAE. In the C57BL/6 mouse strain γSI treatment was able to suppress EAE induced by active immunization with the extracellular domain of MOG but not MOG_{33-55} or $PLP_{178-191}$ peptides. Additionally, genetic reduction of gamma secretase activity in CD4+ T cells did not reduce MOG_{35-55} induced EAE. However, in the B10.PL mouse strain γSI treatment was able to suppress EAE induced by transfer of in vitro T_H1 differentiated CD4+ T cells.

The effect of γSI on CD4+ T cells from these results is unclear because γSI treatment, and genetic reduction of GS activity in CD4+ T cells, failed to suppress EAE severity

induced with the immuno-dominant MOG_{35-55} peptide. This led us to investigate the impact of γSI treatment on autoreactive T cells *in vivo*.

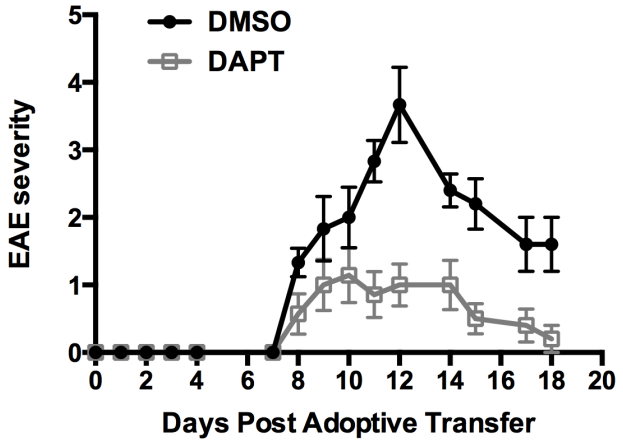

Figure 8 *In vivo γSI treatment reduces adoptive EAE severity*. TCR transgenic CD4+ T cells specific for MBP_{AC1-11} were activated with anti-CD3 and IL-12 in vitro and transferred IP into B10.PL mice. Mice were treated IP with γSI or control the day after adoptive transfer and every other day thereafter. γSI treatment reduced EAE severity.

SECTION 2: IMPACT OF γSI ON AUTOREACTIVE T CELLS *IN VIVO*

A previous study has demonstrated that *in vivo* γSI treatment reduced T cell-mediated inflammation when SJL mice that were previously immunized with $PLP_{139-151}$ for EAE induction were rechallenged with the same peptide [112]. Additionally, reduced IFNγ production was observed from *in vitro* T_H1 differentiation cultures of naive CD4+ T cells isolated from mice fed γSI for three days. These results suggest γSI treatment inhibits T cell mediated immune responses *in vivo*.

To further investigate the impact of γSI treatment *in vivo*, T cells were recovered from γSI and DMSO treated mice in which EAE was induced. T cells were then reactivated with their cognate peptide and assessed by flow cytometry.

γSI Treatment During Active EAE

In active EAE, immunization with emulsified myelin peptide and heat killed *M. tuberculosis* induces a *de novo* immune response. To determine if this response was influenced by γSI treatment, cells were harvested from mice immunized with MOG_{1-125} after the acute phase of EAE. When bulk splenocytes were restimulated with MOG_{1-125} no difference was observed in CD4+ T cell proliferation as assessed by CFSE dilution assay (Figure 9). However when the levels of IFNγ in proliferation culture supernatants were assessed by ELISA, a clear reduction in IFNγ production was observed in cells from γSI treated mice (Figure 10).

Another way γSI may be suppressing EAE is by modulating the trafficking or survival of cells involved in immune activation. To determine if γSI treatment is suppressing EAE by selective depletion of T cells in the periphery, flow cytometry was used to assess the composition of lymph node cell populations. Contrary to expectations, the percentage of lymph node CD4+ T cells substantially increased in γSI treated mice (Figure 11) while CD8+ percentages were unchanged demonstrating that the T cell compartment remains intact.

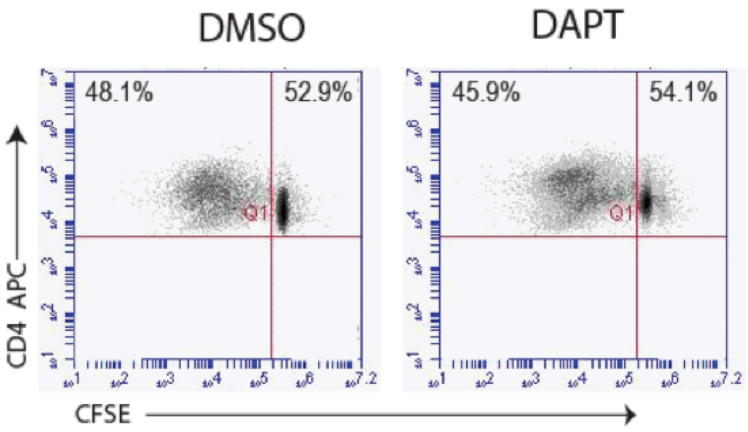

Figure 9 *In vivo γSI treatment does not alter recall proliferation*. Splenocytes recovered from MOG_{1-125} immunized mice were pooled, labeled with CFSE, and restimulated with MOG_{1-125}. No difference was observed in CD4+ proliferation between control and γSI treated groups.

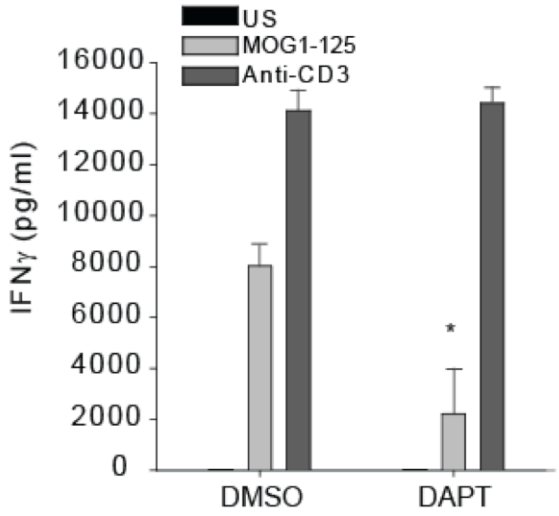

Figure 10 *In vivo γSI treatment reduces recall IFNγ production*. Splenocytes recovered from $MOG_{1\text{-}125}$ immunized C57BL/6 mice were and restimulated with $MOG_{1\text{-}125}$. ELISA of cell culture supernatants revealed a decrease in IFNγ production from MOG-specific cells.

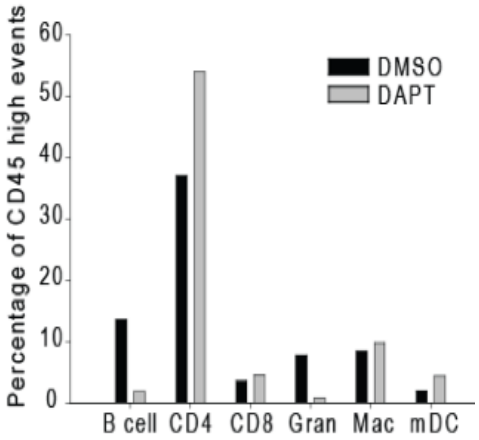

Figure: 11 *In vivo γSI treatment of EAE alters lymph node cellularity*. High expression of CD45 was used to gate mononuclear cells isolated from lymph node homogenates. Treatment with γSI increased the percentage of CD4+ cells whereas essentially no change was observed in CD8+ cells. A substantial reduction in B cells and granulocytes was also observed.

γSI Treatment During Adoptive EAE

In contrast to active EAE where a *de novo* immune response is initiated by immunization *in vivo*, with the adoptive transfer model of EAE myelin specific cells are differentiated *in vitro* to be proinflammatory prior to transfer into naïve mice for disease induction. A previous study has demonstrated that *in vitro* differentiated T_H1 cells are resistant to γSI mediated IFNγ suppression [112]. Therefore any differences observed in cells from γSI treated mice *should* be due to modulation of the immune response in the cells of the recipient but not in the transferred cells. To verify that these *in vitro* results held true *in vivo,* we conducted experiments to assess the impact of *in vivo* γSI treatment on transferred versus host-MBP_{AC1-11} reactive T cells.

To induce adoptive EAE, TCR-transgenic T cells from naïve mice were pushed to differentiate into T_H1 effectors and then transferred into 8-10 week old recipients. Recipient mice were treated with γSI (DAPT) or a DMSO control the day after transfer and every 48 hours thereafter. Splenocytes recovered from mice after the acute phase of T_H1 adoptive EAE exhibited similar levels of proliferation in both control and γSI treated mice when restimulated *in vitro* with MBP_{ac1-11} (Figure 12). When the supernatants of this reactivation culture were assessed for IFNγ production a deficit in cytokine production was observed cells from γSI treated mice (Figure 13). These results suggest that γSI treatment is not suppressing EAE through inhibition of myelin-specific T cell proliferation. However the specific effects of γSI

treatment on T_H1 donor as opposed to *de novo* generation of host MBP_{AC1-11}-specific T cells cannot be determined from this experiment.

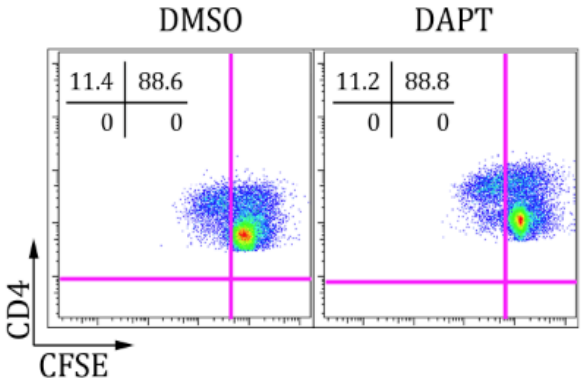

Figure 12 *In vivo γSI treatment does not alter recall proliferation in B10.PL.* Eighteen days post adoptive transfer splenocytes harvested from mice representative of the mean EAE severity of each group were labeled with CFSE and restimulated with MBP_{AC1-11}. No difference was observed in CD4+ proliferation between control and γSI treated groups.

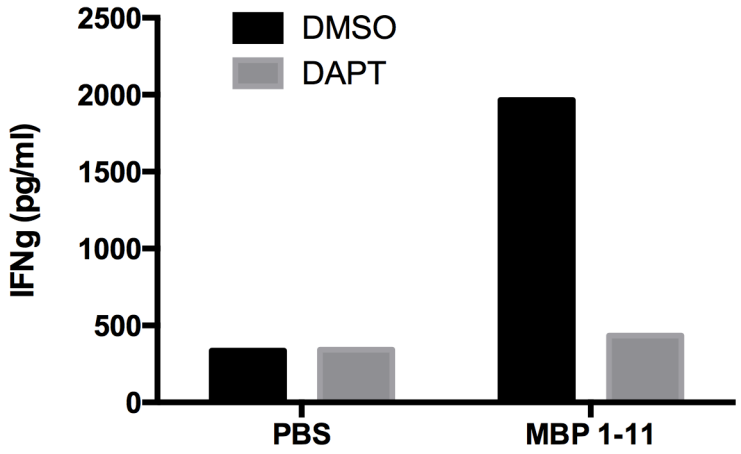

Figure 13 *In vivo γSI treatment reduces recall IFNγ production in B10.PL.* Splenocytes recovered from the recipients of adoptively transferred, in vitro T_H1 pushed, MBP-specific TCR-transgenic CD4+ T cells were restimulated with $MBP_{AC1\text{-}11}$. ELISA of cell culture supernatants revealed a decrease in IFNγ production from $MBP_{AC1\text{-}11}$-specific cells.

To further investigate the effects of *in vivo* γSI treatment on host versus transferred cells, we repeated the previous experiment using congenic markers to discriminate transferred T_H1 cells and their progeny from host cells in the recipient. Specifically, T_H1-pushed $MBP_{ac1\text{-}11}$ specific cells carrying the CD45.2 variant of the CD45 cell surface receptor were activated with $MBP_{AC1\text{-}11}$ and IL-12 for 3 days *in vitro*, then transferred into naïve mice heterozygously expressing CD45.1 and CD45.2. After transfer mice were treated with γSI (DBZ) every other day for eighteen days. Both treatment groups experienced similar EAE severity (mean of DBZ - 2.3, DMSO – 2.8). T cells from the lymph nodes of treatment (n=6) and control (n=5) groups were pooled, then labeled with CFSE and restimulated with $MBP_{ac1\text{-}11}$.

