AUTOREGULATORY CD8 T-CELLS MODULATE CNS AUTOIMMUNE DISEASE BY TARGETING ENCEPHALITOGENIC CD4 T-CELLS

APPROVED BY SUPERVISORY COMMITTEE

Nitin J. Karandikar, M.D., Ph.D.

Carole Mendelson, Ph.D.

Jerry Y. Niederekorn, Ph.D.

Todd Eagar, Ph.D.

DEDICATION

I dedicate this work in honor of my children, wife, siblings and parents.

You are and have always been my strength.

A special dedication goes out to my grandmother, Rosa Bustamante, you have been and always will be the wind beneath my wings.

AUTOREGULATORY CD8 T-CELLS MODULATE CNS AUTOIMMUNE DISEASE BY TARGETING ENCEPHALITOGENIC CD4 T-CELLS

by

STERLING BOLIVAR ORTEGA

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences
The University of Texas Southwestern Medical Center at Dallas
In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas
Dallas, Texas
December, 2012

ACKNOWLEDGMENTS

To my children, Jasmine, Melody and JohnPaul Ortega, thank you for all the strength and joy you have given me during my schooling. To my wife, Lilia Ortega, thank you for your understanding and patience in my endeavor to become a scientist. To my siblings, Roger, Clint, Michael, Emilie, Sterling and Christina thank you for pushing me to be the best. To my parents, Miguel and Dolores Ortega, thank you for raising, educating, and loving me so that I may one day accomplish my goals.

To my cousins Willie, Kelly, Diego, Junior, Ivonne, Pablo, Geovanni, Diani, Elvis, Carolina, Steve, Darling, Evelin, Anthony, Freddy, Jesse, Erika and Stephanie, thank you for reminding me how important family is. To my aunts, Martha, Gloria, Estelli, Aida and uncles, Hernan and Vincente, thank you for inspiring me to become a doctor. To those family members I have lost during my journey in life and science, Rosa and Carlos Bustamante, Bayron Cueva, Maruja, Anibal and Amparo Ortega, you are still very much a part of my life, thank you.

I would like to thank the past and current members of the Karandikar Lab. Beginning with Michael, Xiao, Nathan and Riyaz. Those early years were tough but you guys made it manageable and interesting. To the first mouse gang, Andrew Benagh, Jason Mendoza and Nathan York, I am deeply indebted for your perseverance. Because of you, the EAE model was established in the lab and thus enabled my studies. To those students who have already graduated and moved on to better things, Drs. Vinodh Pillai, Chris Ayer and Ethan Baughman, thank you for all the support and fun times. To the current mouse team, Andrew Tyler, Thomas Lee, Drs. Khrishen Cunnusamy and Venkatesh Kashi, thank you for all your help. To the current MS team, Jorge and Dr. Sushmita Sinha, thank you for your kindness and knowledge you have bestowed on me. To the rotating graduates and green fellow students I was fortunate to train, Alex, Jorge and Ebele, thank you for letting me hone my teaching skills with you. To those who maintain the lab, place the orders and make the lab conducive to research, Rich Grother, Brian Biegler, Jamie Houser, Carlos Aviles, Maycie Garibay, Thomas Lee and Wallace Baldwin, thank you.

To the members of the Racke lab, Anne, Rehanna, Bob and Drs. Michael and Amy Racke, thank you for all your help and encouragement. Thank you to Drs. Mark Siegelman and Pila Estess, your insight and assistance was greatly appreciated. To the members of the Eagar lab, Julie, Lory, Matt and Dr. Todd Eagar, thank you for your assistance and collaborations. To the members of the Anderson lab, Jana Windsor and Dr. Larry Anderson, thank you for your help and support. To the members of the graduate program, Deb, Nancy, Priya and Dr. Nancy Street, thank you for your assistance and strength to carry on with my studies when things were tough.

To the MS group, Drs. Ben Greenberg, Olaf Stuve, Nancy Monson, and Elliot Frohman, thank you for always reminding me the primary reason for our studies, the patients. I would also like to thank my collaborators, Drs. Ashley Simpson, Malu Tansey, Toni Gutierrez and Anne Satterthwaite, thank you for believing in me so that together we may develop new insights in science.

I would like to thank my committee members, Drs. Carole Mendelson, Todd Eagar, and Jerry Niederkorn, for your guidance and mentoring. I would like to give a special thanks to my mentor, Nitin Karandikar. In 2002, you opened the doors of your lsab to me. Since then it has been one great adventure after another. First, as a lab manager and now as a graduate student, I want to thank you for your unwavering confidence in me, even when things looked pretty grim. We had some great times together and I will cherish the memories and teachings you have given me.