The absolute numbers of CD4+ cells recovered from both γSI and control groups were similar. γSI treatment did not appear to have an effect on the proliferation of the CD4+ CD45.1 negative, adoptively transferred cells or their progeny (Figure 14 right panels). However γSI did reduce by half the percentage of CD45.1 negative cells in the lymph node, suggesting γSI may be eliciting a suppressive effect on proinflammatory CNS specific T cells (Figure 14 left panels). Contrary to expectations, the generation of an $MBP_{ac1\text{-}11}$ specific response from the CD45.1+ host cells was nearly doubled in γSI treated mice (Figure 14 center panels).

Summary

These results demonstrate that *in vivo* γSI treatment reduces the ability of autoreactive T cells to produce IFNγ. γSI treatment increased the percentage of CD4+ T cells in the lymphoid compartment, yet no impact was observed on

proliferation following stimulation with self-peptides. Therefore *in vivo* effects were further dissected using congenic markers to track recipient and donor T cells independently. With this method, γSI treatment was observed to enhance auto-antigen proliferation in the recipient while reducing the percentage of self-specific donor cells or their progeny that persisted 18 days post transfer. Because there was such a strong impact of γSI treatment on the generation of a host-derived immune response, we decided to investigate the effects of γSI treatment on the differentiation of naïve CD4+ T cells into effectors *in vitro*.

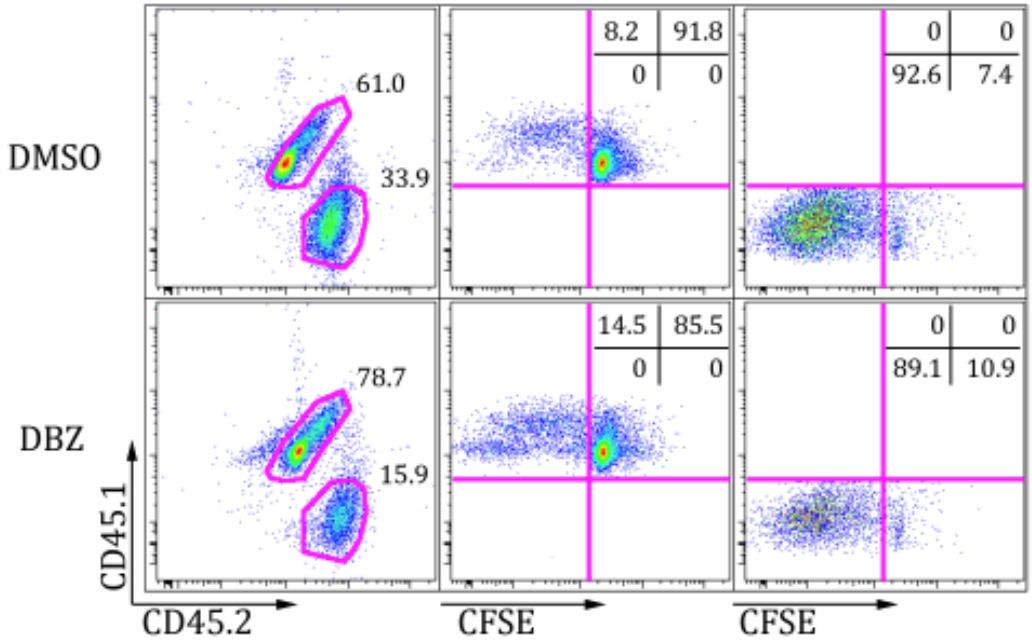

Figure 14 *γSI treatment suppresses survival of myelin-specific T_H1 cells in vivo*. TCR transgenic CD4+ T cells specific for MBP_{AC1-11} were activated with their cognate peptide and IL-12 for 3 days in vitro. 10x10^6 cells were transferred into mice heterozygous for CD45.1/45.2 and treated with 100ug DBZ the day after transfer and every other day thereafter. Lymph node cells were harvested, labeled with CFSE, and restimulated with MBP_{AC1-11}. Cells were pooled from 3 mice per group.

SECTION 3: IMPACT OF γSI ON T_H1 DIFFERENTIATION *IN VITRO*

The affect of γSI on *in vitro* T cell proliferation in the literature is unclear. While the γSI IL-CHO and Compound E were found to reduce proliferation by some [110,124] others found Compound E to enhance proliferation in purified T cell cultures [125] and IL-CHO to have no effect on proliferation [112].

The affects of γSI on *in vitro* T cell activation are also disputed with conflicting results having been reported for IL-2, CD25, and CD69 expression. Some observed repression [55,110,124] while others report instead that Notch engagement represses activation and this repression is overcome by gamma secretase inhibition [125].

Furthermore, γSI treatment of *in vitro* T_H1 culture was reported to reduce IFNγ production, STAT4 phosphorylation, and the expression of Tbet mRNA[112].

To clarify the mechanism by which *in vitro* γSI treatment suppresses T_H1 differentiation, we investigated the modulation of CD4+ T cell activation, proliferation, cytokine expression, transcription factor expression, receptor signaling, and receptor expression in the presence or absence of γSI during T_H1 culture.

Cell Surface Markers of Activation Unaffected

A critical first step required to differentiate naïve T cells into effectors is T cell activation. Impeding T cell activation could be a viable way to reduce active EAE by

inhibiting disease induction or treatment of established disease, as well as reducing adoptive EAE by disrupting activation of naïve host T cells.

To determine if γSI treatment was influencing T_H1 differentiation by disrupting T cell activation, naïve T cells were isolated from the lymph nodes of C57BL/6 mice and stimulated with anti-CD3 to induce activation, either in the presence or absence of γSI for 24 hours. To ascertain CD4+ activation, the expression level of the CD25 and CD69 surface receptors was assessed flow cytometry at the end of culture.

The CD69 receptor is rapidly and transiently expressed on lymphocyte activation, but not detected in resting lymphocytes, [126] and selectively expressed in chronic inflammatory infiltrates and at the sites of active immune responses [127].

CD25 is the alpha chain of the IL-2 receptor. It is a type I transmembrane protein present on activated T cells. CD25 is absent or minimally expressed on resting T and NK cells, but its transcription is potently induced on T cells stimulated via the TCR or with IL-2[128-130].

Twenty-four hours after activation we observed naïve CD4+ T cells to upregulate cell surface expression of both CD25 and CD69. γSI treatment did not have any noticeable effect on T cell activation as determined by percent of cells expressing activation markers or by the average receptor expression level per cell as estimated by mean fluorescence intensity (Figure 15).

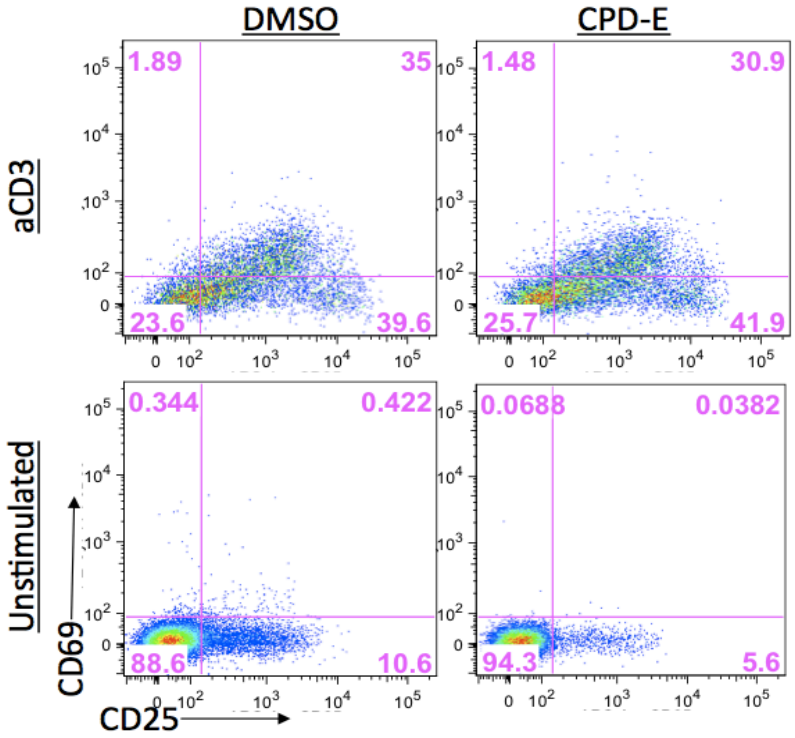

Figure 15. *γSI treatment does not alter CD4+ T cell activation*. Lymph node cells were harvested from a naïve C57BL/6 mouse and treated with Compound-E one hour prior to activation with anti-CD3. CD4+ cells were assessed for early markers of activation after 24 hours of culture.

CD4+ T cell Proliferation

To determine if γSI treatment affects the *in vitro* expansion of T_H1 cells, cell numbers were compared at the end of T_H1 culture using differing methods of activation. CD4+ sorted T cells were activated with anti-CD3 and anti-CD28. Alternately culture of CD25 depleted cells containing both naïve CD4+ T cells and APCs were activated with MBP_{AC1-11}. The number T cell blasts at the end of culture was divided by total number of cells at the beginning of culture to determine the expansion index. γSI treatment enchanced T_H1 expansion in APC free cultures but suppressed T_H1 expansion in cultures contain APCs (Figure 16a). Interestingly, genetic deficiency of PSEN1 in CD4+ T cells enhanced survival *in vivo* (Figure 16b, Appendix 3).

CD4+ IFNγ Production Inhibited

To determine how T_H1 function is affected by γSI we examined IFNγ production *in vitro*. IFNγ is the hallmark cytokine produced by T_H1 cells and is critical for protective immune responses against viral and intracellular bacterial pathogens. IFNγ is crucial to the development of a T_H1 type response, directly promoting B cell isotype switching to IgG2a, and regulation of local leukocyte- endothelial interactions reviewed in [131]. To induce IFNγ production, neuroantigen-specific CD4+ T cells from a mouse carrying a transgenic TCR for MBPac1-11[118] were treated with γSI *in vitro* before activation with their cognate antigen, as specified in the methods section. γSI treatment inhibited IFNγ production in CD4+ T cells as assessed by intracellular flow cytometry (Figure 17). However, this effect was largely overcome when IL-12 or IL-18 were added to culture media (Figure 18a/b).

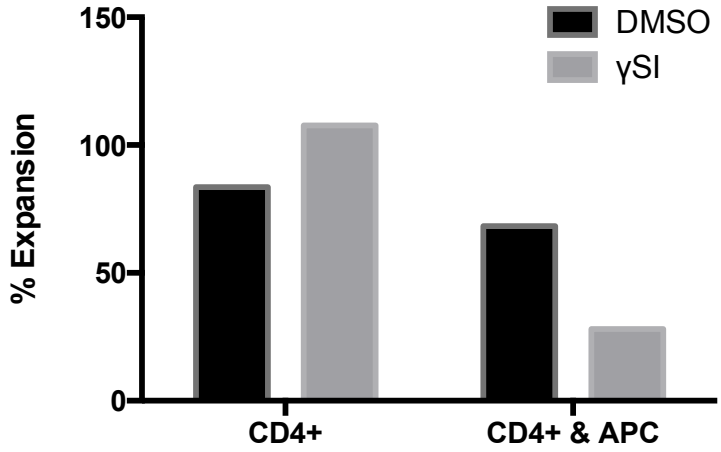

Figure 16a *APC-mediated T_H1 proliferation suppressed by γSI. In vitro* γSI treatment enhanced proliferation of CD4+ sorted T cells activated three days with plate bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (10 μg/ml) but suppressed CD4+ proliferation in CD25 depleted cultures containing CD4+ T cells activated ten days by APCs presenting $MBP_{AC1\text{-}11}$ in MHCII. DAPT and DBZ were used in CD4+ and CD4+ & APC cultures respectively. Percent expansion is calculated as the number of T cell blasts at the end of culture is normalized to the total number of cells at the beginning of culture for each group.

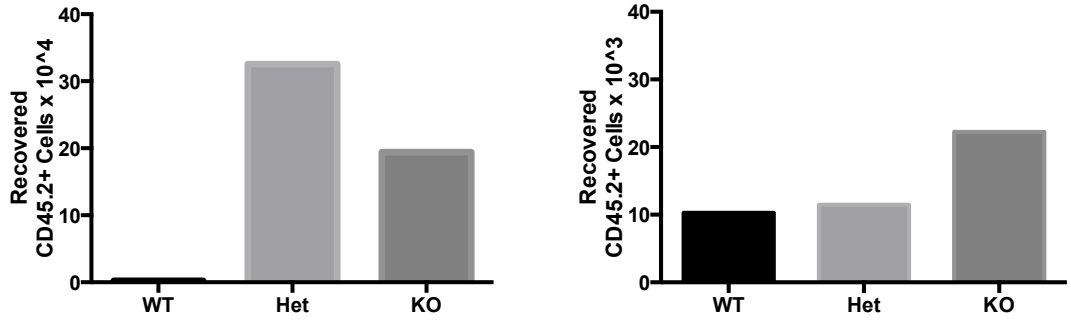

Figure 16b *Genetic PSEN1 deficiency enhances T cell survival*. Naïve CD4+ T cells were harvested from OTII mice in which zero, one or both copies of PSEN1 were disrupted specifically in T cells. 5 x10^6 cells of each genotype were transferred into 1 of 3 naive mice (left panel) or equal numbers of each genotype were pooled and then cotransferred into a single mouse (right panel). Mice were subsequently immunized with 50 μg OVA in CFA. Tens days post immunization draining lymph nodes were harvested and the persistence of transferred cells was calculated by flow cytometry utilizing genotype specific variants of the congenic markers CD45 and CD90.

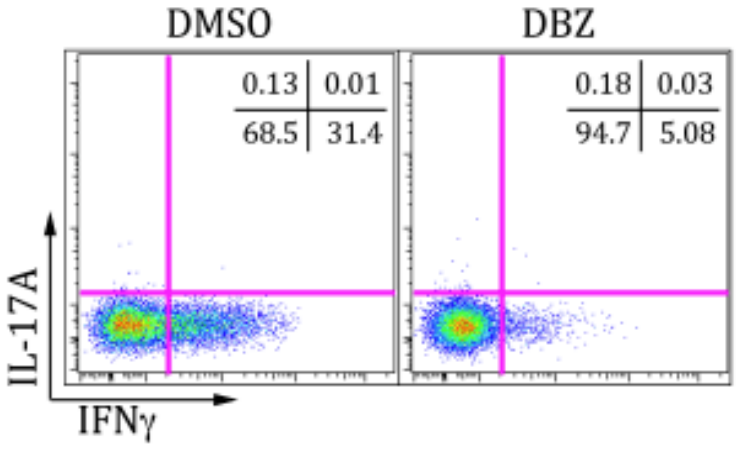

Figure 17 *In vitro γSI treatment inhibits CD4+ IFNγ production*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} in the absence of exogenous skewing cytokines or blocking antibodies.

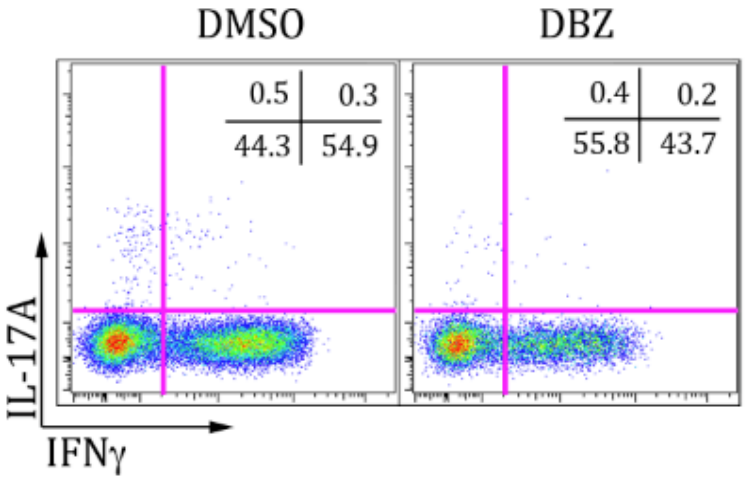

Figure 18a *Exogenous IL-12 lessens γSI mediated IFNγ suppression in vitro*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} in IL-12 supplemented culture media.

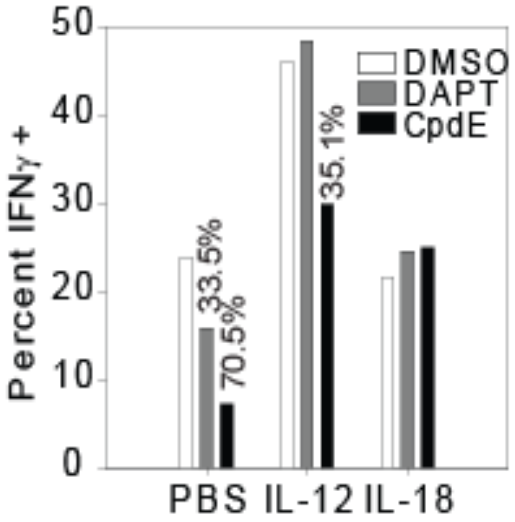

Figure 18b *IL-12 and IL-18 rescue IFNγ production*. Magnetically enriched CD4+ cells from a B6 mouse were activated with αCD3/αCD28 coated, carboxylate-modified latex beads. Cells were treated with γSI and/or T_H1 cytokines. At the end of culture the percentage of IFNγ producing cells was determined by flow cytometry. Percent reduction in IFNγ producers caused by γSI treatment in comparison to DMSO control is shown above individual treatments where applicable.

IFNγ Receptor Signaling Through STAT1 Unaffected

Because IFNγ signaling initiates the process of T_H1 differentiation, STAT1 phosphorylation state was assessed to determine if γSI treatment disrupts IFNγ signaling. The IFNγ receptor is constitutively expressed on the surface of T cells. When the IFNγ receptor encounters its ligand, IFNγ, STAT1 is phosphorylated by the tyrosine kinases (Janus kinase) JAK1 and JAK2. Then pSTAT1 dimerizes and translocates to the nucleus where it binds to the Tbet promoter. Despite reduced levels of IFNγ in γSI treated CD4+ T cells, the STAT1 phosphorylation level in response to IFNγ was unaffected (Figure 19).

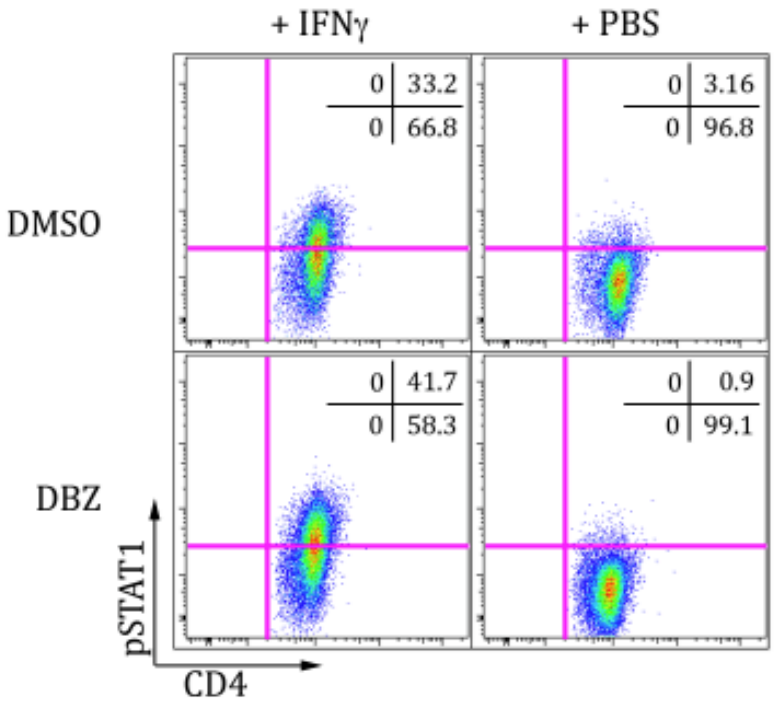

Figure 19 *STAT1 mediated IFNγ signaling is not disrupted by γSI*. Bulk splenocytes were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} in the absence of exogenous skewing cytokines or blocking antibodies. After 3 days of culture cells were rested in fresh medium for 30 minutes then stimulated with IFNγ or PBS for 30 minutes.

γSI Effects on Tbet Expression

To determine how γSI suppresses IFNγ production, expression of the transcription factor Tbet was investigated. Tbet has been identified as a key transcription factor for the development of T_H1 cells and the induction of IFNγ production and IL-12Rβ2 [132] [133]. To determine the kinetics of Tbet expression CD4+ T cells were activated and Tbet levels were monitored for 3 days using intracellular flow cytometry. The majority of T cells were Tbet positive 72 hours post activation (Figure 20). To determine if γSI was altering Tbet expression, lymph node cells and splenocytes were treated with γSI or DMSO control, then activated with MBP_{AC1-11}. Tbet expression was highest 72 hours post activation and γSI treatment reduced both the percentage of cells expressing Tbet and the average level of Tbet expression in Tbet+ cells. By five days post activation Tbet expression had returned to basal levels in lymphocytes and was largely reduced in splenocytes. However, both groups were still found to express elevated levels of IL-2Rα (CD25) (Figure 21). Interestingly, cells that failed to express high levels of Tbet in primary cultures containing γSI also showed reduced levels of Tbet expression when strongly restimulated with anti-CD3 (Figure 22).

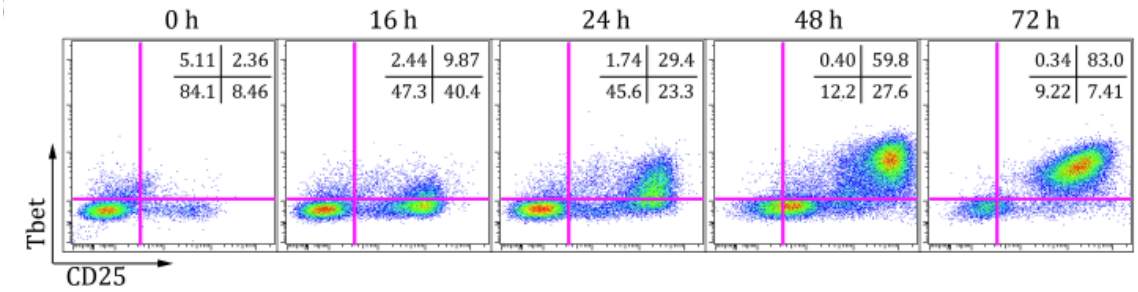

Figure 20 *T_H1 Tbet expression kinetics*. Naïve CD4+ T cells were isolated from $MBP_{AC1\text{-}11}$-specific TCR transgenic mice and activated with $MBP_{AC1\text{-}11}$. The majority of CD4+ cells upregulated both CD25 and Tbet 72 hours post activation.