Finally, I thank you, the patients who have unfortunately succumbed to the debilitating neurological disease call Multiple Sclerosis. It is because of you that I endeavor to become a scientist. It is for you, in the hope that one day we can free you of your disease, that we perform our experiments. To you I say, continue in your hope, for we are diligently working on a therapy.

AUTOREGULATORY CD8 T-CELLS MODULATE CNS AUTOIMMUNE DISEASE BY TARGETING ENCEPHALITOGENIC CD4 T-CELLS

STERLING BOLIVAR ORTEGA, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2012

NITIN J. KARANDIKAR, M.D., Ph.D.

Multiple Sclerosis (MS) is a disease, which presents with neurological dysfunction and is believed to have an immunological etiology. Lesions in the central nervous system (CNS) are characterized by an inflammatory cellular infiltration and demyelination of neuronal axons. It is believed that myelin sheath-targeting CD4 T-cells are important mediators of this disease. While it is known that CD8 T-cells are present, oligoclonally expanded and are the predominant T-cell population in the MS CNS lesion, their antigen specificity and function remains to be elucidated. Using a murine model of MS, experimental autoimmune encephalomyelitis (EAE), we are currently evaluating the role of these poorly studied myelin antigen-specific CD8 T-cells in the

context of this autoimmune disease. We have observed that myelin oligodendrocyte glycoprotein peptide (MOG_{35-55})-specific CD8 T-cells do not mediate EAE, but in fact are capable of suppressing both *de novo* and established clinical disease. Corroborating these data, $CD8^{-/-}$ C57BL/6 mice are now shown to exhibit a more severe EAE. However, the characterization, mechanism of action and cellular targets of these autoreactive regulatory CD8 T-cells are still unknown.

Initial observations revealed that disease ameliorating CD8 T-cells are not unique to MOG_{35-55}-induced EAE, as proteolipid protein peptide ($PLP_{178-191}$)-induced EAE in B6 and SJL mice strains were capable of generating disease ameliorating CNS antigen-specific CD8 T-cells. Autoreactive regulatory (autoregulatory) CD8 T-cells exhibit a central memory phenotype (CCR7+CD62L+CD44-) and produce IFN-γ and perforin. Disease suppression by these cells is dependent on recognition of cognate antigen *in vivo* within the context of MHC Class Ia. These cells do not traffic to the CNS upon transfer into naïve mice; however, they gain CNS access following induction of CNS inflammation, suggesting both a peripheral immune compartment and CNS mechanism of action. Interestingly, autoregulatory CD8 T-cell-mediated suppression is IFN-γ and perforin-dependent and can be augmented by IL-12 *in vitro* modulation.

Next, we asked whether autoregulatory CD8 T-cells could directly target encephalitogenic myelin-specific CD4 T-cells. We now report that treatment with myelin-specific CD8 T-cells results in significantly attenuated adoptive (CD4 T-cell mediated) EAE. Moreover, increased disease severity in $CD8^{-/-}$ mice correlated with an increase in autoreactivity and inflammatory cytokine production by myelin-specific CD4 T-cells. This is reversible upon adoptive transfer of MOG_{35-55}-specific CD8 T-cells. Targeting of encephalitogenic CD4 T-cells by myelin-specific CD8 T-cells is sufficient, as induction of wildtype adoptive EAE in $K^bD^{b-/-}$ recipient mice could be suppressed. *In vivo* proliferation assays revealed a global suppression/cytotoxicity of MOG-specific CD4 T-cells.

These studies define the immune regulatory function of autoreactive CD8 T-cells in EAE. Our results demonstrate that autoregulatory CD8 T-cells have an important disease ameliorating role in EAE, which is a disease of perturbed immune regulation. Understanding this arm of the adaptive immune system offers a promising strategy for immunotherapeutic intervention of MS.

TABLE OF CONTENTS

PUBLICATIONS

In preparation

Ortega SB., Mendoza JP., Cunnusamy K., Tyler, AF., Kashi VP., Benagh A., Karandikar NJ., Cytotoxic autoreactive regulatory T-cells modulate CNS autoimmune pathology by targeting encephalitogenic CD4 T-cells. (2012) *Final draft in preparation.*

Ortega SB., Mendoza JP., Kashi VP., Tyler, AF., Benagh A., Karandikar NJ., CD4 and CD8 myelin determinant requirements of autoregulatory CD8 T-cell generation. (2012) *In preparation.*

Ortega SB., Mendoza JP., Ayers CL., Garibay MM., Baldwin WH., Racke MK., Frohman EC., and Karandikar NJ., Discriminating autoreactive T cell responses from MS patients and healthy controls using multiparametric flow cytometry. (2012) *In preparation.*

Mendoza JP., Firan M., Tyler AF., **Ortega SB.,** York NR., Benagh A., and Karandikar NJ., CD8+ T cells are required for Therapeutic Modulation of Autoimmune demyelinating disease (2012) *Submitted.*

Guitierrez T., Mayeux JM., **Ortega SB.,** Karandikar NJ., Li Q., Rakeja D., Zhou J., and Satterthwaite AB., IL-21 is required for IgG autoantibodies but not kidney damage in lyn-/- mice (2012) *Submitted.*

Prior publications

Biegler BW., Yan SX., **Ortega SB.,** Tennakoon DK., Racke MK., and Karandikar NJ., Clonal composition of neuroantigen-specific CD8+ and CD4+ T-cells in multiple sclerosis, *J Neuroimmunol. 2011 May:234(1-2):131-40*

Baughman EJ., Mendoza JP., **Ortega SB.,** Ayers CL., Greenberg BM., Frohman EM., Karandikar NJ., Neuroantigen-specific CD8+ regulatory T-cells function is deficient during acute exacerbation of multiple sclerosis. *J Autoimmun, 2011 Mar;36(2):115-24*

York NY., Mendoza JP., **Ortega SB.,** Benagh A., Firan M., Karandikar NJ., Immune regulatory CNS-reactive CD8+ T cells in Experimental Autoimmune Encephalomyelitis. *J Autoimmun. 2010 Aug;35(1):33-44*

Sathe A., **Ortega SB.,** Mundy DI., Collins RH., Karandikar NJ., *In vitro* methotrexate as a practical approach to selective allodepletion.*Biol Blood Marrow Transplant. 2007 Jun;13(6):644-54. Epub 2007 Mar 23*

Pillai V., **Ortega SB.,** Wang CK., Karandikar NJ., Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol. 2007 Apr;123(1):18-29. Epub 2006 Dec 19*

Biegler BW., Yan SX., **Ortega SB.,** Tennakoon DK., Racke MK., Karandikar NJ., Glatiramer acetate (GA) therapy induces a focused, oligoclonal CD8+ T-cell repertoire in multiple sclerosis. *J Neuroimmunol. 2006 Nov;180(1-2):159-71. Epub 2006 Aug 28*

Tennakoon DK., Mehta RS., **Ortega SB.,** Bhoj V., Racke MK., Karandikar NJ., Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J Immunol. 2006 Jun 1;176(11):7119-29*

Crawford MP., Yan SX., **Ortega SB.,** Mehta RS., Hewitt RE., Price DA., Stastny P., Douek DC., Koup RA., Racke MK., Karandikar NJ., High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood. 2004 Jun 1;103(11):4222-31. Epub 2004 Feb 19*

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Ab	Antibody
ADEM	Acute disseminated encephalomyelitis
APCs	Antigen presenting cells
APC	Allophycocyanin
AT-EAE	Adoptively transferred EAE
B-cell	Bone marrow-derived lymphocyte
β2m	Beta-2-microglobulin
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
CD	Cluster of differentiation
CFA	Complete freund's adjuvant
CFSE	5-(and 6-) carboxyfluorescein diacetate succinimidyl ester
CNS	Central nervous system
ConA	Concanavalin A
CSF	Cerebrospinal fluid
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DMA	Disease-modifying agent
DMTI	Diabetes mellitus type I
DMSO	Dimethyl sulfoxide
DC	Dendritic cell
dH_20	Distilled water
FACS	Fluorescence-activated cell sorting
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetate
ER	endoplasmic reticulum
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
GA	Glatiramer acetate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
H&E	Hematoxlin and eosin
IBD	Inflammatory bowel disease
IDO	indoleamine-pyrrole 2,3-dioxygenase
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IFN-γR	Interferon gamma receptor
i.p	Intraperitoneal
i.v.	Intravenous
Ig	Immunoglobulins
IL	Interleukin