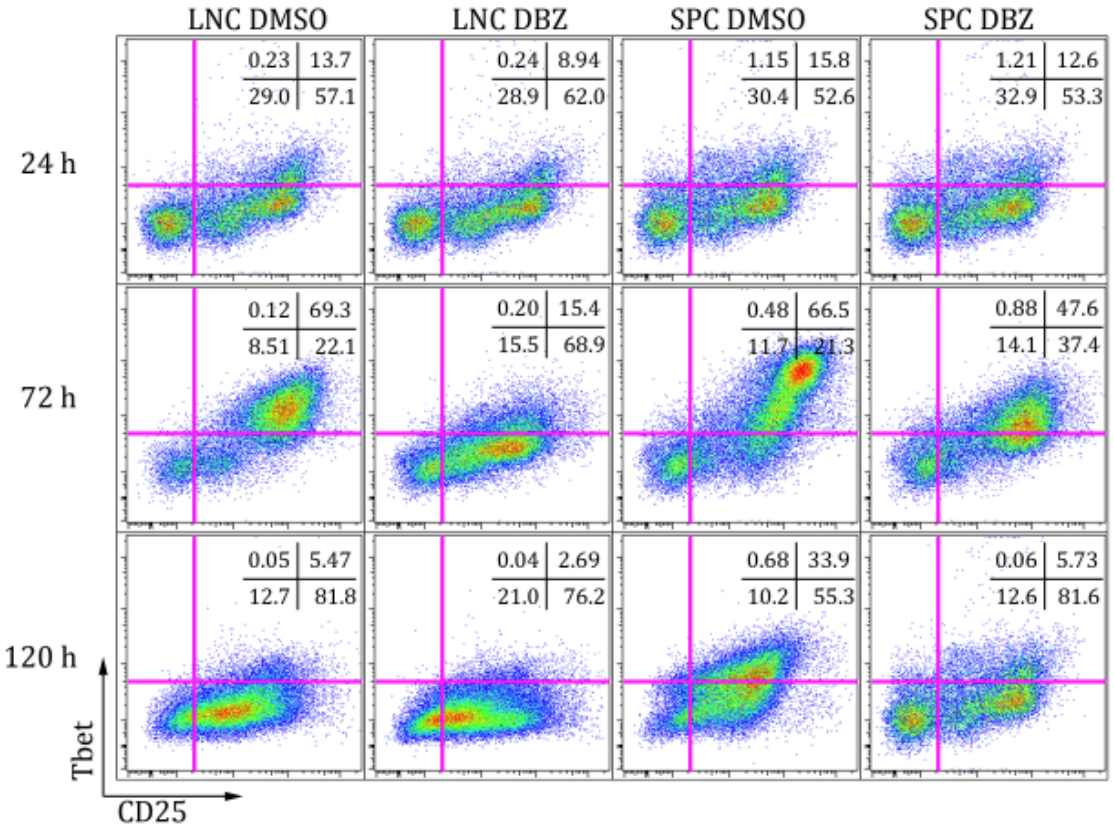

Figure 21 *In vitro γSI treatment suppresses Tbet expression*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice and activated with MBP_{AC1-11} with IL-12. Tbet expression peaked at 72 hours and subsided by 120 hours post activation. γSI treatment suppressed te activation induced upregulation of Tbet in both lymph node cells (LNC) and splenocytes (SPC).

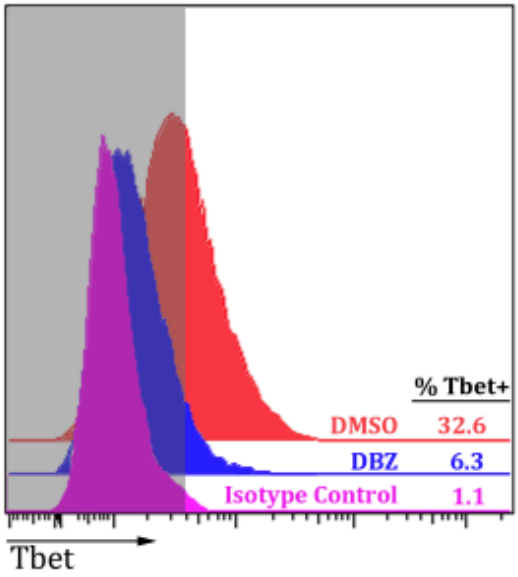

Figure 22 *In vitro γSI treatment suppresses Tbet during 2° activation*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, treated with γSI or control, and activated with MBP_{AC1-11}. After 3 days of activation cells were rested in fresh media containing IL-2 for 2 days then reactivated with MBP_{AC1-11}. Tbet expression of CD4+, CD25+ cells was assessed 3 days after reactivation. γSI or control was added to cultures daily.

γSI Effects on IL-12 Signaling

γSI Treatment Impedes IL-12 Receptor Expression

The IL-12 cytokine is produced by dendritic cells[134] and macrophages in response to antigen stimulation and is involved in differentiation of naïve T cells into T_H1 cells[135]. The IL-12 receptor contains two subunits IL-12Rβ1 and IL-12Rβ2. IL-12Rβ1 has a low affinity for IL-12 and is expressed constituently on T cells. On the cell surface IL-12Rβ1 dimerizes with IL-12Rβ2 to form the high affinity IL-12 receptor. IL-12Rβ2 is not expressed on naïve, resting, T cells but is induced upon antigen activation through the T cell receptor [136]. When naïve T cells were activated in the presence of γSI they failed to express IL-12Rβ2 (Figure 23)

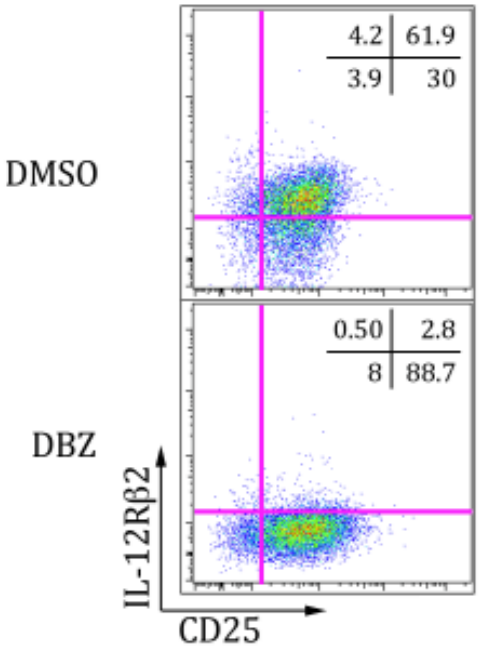

Figure 23 *In vitro γSI treatment suppresses CD4+ expression of IL-12 receptor*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, treated with γSI or control, then activated with MBP_{AC1-11} for three days. γSI treated CD4+CD25+ cells failed to express IL-12Rβ2.

γSI Treatment Prevents IL-12 Signaling via STAT4

Phosphorylation of the transcription factor STAT4 in response to IL-12 signaling in CD4+ cells is required for IFNγ production [137,138]. In addition to directly inducing IFNγ, STAT4 contributes to T_H1 differentiation by inducing Tbet expression [139]. To determine the impact of γSI treatment on STAT4 signaling, naïve CD4+ T cells treated with γSI during activation were washed and rested in fresh media and then stimulated with IL-12. As compared to an unstimulated control, DMSO cultured T cells showed an increase in the percentage of cells containing pSTAT4 and an increase in the MFI of pSTAT4 staining. By comparison, γSI-treated cells showed a marked reduction in the percentage of pSTAT4 positive cells (Figure 24 left side) and only a modest increase in MFI (Figure 24 right side). Thus, it appears that IL-12 induced STAT4 phosphorylation is reduced in cells cultured in the presence of γSI.

Summary

Here we have shown *in vitro* γSI treatment of naïve CD4+ T cells suppresses T_H1 effector differentiation. While markers of activation were largely unchanged the hallmark T_H1 transcription factor Tbet and cytokine IFNγ were reduced. IFNγ signaling through phosphorylation of STAT1 was unaffected by γSI treatment suggesting the IFNγ receptor is likewise unaffected. Additionally, IL-12 receptor expression was suppressed and IL-12 signaling through phosphorylation of STAT4 was reduced. However exogenous IL-12 was able to alleviate γSI suppression of IFNγ, suggesting some level of IL-12 signaling persists. To determine if γSI

modulation of T_H1 differentiation solely accounts for the observed suppression of active EAE by γSI treatment, we examined the effects of γSI on other CD4+ T cell subsets involved in EAE pathogenesis, particularly T_H17 effectors.

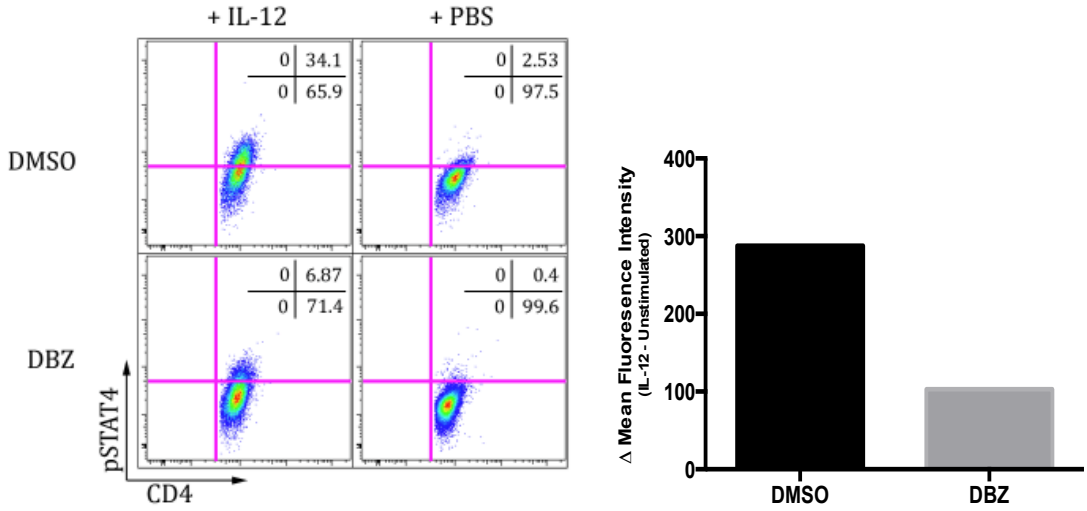

Figure 24. *In vitro γSI treatment prevents STAT4 mediated IL-12 signaling*. Bulk splenocytes were isolated from $MBP_{AC1\text{-}11}$-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with $MBP_{AC1\text{-}11}$ in the absence of exogenous skewing cytokines or blocking antibodies. After 3 days of culture cells were rested in fresh medium for 30 minutes then stimulated with IL-12 or PBS for 30 minutes. Phosphorylation state of STAT4 in CD4+ cells was assessed via flow cytometry. Percentage of CD4+ pSTAT4+ cells of IL-12 stimulated versus PBS control (flow plots on the left), change in pSTAT4-PE mean fluorescence intensity of IL-12 stimulated versus PBS control (bar graph on the right).

SECTION 4: IMPACT OF γSI ON T_H17/T_{reg} DIFFERENTIATION

In vivo γSI Treatment Inhibits T_H17 Differentiation

Given the importance of T_H17 T cells in EAE, we speculated that one way in which γSI impacted EAE was by inhibiting the differentiation of T_H17 effector T cells. Previously, *in vitro* γSI treatment has been shown to reduce IL-17A, IL-17F, and IL-21 in naïve CD4+ mouse cells treated with IL-CHO and to reduce IL-17A, IL-17F, and IL-22 in naïve CD4+ human cells treated with Compound E as measured by ELISA[114]. Additionally, IL-17 and RORγt mRNA expression as well as CSL-mediated notch binding to their respective promoters was found to be decreased with *in vitro* γSI treatment. Furthermore splenocytes but not CNS infiltrating cells from SJL/J mice fed γSI (LY-(411,575)) and immunized with $PLP_{139-151}$ had reduced production of IFNγ and IL-17 in response to recall activation. However a trend toward increased CD4+ and decreased CD8+ infiltration into the CNS was observed.[114]

When Eagar Lab members immunized C57BL/6 mice with MOG_{1-125} and then treated them with γSI or DMSO *in vivo*, the γSI treated group experienced a much less severe disease course (Figure 4). Upon restimulation with MOG_{1-125}, cells from γSI treated mice had decreased production of T_H17 associated cytokines IL-17 (Figure 25) and IL-6 (Figure 26) as measured by ELISA of cell culture supernatants.

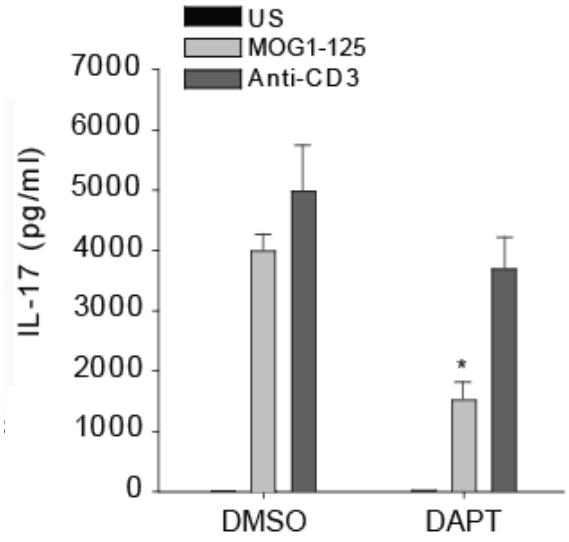

Figure 25 *In vivo γSI treatment reduces recall IL-17 production*. Splenocytes recovered from $MOG_{1\text{-}125}$ immunized C57BL/6 mice were and restimulated with $MOG_{1\text{-}125}$. ELISA of cell culture supernatants revealed a decrease in IL-17 production from MOG-specific cells.