Li	Invariant chain
ln	Lymph node
MBP	Myelin basic protein
MHC	Major histocompatability complex
mins.	minutes
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MX	Mitoxantrone
µg	Micrograms
µl	Microliters
µM	Micromolar
NO	Nitrous oxide
PBS	Phosphate-buffered saline
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death 1
PE	Phycoerythrin
PerCp	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PLP	Proteolipid protein
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PRR	Pattern recognition receptors
PTX	Pertussis toxin
RA	Rheumatoid arthritis
RAG	Recombinase-activating genes
RRMS	Relapsing-remitting MS
rpm	Revolutions per minute
rt	Room temperature
SEB	Staphylococcus enterotoxin B
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
spl	Spleen
SPMS	Secondary or chronic progressive MS
T-cell	Thymus-derived lymphocyte
TAP	Transporter associated with antigen processing
Tc	Cytotoxic T-cell
TCR	T-cell Receptor
Th	Helper T-cell
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Ts	Suppressor T-cell
Treg	CD4 T-regulatory cell
vs.	Versus

CHAPTER 1: GENERAL INTRODUCTION

1.1: Principles of the Immune System

The role of the immune system is to protect the host from both foreign, as well as altered-self pathogens. This is accomplished by the concerted effort of both the innate and adaptive immune components. The response by the innate immune component, although rapid, is not pathogen-specific and confers no immunological memory. In contrast, the adaptive immune system is initially delayed, but can generate specificity and immunological memory, which augments its ability to respond to subsequent immune challenges.

This general introduction will summarize the components of the immune system, maintenance of immunological tolerance and current hypothesis for the generation of autoimmunity, in order to facilitate the reader in understanding the role of autoregulatory CD8 T-cells in an autoimmune CNS disease. A brief introduction including the etiology, pathogenesis and current regimen of MS therapies will be included, so that the reader may appreciate the urgency in studying autoregulatory CD8 T-cells.

1.1.1: The Innate Immune System

In order to combat a plethora of pathogens, the immune system has developed an effective arsenal of both cellular and molecular mechanisms. These immune components provide for an almost unlimited repertoire of receptors that are capable of recognizing and eliminating many forms of foreign pathogens. In fact, the innate immune system, the first responder, uses an array of components such as pattern recognition receptors (PRR), the complement system and phagocytes in order to coordinate and effectively quench an infection.

PRR are receptors displayed on innate immune cells, which recognize conserved structures on pathogens [1]. A well-studied set of receptors known as Toll-like receptors (TLR) can recognize bacterial or viral components. Upon TLR engagement, a signaling cascade resulting in the activation of transcription factors can promote the expression of cytokines, chemokines and other inflammatory mediators [2]. Each of these components contributes to an effective means of combating the ever-waging war between human and pathogen. Unfortunately, this component of the immune system suffers from several deficiencies, such as an inability to remember and adapt to evolving pathogens. Thus, the second arm of the immune system has evolved to overcome these deficiencies.

1.1.2: The Adaptive Immune System

The adaptive immune system is separated into the humoral and cell-mediated components. In the humoral unit, B-cells recognize foreign antigens by producing surface immunoglobulins (B-cell receptors), which can recognize three-dimensional structures on the surface of pathogens. Upon recognition, the B-cells become activated and can mature into plasma cells, which produce and secrete antibodies. These antibodies can recognize foreign antigen from pathogens, form antigen-antibody complexes, and in a coordinated effort with the innate immune system, eliminate the pathogen.

The cell-mediated component is composed of T-cells. T-cells express on their surface proteins called T-cell receptors (TCR), which are theoretically capable of recognizing small peptides derived from antigens expressed by pathogens. Depending on their surface expression, T-cells can be subdivided into T helper cells (Th), which express CD4 surface protein or cytotoxic T-cells (Tc) that express CD8 surface protein. CD4 T-cells produce cytokines, which

avails them the ability to either potentiate the humoral immune response or enhance the cytotoxicity of Tc. CD8 T-cells interrogate cells and assess if they are either infected or transformed.

1.2: Antigen Presentation to T-cells

In order for a T-cell to acquire effector function, it must first go through a process known as antigen presentation, whereby they are presented a peptide in the context of an MHC (major histocompatibility complex) molecule. This process is performed by antigen presenting cells (APCs), which can use either use MHC-I to present to CD8 T-cells or MHC-II to present to CD4 T-cells.

The MHC-I presentation involves the degradation of pathogen-derived proteins within the cytoplasm using proteasomes complexes. This is followed by transport into the endoplasmic reticulum (ER) via transporter associated protein (TAP) [3]. An 8-10 amino acid peptide is loaded onto the MHC-I complex, which is composed of two polypeptide chains, α and β2-microglubulin (β2m) [3]. The complex of peptide and MHC-I molecule is now displayed on the surface of the APCs, where the CD8 T-cell can interact. If the TCR on the CD8 T-cell recognizes the peptide and the APCs displays a proper cohort of costimulatory molecules, the CD8 T-cell will become activated and divide and/or produce effector cytokine molecules [4]. The process whereby a specific CD8 T-cell recognizes a peptide and proliferates (all having the same antigen specificity) is called clonal expansion.

The MHC-II pathway presents peptides of pathogenic bacteria found in the extracellular environment [5]. The α and β chain of the MHC-II molecule assemble in the ER and along with invariant chain (Li), are transported to a vesicle of the endocytic pathway, where the extracellular peptide (15-24 amino acids in length) will replace the Li and complex with MHC-II molecule.

The peptide-loaded MHC-II complex is then transported to the cell surface where specific CD4 T-cells can now recognize it. Similar to CD8 T-cells, if appropriate recognition of antigen and co-stimulation occurs, the CD4 T-cell is activated and proliferation and/or cytokine production begins. [6]

1.3: T-cell Differentiation

Upon activation and depending on the local cytokine milieu, T-cells can differentiate into one of several different subsets. For instance, during antigen presentation, if the APCs secrete IL12, a CD4 T-cell will differentiate into a Th1 cell. Th1 cells produce IFN-γ, a pro-inflammatory cytokine that aids in the activation of macrophages. Upon activation, macrophages destroy microbes either through the production of reactive oxygen species (ROS) or phagocytosis [7]. IFN-γ also promotes cell-immunity by activating CD8 T-cells in order to eliminate any intracellular pathogens [4]. During an inflammatory response, there is significant tissue damage due to the toxic effects of the cytokines released during an immune attack [8]. Thus, Th1 cells were the first cells implicated in CNS autoimmunity in human and mice.

If APCs produce IL4 during antigen presentation, a Th2 cell will develop and produce IL4, IL5, and IL13 [9] in order to augment humoral immunity and combat extracellular pathogens such as helminthes [8].

Th17 cells, is a third recently discovered subset that is produced when IL6 and TGF-β is secreted by APCs [10]. Still understudied, these cells are believed to function in an anti-microbial capacity at the epithelial/mucosal barriers by secreting IL17 and IL22 [11]. Recent observations have implicated Th17 cells as possible co-contributors to CNS autoimmunity [10].

1.4: Immune Tolerance

The immune system has developed a potent system that enables the identification and eradication of pathogens. However, this system is not without its flaws; and if left uncontrolled, could become detrimental. In fact, this is highlighted by the existence of several immune regulatory checkpoints, both independent and redundant mechanisms involved in the suppression of unwanted immune responses. There are two components used in suppressing unwanted immune response. The first is called central tolerance and is implemented during thymic lymphocyte development. The second is called peripheral tolerance and it involves checkpoints initiated during the maturation phase of a lymphocyte, within the secondary lymph node and peripheral tissues.

1.4.1: Central Tolerance

Within primary lymphoid tissues, the generation of thymocytes is a random process, which can result in the development of thymocytes expressing receptors to both foreign, as well as self-peptides. For this reason, thymocytes must go through a two-step selection process cumulatively termed “central tolerance”. The final outcome of this process is the generation of T-cells capable of recognizing foreign peptide within the context of self major histocompatibility complex (MHC) molecules, but not capable of recognizing self-peptide. The first step, termed positive selection occurs within the thymic cortex and it involves the interaction between thymic epithelial cells and T-cell. If the T-cell is capable of binding sufficiently with MHC Class I or II molecules, the cell receives a pro-life signal and survives, whereas T-cells with no binding die off of “neglect”. During negative selection, occurring within the cortico-medullary junction and medulla, medullary thymic epithelial cells display self-antigen to the T-cells. If the T-cell

recognizes the self-antigen with a high affinity, apoptosis is induced and the self-reactive T-cell is removed from the lymphocyte repertoire. It is postulated that failure in the negative selection process results in the induction of autoimmune disease.

Approximately 97% of T-cell precursors, which enter central tolerance are removed by either the positive or negative selection. Expression of self antigen is regulated by autoimmune regulator protein (AIRE) within the thymus and failure of this AIRE gene results in significant autoimmunity [12]. Because not every self-protein can be expressed in the thymus and many self-reactive T-cells only bind mildly to self-antigen, a secondary method of tolerance known as "peripheral tolerance" has also evolved.