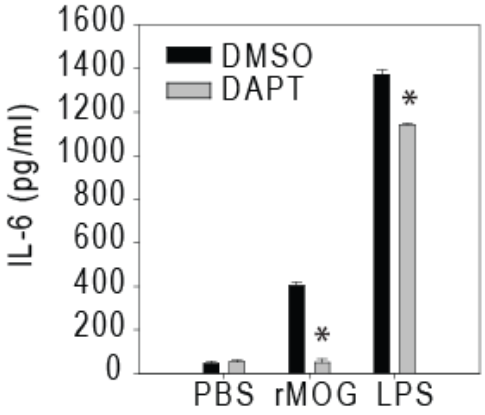

Figure 26 *In vivo γSI treatment reduces recall IL-6 production*. Lymph node cells recovered from C57BL/6 mice immunized with recombinant MOG protein (rMOG) and treated with in vivo with γSI or DMSO were restimulated with rMOG or LPS. ELISA of cell culture supernatants revealed a decrease in IL-6 production.

In vitro γSI Treatment Inhibits T_H17 Differentiation

To determine if γSI disrupted $T_H17$differentiation *in vitro*, lymph node cells were isolated from naïve mice transgenic for TCR specific for MBP_{ac1-11}. CD25+ cells were then removed by magnetic separation to eliminate any previously activated effector T cells or T regulatory cells from the culture. Cells were then treated with γSI and activated with MBP_{ac1-11} along with IL-6 and TGFβ to promote T_H17 differentiation. IL-23 was also added during culture to promote T_H17 expansion. Once cells had fully divided out and reached a resting state they were reactivated with anti-CD3 for three days and examined using flow cytometry.

CD4+ T cell Activation Unaffected

To determine if γSI influenced *in vitro* activation of T_H17 cells, the expression levels of CD25 and CD40 ligand (CD40L or CD154) were assessed. CD40L is a type II transmembrane protein of the TNF superfamily that serves as a marker of acute activation transiently expressed by activated T cells and activated B cells, as well as a variety of cells in the innate arm of the immune system [140]. CD40L binding to CD40 promotes dendritic cell cytokine production, expression of costimulatory molecules, and cross-presentation of antigen [141]. Additionally, CD40 signaling in B cells promotes germinal center formation, immunoglobulin isotype switching, somatic hypermutation, and the formation of long-lived plasma cells and memory B cells [142]. It has been shown that the CD40 pathway is essential for the survival of many cell types including germinal center B cells, DCs, and endothelial cells under normal and inflammatory conditions [143]. No reduction in the percentage of CD4+ T

cells positive for the activation markers CD25 and CD40L was observed with γSI treatment (Figure 27 left panel).

IL-17 Production Suppressed

To determine if γSI treatment affected T_H17 differentiation *in vitro* we examined the production of IL-17A, the signature cytokine produced by T_H17 cells. IL-17A (often referred to as just IL-17) is a pro-inflammatory cytokine that acts to induce neutrophil recruitment and inflammation [144,145]. IL-17A also plays an essential role in host defense against many microbial infections and is implicated in various autoimmune diseases [146-150]. When naive T cells were treated with γSI during *in vitro* T_H17 differentiation, IL-17A production was inhibited (Figure 27 right panel).

T_H17 Expansion Suppressed

As was observed with T_H1 T cells, γSI treatment resulted in a reduction in the extent of T_H17 cell expansion (Figure 28).

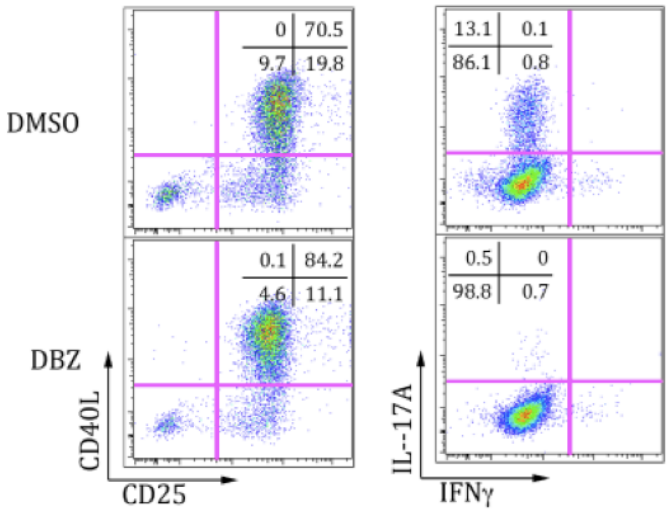

Figure 27 *In vitro γSI treatment suppresses IL-17 production in CD4+ cells*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} in T_H17 favoring medium containing IL-6, TGFβ and blocking antibodies against INFγ and IL-4. γSI treated CD4+ cells displayed no reduction in surface activation markers (left panel) but an absence of IL-17A production (right panel).

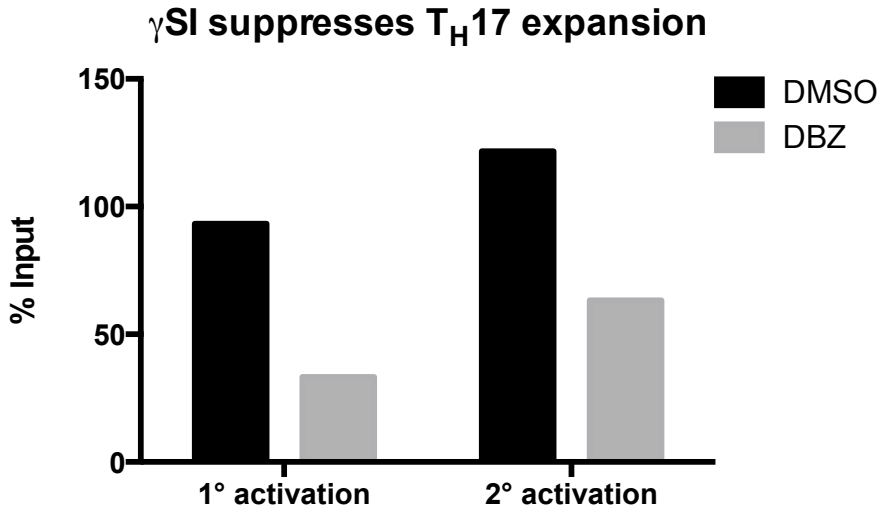

Figure 28 *γSI suppresses T_H17 expansion*. CD25 depleted splenocytes were cultured in T_H17 conditions for adoptive EAE. γSI treatment suppressed T_H17 expansion during primary activation with $MBP_{AC1-11.}$ The number of cells in both groups increased from the end of primary actication to the end of secondary activation. However, relative suppression of γSI treated cells in comparison to DMSO control persisted through secondary activation with plate-bound anti-CD3 and anti-CD28.

RORgt Expression Suppressed

To determine the cause of IL-17 reduction in γSI treated T_H17 cultures, retinoic-acid-receptor-related orphan nuclear hormone receptor gamma t (RORγt) expression was examined. RORγt is a member of the ROR family [151] that is exclusively expressed in cells of the immune system [152]. RORγt binds ROR response elements in conserved non-coding regions of the *IL-17* promoter to induce transcription [153,154]. RORγt along with RORα are master regulators that drive T_H17 cell lineage differentiation. RORα is mostly redundant to RORγt in terms of expression and most functions [155]. T cells lacking both RORγt and RORα are unable to differentiate into T_H17 cells [153]. γSI treatment of T_H17 cell cultures reduced RORγt expression in CD4+ CD25+ cells (Figure 29 and Figure 32)

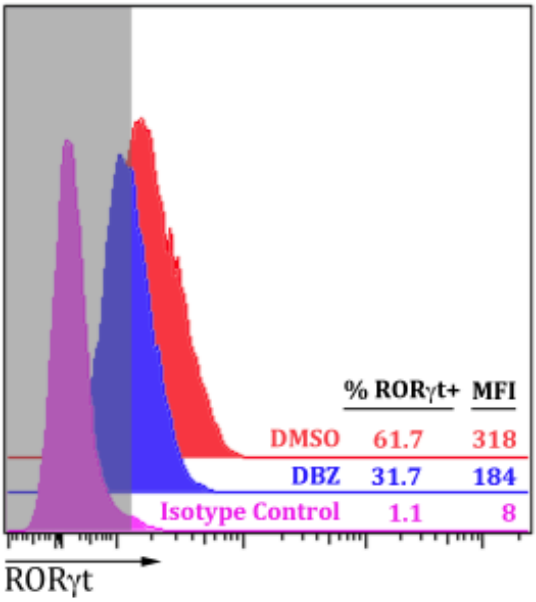

Figure 29 *In vitro γSI treatment suppresses RORγt expression in CD4+ cells*. Naïve CD4+ T cells were isolated from $MBP_{AC1\text{-}11}$-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with $MBP_{AC1\text{-}11}$ in T_H17 favoring medium containing IL-6, TGFβ and blocking antibodies against INFγ and IL-4. γSI treatment resulted in a decrease in the percentage of CD4+ T cells expressing RORγt+ as well as a decrease in average RORγt expression per cell as measured by mean fluorescence intensity (MFI) CD4+.

IL-23 Receptor Signaling Through STAT3 Inhibited

Expression of RORγt is facilitated by cytokine-mediated activation of the signal transducer and activator of transcription 3 (STAT3) [155-158]. STAT3 is a transcription factor that serves critical functions in development, cell growth, and homeostasis in a variety of tissues [159,160]. STAT3 also plays a critical role in the differentiation of CD4+ T cells into T_H17 cells [161-165] by facilitating the expression of IL-17 [154] and IL-23R [158,166]. T_H17 associated cytokines IL-6, IL-21 and IL-23 activate STAT3 and promote immunity against extracellular bacteria and fungi [157,167-169]. To determine if disruption of cytokine signaling was the basis for γSI mediated reduction of RORγt, STAT3 phosphorylation in response to IL-6 and IL-23 was assessed. IL-6 mediated phosphorylation of STAT3 was intact in γSI treated cells (Figure 30 left panel) but IL-23 mediated phosphorylation was abrogated (Figure 30 right panel), suggesting that γSI is affecting the IL-23 receptor.

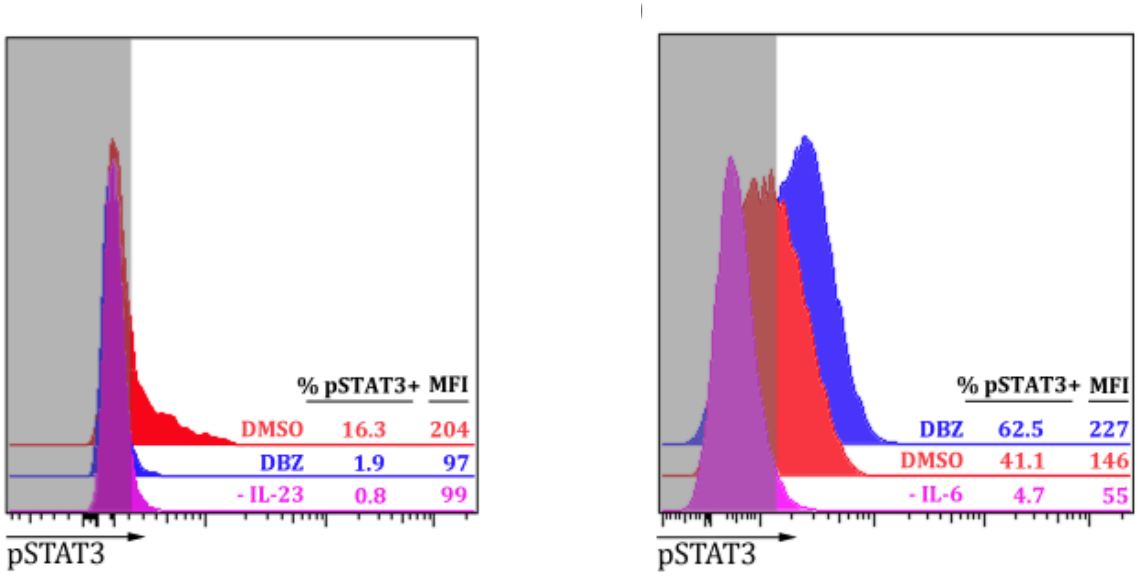

Figure 30 *In vitro γSI treatment interferes with IL-23 but not IL-6 signaling*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} under conditions favoring T_H17 differentiation. After 3 days of culture cells were rested in fresh medium for 30 minutes then stimulated with IL-6 (left panel) or IL-23 (right panel) for 30 minutes. Controls receiving PBS containing no cytokines are labeled as –IL-6 and –IL-23 respectively. Phosphorylation state of STAT3 in CD4+ cells was assessed via flow cytometry.