1.4.2: Peripheral Tolerance

Induction of immunological tolerance after T-cells have matured and entered the periphery is called peripheral tolerance. This method of tolerance induction becomes understandably important when one recognizes the presence of numerous self-reactive T-cells in the mature T-cell population. Peripheral tolerance uses intrinsic, as well as extrinsic mechanisms.

Intrinsically, autoreactive T-cells can be tolerized by three mechanisms: 1) apoptosis, 2) anergy and 3) skewing. Activation of a T-cell by the binding of the TCR with the MHC/peptide complex is dependent on the avidity and duration of binding. When this interaction is unstable and/or of short duration, the T-cell undergoes apoptosis. Anergy mediated tolerance can be induced by the presentation of antigen to T-cells without the proper cohort of costimulatory molecules thus inducing a state of anergy in self-reactive T-cells [13]. This is probably a very ubiquitous method of immune tolerance as self-antigen is continuously presented to T-cells in the absence of inflammatory signals, e.g. during a healthy state of immune surveillance.

Distinctive lineages of T-cells develop depending on the cytokine milieu during primary activation. Induction of a Th1 produces a cell, which secretes IFN-γ, a cytokine that can inhibit Th2 cells. On the other hand, Th2 cells secrete IL4, TGF-β and IL10 (which can inhibit Th1 cells). Regulatory T-cells, which secrete IL10 termed Tr1 or TGF-β termed Tr3, are also capable of modulating immune responses. Cumulatively, the intrinsic mechanisms induce tolerance by controlling the magnitude and class of immune response.

Extrinsic mechanisms involved in tolerance induction include NKT cells, CD4 Tregs and Qa-1-dependent CD8 T-cells. NKT cells are natural killer cells which express a specialized α/β T-cell receptor composed of the invariant alpha chain (Vα24-JαQ) and various other Vβ chains [14]. NKT cells are capable of recognizing glycolipids and upon presentation on CD1d (MHC molecule) by APCs, these cells can secrete IL4, IL10, IFN-γ and TGF-β, as previously mentioned, all cytokines capable of differentially modulating immune responses. Cells specialized in the suppression of effector T-cells, i.e. autoreactive T-cells, are termed regulatory T-cells. Although not the first described, but definitely the most studied, CD4+CD25+Foxp3+ T-cells have been observed in both mice and humans [15]. These cells have been shown to use IL10, TGF-β, consumption of IL2, and modulation of APCs as possible mechanisms in the suppression of autoreactive T-cells [16]. Qa-1-dependent CD8 T-cells are T-cells which can modulate an immune response by either killing or suppressing a pathogenic T-cells. The pathogenic cells are targeted by their display of an MHC Class Ib molecule, Qa-1 in mice (HLA-E in humans), and self-peptide complex[14]. Upon TCR and MHC/peptide binding, these CD8 T-cells have been shown to delete target cells either by a perforin-dependent mechanism or suppress target cells by IFN-γ production [17].

As certain self-antigens are not expressed thymically and hence central tolerance cannot be induced upon T-cells that are reactive to these antigen, an additional passive form for the maintenance of tolerance has evolved which is termed "ignorance". In this case, the self-antigen is sequestered in immune privileged sites, thus made inaccessible to T-cell surveillance and thereby impede presentation of self-antigen to autoreactive T-cells. Termed immune privileged sites, the brain, anterior chamber of the eye, testes and fetus, these locations have a limited infiltration of immune cells. The limitation is largely in part, because of physical barriers involving blood-tissue-barriers incorporating tight junctions between the endothelial cells. Additionally, within these tissues, there are several other intrinsic mechanisms, which inhibit immune responses including the use of Fas-ligand expression in order to induce apoptosis or anti-inflammatory cytokines such as TGF-β and IL10.

In summary, all these individual components acting in a synergistic manner are involved in the attenuation of any immune response that might be directed against self-antigen. When one or more of these tolerance mechanisms fail, autoimmunity ensues and if untreated can eventually lead to tissue pathology.

1.5: Autoimmunity

An immune response is initiated during an infection. Once pathogens have been eliminated, the immune response is quenched by contraction of the effector population. In cases where either the pathogen is not completely removed (chronic inflammation) or due to the resemblance of pathogen and self-proteins (molecular mimicry), self-reactive immune cells, which have escaped both the central and peripheral immune tolerance, are inappropriately

activated. This combination of events can result in an inappropriate immune response culminating in autoimmune pathology [18].

Depending on the antigen-specificity of the autoreactive T-cells, the tissue destruction could be wide spread, such as that seen in systemic lupus erythematous (SLE), where the T-cells are believed to be targeting chromatin or the splicesome complex [19]. Due to the abundance of target antigen, clinical presentation includes damage to the heart, joints, lungs, skin, blood vessels, liver, kidney and nervous system [20].

In contrast, when the T-cells are directed against an antigen expressed in only a few tissues, such as the beta cells of the pancreas, a more focused organ-specific autoimmune disease develops, type I diabetes (DMT1). The clinical presentation includes a recurrent or persistent hyperglycemia, reflecting the more focused response [21],[22]. Another organ-specific autoimmune disease is multiple sclerosis, a CNS disease where the myelin that insulates the axons of nerves is destroyed by a hypothesized aberrant immune response [18]. In this study, we will focus on the debilitating neurological disease known as multiple sclerosis.

1.6: Multiple Sclerosis

"… the chief curse of the illness… I must ask constant services of people I love most closely…it is an illness accompanied by frustration…it is illness that inflicts awareness of loss…sporadically it is, in its manifestations, a disgusting disease"

Brigid Brophy, 1929-1995

Multiple sclerosis is a debilitating chronic neurodegenerative disease with a hypothesized aberrant immune response with a multifactorial epidemiology. Afflicted individuals can develop neurological deficits such as hypoesthesia (loss of sensitivity) and paraesthesia (prickling or numbness), ataxia (difficulty in coordination and balance), dysarthria (difficulty in speech) and

optic neuritis. First described in 1868 by Jean-Martin Charcot, MS is the most common CNS disease in young adults [23],[24].

1.6.1: Statistics and Epidemiology

There are over 2.5 million individuals world-wide afflicted with MS, and of these, over 400,00 are Americans. Diagnosis of MS typically occurs between the third and fourth decade of life, although there are reports of pediatric MS [25]. MS is more common in young adult women, with the trend increasing to 3:1 in the latest analysis [26]. In children, girls are more likely to get MS, while older individuals (+50 years of age) the risk is almost equal [27]. The prevalence ranges between 2-150 per 100,000 individuals (0.002%-0.15%), depending on the country or specific population [28]. Within the United States population, 1 in every 1110 individuals are diagnosed with MS (~0.09% fraction) (Figure 1). Monozygotic twins have a 30% risk of MS, while in siblings of two affected parents, this decreases to 23%, and with one affected parent this drops to 13%. First-degree siblings have a risk factor of 5% [29].

Immune related genes also contribute to the risk factor for MS. For instance, the markers DR15 and DQ6 share a strong association with MS [30]. A strong association has also been seen in the DR4 marker of northern Europeans, Sardinians and Mediterranean groups [31]. Increased susceptibility has also been demonstrated in association with single nucleotide polymorphic markers for interleukin-2 and interleukin-7 receptor α chains [32]-[35]. Conversely, decreased risk (protective effect) has been shown with the HLA-C5 [36]and HLA-DRB1*11 allele [37],[38].

Environmental factors have also been shown to have an effect on the prevalence of MS. The distance from the equator increases the risk factor, but this is more dependent on where the

individual lived during their early life (less than 15 years of age) [39]-[42]. Viral exposure during early life also plays a role in MS susceptibility. MS patients have an increase in measles, mumps, rubella or Epstein infections, as compared to controls [43]. Interestingly, new studies reveal an increase in B-cells infected with Epstein-Barr virus accumulating in chronic MS lesions [44].

Association of MS to other autoimmune disease (e.g. autoimmune thyroid disease) shows only a slight correlation [45]. Prevalence of MS is not isolated to one individual causative factor. Interplay between both genetic, as well as environmental agents leads to an increase in MS susceptibility.