Adoptive T_H17 EAE Suppressed

Because γSI treatment suppressed T_H17 differentiation *in vitro*, we then transferred *in vitro* γSI-treated, T_H17-pushed, T cells into naïve mice to determine if their ability to cause EAE was also diminished. Mice that received control treated T_H17 cells experienced acute and very severe EAE whereas mice that received γSI treated cells displayed very mild symptoms or no EAE whatsoever (Figure 31).

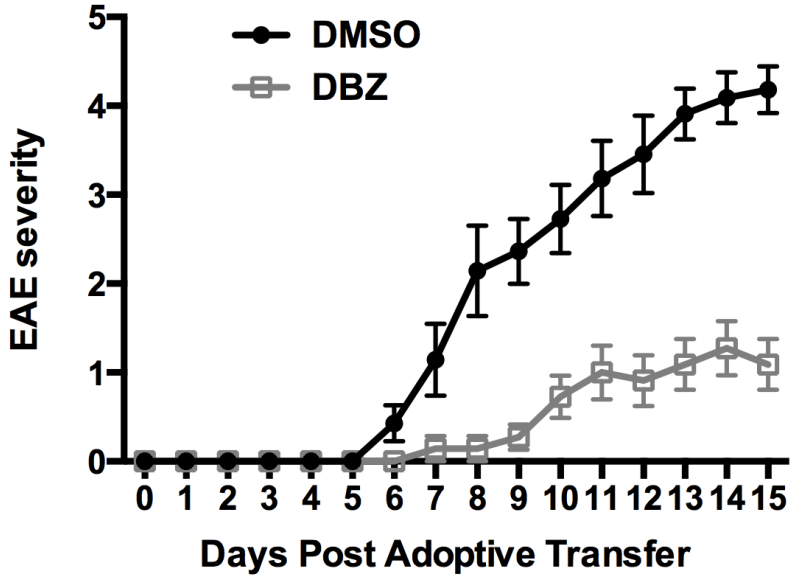

Figure 31 *In vitro γSI treatment during T_H17 differentiation impairs adoptive transfer of EAE*. CD25 depleted bulk splenocytes and lymph nodes cells were isolated from $MBP_{AC1\text{-}11}$-specific TCR transgenic mice, pre-incubated with γSI or control for 30 minutes then activated with $MBP_{AC1\text{-}11}$ in media favoring T_H17 differentiation for 10 days. Depicted are the pooled results of two separate experiments with a combined n=11 for each treatment group and a p-value of 0.0366.

In vitro γSI Treatment Promotes FoxP3 Expression

Proportion of FoxP3+ Cells Increased

In our models, γSI prevented T_H1 and T_H17 effector differentiation but it did not alter T cell activation. We next sought to determine whether γSI treatment prevented T_H differentiation or if it would redirect T cells to adopt an anti-inflammatory or T_{reg} phenotype. A previous study investigating the affect of gamma secretase inhibition reported that IL-CHO reduced FoxP3 expression in C57BL/6 cells and that the suppressive ability of these induced T_{regs} diminished. However feeding mice chow containing γSI (LY-441,575) did not affect Foxp3 expression in nTegs[170].

Because treatment of naïve CD4+ T cells with γSI inhibited the differentiation of IL-17A producing T_H17 cells, the expression of Foxp3 was assessed to determine if γSI treatment is pushing these cells down the alternative path toward T_{reg} differentiation. CD25+ cells had been depleted prior to activation to cell culture so Foxp3+ cells represent newly induced iT_{regs} and not T_{regs} expanded from previously generated nT_{regs} or iT_{regs}. Indeed when γSI is included in T_H17 culture, the Foxp3+ population is greatly enhanced while RORγt expression in Foxp3- cells is curtailed (Figure 32 left side). Surprisingly γSI treatment also induced Foxp3+ cell generation by ten-fold in T_H1 differentiation cultures where no exogenous TGFβ is added (Figure 32 right side). This suggests that γSI treatment may bypass the need for TGFβ signaling or induce the production of TGFβ from APCs or T cells.

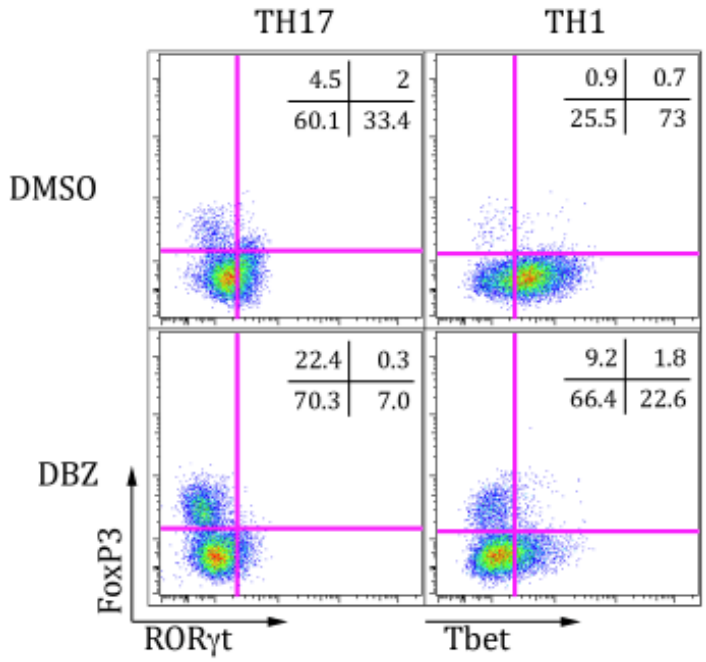

Figure 32 *In vitro γSI treatment induces FoxP3 expression*. Naïve CD4+ T cells were isolated from MBP_{AC1-11}-specific TCR transgenic mice, incubated with γSI or control for 30 minutes, then activated with MBP_{AC1-11} under conditions favoring T_H17 or T_H1 differentiation for 8 days. γSI treatment reduced expression of proinflammatory transcription factors RORγt and Tbet in both T_H17 and T_H1 cultures respectively while drastically increasing the percentage of cells expressing the pro-regulatory transcription factor FoxP3 in both groups. Gated on CD4+ CD25+

IL-2 Production Increased

TGF-β-induced expression of FoxP3 is strictly dependent on the cytokine IL-2 but once FoxP3 is induced other cytokines maintain this expression [171,172]. However T_{reg} cells do not produce IL- 2. In contrast to naive T cells, the IL- 2 proximal promoter in T_{reg} cells does not undergo chromatin remodeling upon TCR activation [173]. CD4+ and CD8+ T cells are the primary source of tissue IL-2 as IL-2 is rapidly and transiently produced upon engaging the TCR and costimulatory molecules such as CD28 on naive T cells [174-176]. Activated dendritic cells (DCs), natural killer (NK) cells, and NKT cells can also produce IL-2 [177-179].

To determine if T cell IL-2 production is altered by γSI, naïve T cells were activated for three days under T_H1 conditions and treated with DBZ, DAPT or DMSO control. At the end of culture cells were reactivated and IL-2 production was measured via intracellular flow cytometry. γSI treated cells displayed an increased capacity for IL-2 production (Figure 33). Increased IL-2 production may support the generation of Foxp3+ T_{regs} which are unable to produce IL-2 themselves.

Summary

These results demonstrate that the effects of γSI treatment are not restricted to suppression of T_H1 effectors. When restimulated *in vitro*, cells recovered from mice treated with γSI *in vivo* showed reductions in T_H17 associated IL-17A and IL-6 cytokines. Markers of T cell activation were not reduced in T_H17 cells with γSI treatment, but a possible effect on proliferation or survival was observed. Expression of the hallmark T_H17 transcription factor RORγt was reduced with γSI

treatment of CD4+ T cells, as was IL-23 responsiveness measured by STAT3 phosphorylation. However the ability of IL-6 to signal via pSTAT3 remained intact, suggesting IL-23 receptor expression may be affected. Additionally, the percentage of cells expressing of the hallmark T_{reg} transcription factor FoxP3 was noticeably increased in both T_H1 and T_H17 differentiation cultures after treatment with γSI. Treatment with γSI also increased the percentage of T cells expressing IL-2, a cytokine necessary for T cell survival, possibly sustaining the increased population of FoxP3+ cells, as T_{reg} cells are unable to produce their own IL-2.

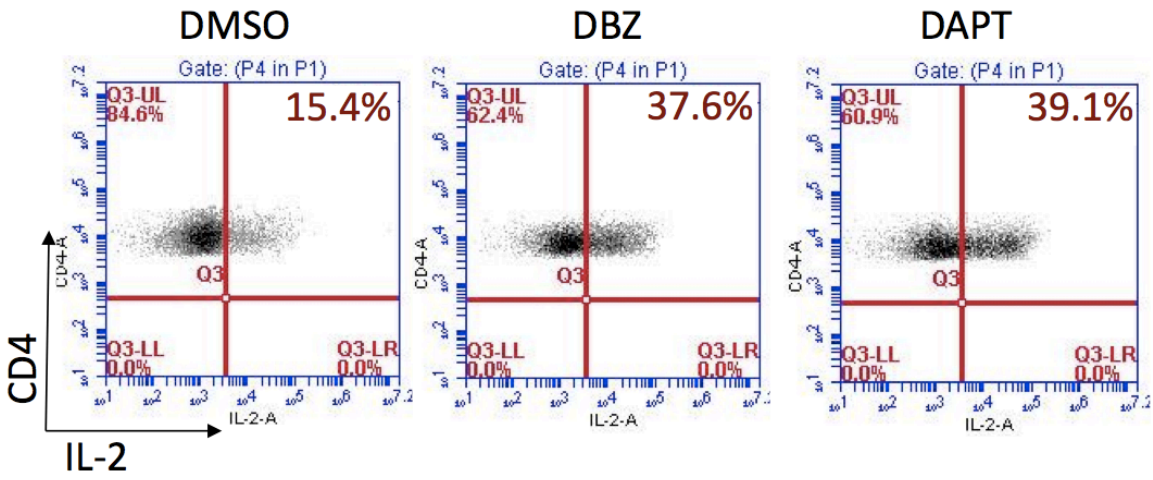

Figure 33: *γSI treatment enhances IL-2 production*. Naïve splenocytes were activated with aCD3 for 3 days and treated with γSI then restimulated and stained for IL-2 production. Both DBZ and DAPT treated CD4+ T cells showed a substantial increase in IL-2 produciton.

CHAPTER FOUR

Discussion

γSI as a possible MS Therapy

MS is a multi-factorial disease comprised of both genetic and environmental factors. A disease such as MS is difficult to cure, or even manage, as no single therapy is likely to be effective in a majority of patients. Due to this, a number of therapeutics have been developed to slow disease progression in MS. While some therapies like glucocorticoid treatment are general immuno-suppressants, even highly targeted MS therapeutics achieve efficacy via generalized mechanisms such as the prevention of immune surveillance in the brain with Natalizumab, or the depletion of an entire arm of the adaptive immune system as with off-label use of Rituximab. Serious side effects resulting from new infections or reactivation of latent viruses are a major concern with these therapies. For these reasons, methods that work to restore tolerance by downregulating the function of T_H1 or T_H17 effectors while increasing the balance of T_{regs} would ultimately be the most beneficial. Our studies have shown that gamma secretase inhibition is capable of producing these effects on T cells *in vitro*, and that *in vivo* γSI treatment suppresses the severity of EAE, the mouse model of MS. Therefore, pharmacologic inhibition of gamma secretase is intriguing as a possible MS therapeutic.

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γSI Suppression of EAE

The EAE models I used suggest that the role of GS in EAE and MS is more complicated than I originally expected when starting this project.

Adoptive EAE

The adoptive transfer model of EAE is useful in determining efficacy of treatment on established disease as fully differentiated effectors are usually transferred. This model is also useful in dissecting the impact of treatment on T_H1 and T_H17 subsets individually.

Uniquely we demonstrated γSI treatment suppresses adoptive EAE. The treatment of neuroantigen-specific T cells during *in vitro* T_H17 differentiation prior to transfer was able to suppress disease. We also found that *in vivo* γSI treatment post-transfer of fully differentiated T_H1 effectors suppressed EAE. These treatments are likely modulating disease via different mechanisms as the sensitivity of naïve T cells to γSI-mediated suppression disappears by 4 days post activation [112,114]. Indeed, when we used congenic markers to track adoptively transferred cells during *in vivo* γSI treatment, a differential response between donor and recipient cells was observed. Proliferation of donor cells was unchanged but their survival *in vivo* appeared to be decreased whereas cells from the recipient exhibited increased proliferation in response to antigen restimulation.

Active EAE

The immunization model is useful in dissecting effects on factors are required for the *generation* of an immune response including non-t cell factors such as antigen presentation and costimulation provided by antigen presenting cells.

We observed *in vivo* γSI treatment reduced EAE in a C57B6/J mice immunized with MOG_{1-125}. We found a reduction of IFNγ and IL-17 cytokine production in cells recovered from *in vivo* treatment groups upon recall activation. MOG_{1-125} represents the extracellular domain of the MOG protein and it was grown in insect cells to produce the proper glycosylation. Interestingly, B cell depletion with Ritximab in mice carrying a humanized CD20 was also able to reduce EAE severity in rMOG immunized mice[180,181]. In contrast to the MOG_{1-125} model, B cell depletion in EAE models induced by MOG_{35-55} have increased EAE severity. Interestingly, these experiments demonstrated an anti-inflammatory role for a regulatory B cell subset that produces IL-10[180]. Similarly, when we immunized mice with short T cell epitopes of MOG or PLP, EAE severity was not reduced with γSI treatment. In fact, we observed disease trended toward more severe when γSI treated mice were immunized with MOG_{35-55} or $PLP_{178-191}$. Additionally, we observed a decrease in lymph node B cells with γSI treatment further supporting the role of B cells in γSI mediated EAE suppression.

Model of γSI treatment on EAE

Our studies demonstrated γSI treatment was effective in reducing the severity of acute autoimmune demyelination. Based on previous published data using the MOG_{1-125} model of EAE it seems likely that disease suppression was a result of γSI effects on B cells. Additional support for the view that γSI can indirectly impact T cell differentiation by altering APC function can also be seen in our *in vitro* activation and T_H1 differentiation models where γSI had distinct effects on T cells depending on whether they were purified or cultured together with APCs.

However, there is some evidence suggesting γSI acts on T cells directly as NICD is recruited to CSL binding DNA at the promoters of IFNγ[182], Tbet[112], RORγt and IL-17A[114]. Additionally, antibody blockade of DLL4 was found to reduce MOG_{35-55}-induced EAE severity[117]. We have also shown that γSI can suppress IFNγ production in purified T cell cultures *in vitro* as well as in the *in vivo* model of adoptive EAE, a model in which B cells are not known to play a role. It will be interesting to examine the functions of B cells in more depth in this model.

Genetic PSEN deficiency

T cell-specific gene deletion can be accomplished using CD4-cre and a floxed gene of interest. Our studies examined the effect of PSEN1 deficiency on EAE. One of the known substrates of PSEN is notch and notch expression is upregulated during T cell activation. The extent of notch signaling can be assessed by expression

of HES-1, a downstream target of notch engagement. Using HES-1 expression as a marker for PSEN activity, we determined that knockout of a single allele of PSEN1 reduced enzymatic activity to levels similar to that of unactivated T cells. However when mice with a conditional PSEN1 deficiency in CD4+ cells were immunized with MOG_{35-55} no difference in EAE severity was observed. The lack of phenotype we observed may be a result of gene copy number, MOG peptide immunization or a combination of the two.

γSI treatment on Immune Activation

A hallmark of the adaptive immune system is the generation of diverse immune receptors by T cells for the anticipated encounter with rapidly evolving pathogens. T cells rapidly proliferate upon activation through the TCR as well as costimulation provided by molecules upregulated on APCs in response to inflammatory stimuli. These activation signals are necessary for the differentiation of naïve CD4+ T cells into effector T cell subsets [183]. T effector subsets are characterized by the production of specific cytokines and effector functions [30,31]. The polarization of naïve T cells to a particular effector subset is generally determined by the local cytokine milieu and composition of the innate immune cell populations present during initial activation[184].

Initial reports of γSI treatment on T cell activation, the time when T cells become sensitive to skewing conditions, yielded conflicting results. Some reported that antibody-mediated CD3 and CD28 T cell proliferation was reduced with γSI

treatment [110,124] whereas others reported notch signaling actually suppressed antibody-stimulated T cell proliferation and that γSI treatment relieved this suppression resulting in increased proliferation [125]. Eagar et al. 2004 also reported that when APCs were included in culture, γSI treatment suppressed proliferation.

We also observed differential effects of γSI on in vitro T cell cultures depending upon whether TCR and costimulatory signals were induced by antibodies or by APC-displayed peptide:MHC complex. Our studies found that in the absence of APC costimulation, γSI treatment enhanced T cell proliferation. Conversely, in unsorted splenocyte cultures, γSI treatment reduced proliferation of TCR-transgenic T cells activated by MBP_{AC1-11} loaded APCs, supporting previous observations by Eagar et al. 2004.

This points to the fact that GS-sensitive signals are being delivered by APCs which may differ from T cell to T cell signals. It has recently been shown that Notch signaling increases the longevity of activated T helpers [185]. Therefore the mechanism by which γSI enhances T cell proliferation in the absence of APCs remains unclear, and may not be notch mediated.

On a molecular level, T cell activation is regulated by duration and quantity of TCR-ligand engagement [186]. APC presentation of fewer than ten MHC-peptide complexes are able to form a immunological synapse capable of activating T cells [187]. Both the formation and the maintenance of the immunological synapse are dependent on TCR engagement and TCR-mediated signaling[188]. Ligand-driven formation of TCR clusters is required for effective activation [189], with a prolonged

exchange of activation signals at the immune synapse necessary to produce the full effector response in terms of proliferation and cytokine release of the CD4+ T-cells [188]. Costimulation by APCs is critical as 10 to 50 times more antigen is required to activate T-cells to the same level without coreceptor engagement[187,190]. In artificial systems of T cell activation ~100-1000 fold the amount of antibody to CD3 is required for equivalent activation as to when APCs are included [186]. Many studies examining the effect of γSI treatment on T helper differentiation use antibodies to CD3 and CD28 to activate naïve, CD4+ sorted, T cells *in vitro.* In contrast, for our studies we activated neuroantigen-specific TCR-transgenic T cells with peptide:MHCII displayed by APCs. These distinct methods of activation are not trivial in regards to T cell activation and proliferation as discussed previously. As such, this difference should be considered when comparing our results to those reported by other groups.

γSI suppresses T_H1 differentiation in vitro

The T_H1 and T_H2 effector subsets were the first to described[30] and both have subsequently been well characterized. The principle function of T_H1 cells is in promoting phagocyte-mediated defense against infections, particularly intracellular microbes, while T_H2 cells promote IgE production as well as eosinophil and mast cell-mediated immune reactions against helminthic infections. T_H2 activation by non-pathogenic material manifests as food and environmental allergies, whereas inappropriate T_H1 responses can develop to self-antigens, resulting in organ-specific

autoimmune pathogenesis. EAE was first described as a T_H1-mediated autoimmune disease[191], leading us to examine the effects of γSI inhibition on T_H1 differentiation.

Interestingly, Ong et al. 2008 reported that conditional knockout of both PSEN1 and PSEN2 in CD4+ T cells did not affect Tbet expression[192]. However, our studies using γSIs to inhibit PSEN function demonstrated a clear suppression of multiple aspects of the T_H1 differentiation program, which may be due to the effects of γSI on APCs included in differentiation cultures. We found γSI treatment reduced surface expression of IL-12Rβ2, levels of intracellular IFNγ, Tbet, and pSTAT4. These results support and expand upon previous results reported by Minter et al., 2005. Particularly notable is the suppression we observed in IL-12Rβ2 expression, as IL-12 signaling is required for stabilization of the T_H1 program [193].

γSI suppression of T_H17 differentiation

EAE was initially described as a T_H1 disease, but in 2005 the T_H17 linage was first described[31,32] and quickly proved to be a major factor in a number of autoimmune diseases. In contrast to IFNγ knockout mice where EAE severity increases, IL-17A ablation results in reduced EAE severity and improved recovery[52].

In addition to T_H1 suppression we also found γSI treatment suppresses many factors required in T_H17 differentiation. We observed that γSI treatment lowered expression of RORγt, intracellular IL-17A, and IL-23 induced pSTAT3. These results support and expand upon a previous study by Keerthivasan et al., 2011. Uniquely we found that IL-23 signaling through STAT3 phosphorylation was disrupted by γSI

treatment. It seems likely that γSI prevents the upregulation of the IL-23R subunit, which in conjunction with IL-12Rα forms the IL-23 receptor. Unfortunately we were unable to confirm IL-23R surface expression on γSI treated T_H17 cells by flow cytometry due to the quality and availability of antibodies against the murine protein. TGFβ is a common cytokine in differentiation of T_H17 and T_{reg} cells, and it has been proposed that the balance between T_{reg} and T_H17 generation in the periphery is controlled by local levels of IL-2 and IL-6 as reviewed in Dardalhon et al. 2008[194]. Some of our earliest results showed γSI treatment led to increased IL-2 production (Figure 33). This result in conjunction with our finding of reduced T_H17 differentiation led us to examine the possibility that γSI treatment was promoting T_{reg} differentiation.

γSI treatment promotes FoxP3 expression

T_{regs} play a crucial role in suppressing autoimmunity as exemplified by genetic mutations of FoxP3, the hallmark transcription factor of T_{regs}, that cause severe multi-organ autoimmune syndromes in humans and a fatal lymphoproliferative disorder in mice. Some T_{regs} are naturally produced in the thymus, but naïve T cells can also differentiated into T_{regs} in the periphery. The cytokines IL-2 and TGFβ are important in T_{reg} generation *in vivo* and can be used to differentiate naïve T cells into T_{regs} *in vitro*.[195] It has been speculated that T_{regs} perform many roles as reviewed by Corthay.[196] Of particular significance to our studies are the proposed functions of T_{regs} to regulate of the effector class of the immune response, provide feedback control of the magnitude of the immune

response by effector T_H cells, and prevent of autoimmune diseases by establishing and maintaining immunologic self-tolerance.

Because γSI treatment indiscriminately suppressed differentiation of two distinct T effector subsets, we decided to look for a common factor that could be responsible for suppression of both. While Samon et al., 2008 reported γSI treatment inhibition of T_{reg} differentiation, here we report the skewing of T effector polarization toward FoxP3+ expressing T cells in both T_H1 and T_H17 promoting cultures. Our results are not mutually exclusive as different culture conditions were used. However, a more recent study using a DLL4 blocking antibody to inhibit notch signaling also observed an increase in T_{reg} differentiation, supporting our results [117].

It is possible that the increased FoxP3 expression observed with γSI treatment is due to increased production of TGFβ from APCs or T cells in these cultures. TGFβ has been shown to inhibit IFNγ signaling as well as the induction of IRF-1 and Tbet mRNA in primary murine CD4+ T cells[197]. Induction of TGFβ by γSI could also explain increased FoxP3+ expression in T_H17 cultures as differentiation is dependent upon TGFβ concentration. High levels of TGFβ favor the expression of FoxP3, in turn repressing RORγt, whereas low levels of TGFβ favor RORγt expression[198], possibly indirectly through suppression of the T_H1 transcription factor Tbet [197]. Conversely, it is possible that gamma secretase inhibition bypasses the need for TGFβ signaling in T_{reg} differentiation altogether, allowing IL-2 signaling to solely promote expression of FoxP3.

Unfortunately our preliminary experiments to assess the suppression capacity of these FoxP3+ cells generated under pro-inflammatory differentiation conditions were inconclusive, and further examination of the phenotype of these cells needs to be conducted.

Our studies demonstrated that γSI treatment upregulates FoxP3 in neuroantigen-specific cells. There is some evidence to support the hypothesis that T_{reg} suppression of inflammation is antigen specific[199,200]. Recently it has also been shown that T_{regs} need to express the same transcription factor of the T effector causing inflammation in order to suppress, presumably by being able to respond to the same chemokine signals and migrate to the proper location[201]. In our studies there was little to no overlap in FoxP3 and Tbet or RORγt expression. But that result may have been due to the late time point post-activation that we examined. Another group using DNMAML to genetically repress notch signaling observed no deleterious effect on chemokine-induced migration.[202] Therefore, it remains possible that γSI-induction of FoxP3+ cells under proinflammatory conditions is an ideal mechanism for generating suppressor cells *in vitro*. As such our studies define a method for generation of T_{regs} which may be useful in cell-based immunotherapy of autoimmune disease.

Conclusions

Taken as a whole our studies suggest that γSI treatment favors the generation of antigen-specific non-inflammatory T cells in the host, resulting in the reduction EAE severity. Here I have characterized the effects of γSI on T_H1 and T_H17 differentiation. As this study was nearing completion it became obvious that antigen-presenting cells were playing a critical role in γSI mediated inhibition. *In vivo* results from immunization-induced EAE as well as *in vitro* differentiation cultures both suggest that APCs are important for γSI mediated suppression. *In vivo* γSI treatment of peptide-immunized mice tended to increase EAE severity while purified T cell cultures lacking APCs were resistant to γSI-induced suppression of differentiation *in vitro*.

From our studies it is clear that the addition of γSI during CD4+ T cell activation did not interfere with the initial steps of T_H polarization by IFNγ or IL-6 for T_H1 and T_H17 differentiation respectively. However results showed that receptor expression and STAT signaling of IL-12 and IL-23 was disrupted (Figure 34). Because IL-12Rβ2 expression is rapidly upregulated during T_H1 polarization, it is likely that the ensuing FoxP3 expression in γSI treated T cells is a result of, as opposed to the driver for, γSI repression of pro-inflammatory fate acquisition. However, the full scope, magnitude, and timing of γSI-induced alterations on TCR-activated, CD4+, T cell gene expression have yet to be characterized. Of particular interest will be the expression and binding of transcription factors influencing IL-

12Rβ2 and IL-23 expression as well as aspects of the TGFβ signaling pathway leading to FoxP3 expression.

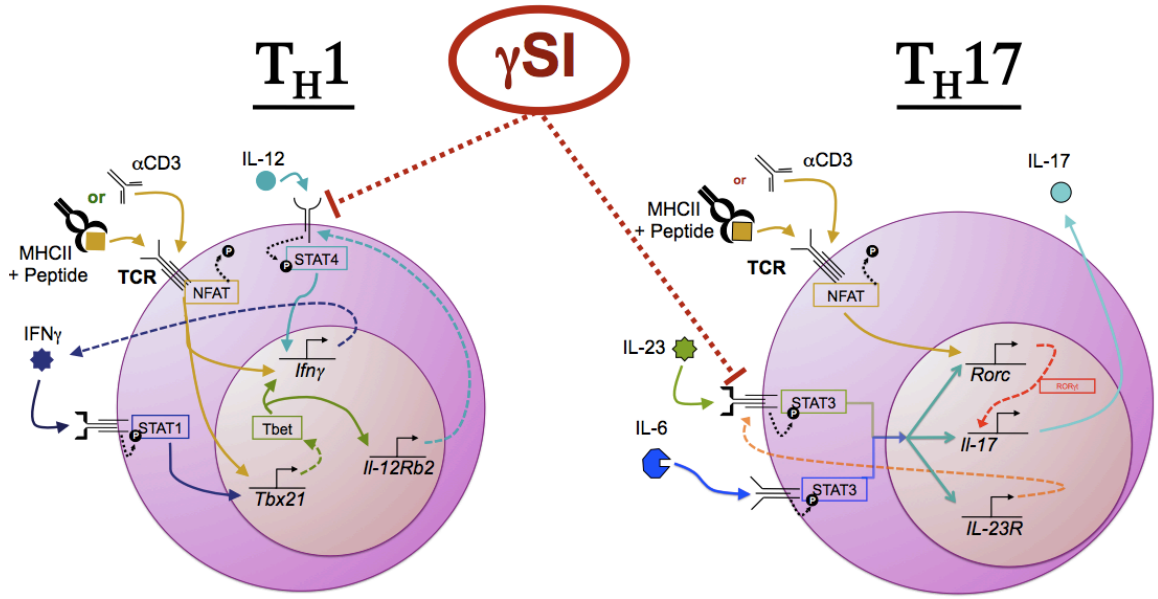

Figure 34 *Model of γSI action*. The proposed model of γSI action on T_H differentiation is via the disruption of IL-12/IL-23 signaling in the early stages of T cell activation during T_H1 and T_H17 formation respectively. Specifically, γSI are theorized to disrupt IL-12Rβ2 and IL-23 expression and their subsequent association with the IL-12Rβ1 subunit preventing the formation of the high affinity IL-12 or IL-23 receptors.

Additionally, our studies point toward γSI action on B cells as a mechanism of suppression of T effector differentiation. Many studies have been conducted on the contribution of B cells to EAE pathogenesis as reviewed in Mann et al. 2012.[203] B cells have the capacity to function as antigen presenting cells. Additionally a recent study has reported genetic presenilin deficiency impairs B cell responses to LPS and B-cell receptor stimulation [204]. It will be interesting to see how γSI impacts B cell function.

Clinical Translation

Unlike many experimental therapies, γSI treated T cell adoptive immunotherapy can easily and rapidly be adapted to human use. Multiple γSIs have made it through the safety phase of clinical trials, only failing in meeting efficacy endpoints in late phase Alzheimer's disease. Autologous cell transfer of regulatory T cells has already been tested in several autoimmune disorders, including type 1 diabetes, as well as graft-versus-host disease [205,206]. However, cellular manipulation, purification, and stabilization of T_H phenotype remain obstacles to the widespread use of such therapies. Once these obstacles are overcome, γSI-induced T_{reg} transfer could prove a safe and effective therapy.

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APPENIDIX 1: Frequently Used Flow Panels

Transcription Factors
CD4-PacBlue 0.25 μl/sample Biolegend
CD25-Fitc 0.25 μl/sample eBioscience
Tbet-PE 5 μl/sample eBioscience
RORγt-APC 2.5 μl/sample eBioscience
FoxP3-Alexa700 5 μl/sample eBioscience

Cytokines
CD4-PacBlue 0.25 μl/sample Biolegend
CD25-APC 0.25 μl/sample eBioscience
IL-17A-Fitc 1 μl/sample eBioscience
IFNγ-PE-Cyanine7 0.5 μl/sample eBioscience
GM-CSF-PE or IL-4-PE 1 μl/sample eBioscience

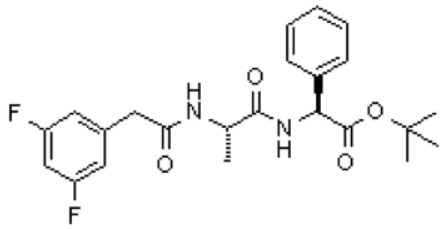
pSTATs
CD4-Pacific Blue eBioscience
CD25-PE-Cy7 eBioscience
pSTAT1-Alexa 647 BD Biosciences
pSTAT3-Alexa 647 BD Biosciences
pSTAT4-PE BD Biosciences

In vivo CFSE dilution
CD45.2-BV421 Biolegend
CD45.1-PE-Cy7 eBioscience
FoxP3-Alexa700 eBioscience
CD4-APC eBioscience
CD25-PerCP-Cy5.5 eBioscience

Other
IL-12Rβ2-PE R&D Systems
IL-23R-PerCP R&D Systems
IL-23R-PE BD Biosciences

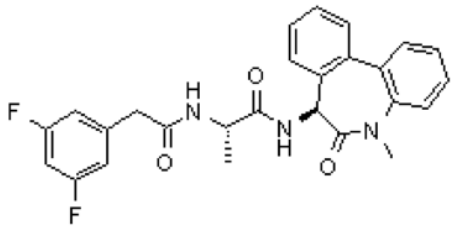
APPENDIX 2: Chemical structures of gamma secretase inhibitors

DAPT



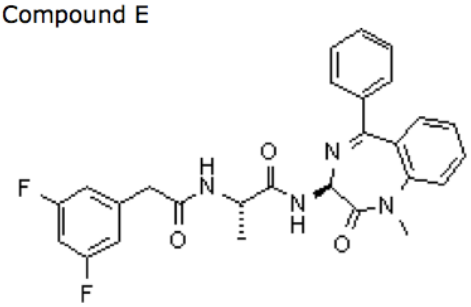
CAS 208255-80-5
C23H26F2N2O4 MW 432.46

γ-Secretase Inhibitor DBZ



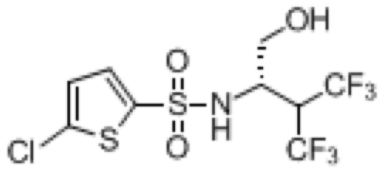
CAS 209984-56-5
C26H23F2N3O3 MW 463.49

γ-Secretase Inhibitor BZ
Compound E



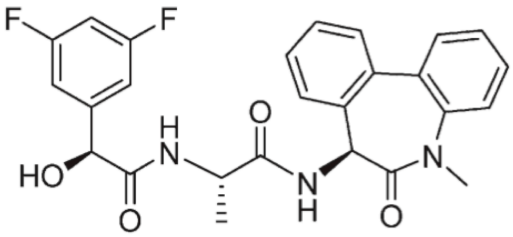
CAS 209986-17-4
C27H24F2N4O3 MW 490.51

Begacestat
GSI 953

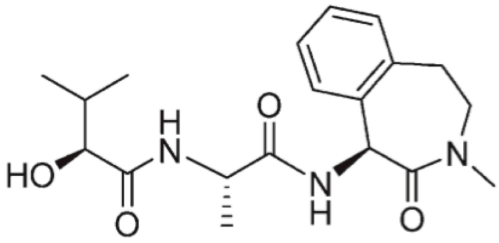


CAS 769169-27-9
C9H8ClF6NO3S2 MW 391.74

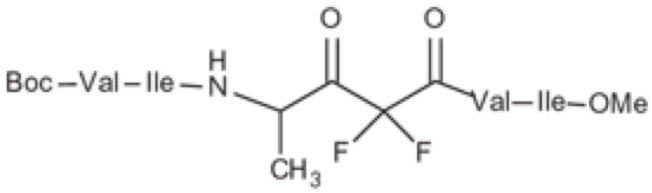
LY-441,575



Semagacestat or LY-450,139



MW167



Not pictured: IL-CHO – a leucine aldehyde with iodine bonded to the amine group.
Images courtesy of Axon-Medchem and Kreft et al., 2009[207].

APPENDIX 3: Recovery of PSEN1 deficient T cells activated *in vivo*

Table 1: PSEN1 deficient T cells transferred individually into mice.

	Cell Count	%CD4+	# CD4+	# Cd45.2	%CD45.2	%CD44+
WT (CD90.2)	4.40E+07	31.0%	1.36E+07	2196	0.01610%	48.60%
Het (CD90.1)	8.10E+07	28.5%	2.31E+07	325499	1.41000%	86.90%
KO (CD90.1/2)	6.70E+07	22.3%	1.49E+07	194233	1.30000%	88.30%

Table 2: Pooled genotypes transferred into a single mouse.

	Cell Count	%CD4+	# CD4+	# Transgenic	%Transgenic	%CD44+
Total (CD45.2)	6.50E+07	28.3%	1.84E+07	47643	0.259%	89.68%
WT (CD90.2)				10291	0.056%	82.10%
Het (CD90.1)				11482	0.062%	93.80%
KO (CD90.1/2)				22249	0.121%	91.40%