Figure 1: US Patients with Diagnosis in a Given Year

	male	female	all
fraction of US population	1 in 1660 ≈ 0.06%	1 in 890 ≈ 0.11%	1 in 1110 ≈ 0.09%
number of US patients	107 400 per year	276 600 per year	384 000 per year
average patient age	49 years	48 years	48 years
diagnosis sample size	76 visits	237 visits	313 visits

(estimates based on 131 748 patient visits to healthcare providers from NAMCS and NHAMCS, weighted for USA demographics, 2006 to 2007)

1.6.2: Clinical Course of MS

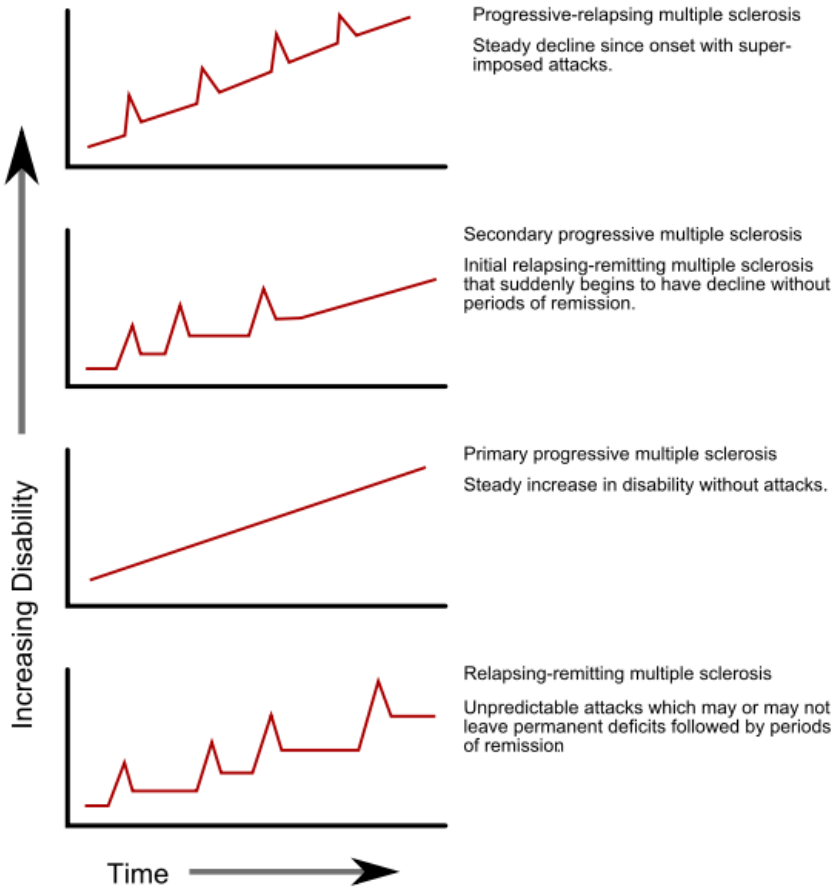
MS is divided into four subtypes. The first, remitting relapsing MS (RRMS) subtype (Figure 2) is characterized by an initial clinically isolated syndrome (CIS). This is followed by a subacute clinical phase, where over a period of months to years, there are no new signs of

disease activity (remission). The chance of a second episode (relapse) increase from 50% at 2 years to 82% at 20 years [46]. 80% of MS patients are within this subtype, but eventually 65% convert to the secondary progressive form (SPMS), whereby recovering from each event is incomplete and symptoms accumulate. The mean conversion time from RRMS to SPMS is 19 years [47]. 10-15% of patients (~40 years of age) initially present with clinical progression which does not remit or relapse and is termed primary progressive MS (PPMS) [27]. A minor subtype of MS, known as progressive relapsing MS is described as a combination of RRMS and PPMS, whereby the patient has a steady neurological decline but also suffers from clear superimposed attacks [48].

The clinical course of MS can be affected by viral exposure which doubles the risk of relapse [49], while parasitic infections reduce the relapse rate [50],[51]. During pregnancy, MS patients have a reduction in the relapse rate but there is a three-fold increase during the puerperium [52]. Stress and core hyperthermia increases the risk of relapse [53].

Although controversial, life expectancy of people with MS does seem to be decreased. Death from MS is usually attributed to complications from the neuronal dysfunction leading to infections of the skin, chest and bladder. Life expectancy of MS patients has been shown to be reduced by 5-10 years [25],[54].

Figure 2: Clinical Disease Progression in MS Subtypes



1.6.3: Diagnosis of MS

Depending on where the sclerotic plaques form in the CNS, clinical presentation can involve motor, sensory, visual and autonomic systems. Clinical tools used in the diagnoses include the assessment of neurological function, which depending on their time and location may be sufficient for diagnoses. MRI assessment of white matter may reveal focal or confluent abnormalities, which is present in only 95% of patients, but this alone is not sufficient, since older individuals (50+ years) have similar white matter cerebral lesions but are clinical disease negative [29]. A prolonged latency in evoked potentials is another indicator of clinical disease.

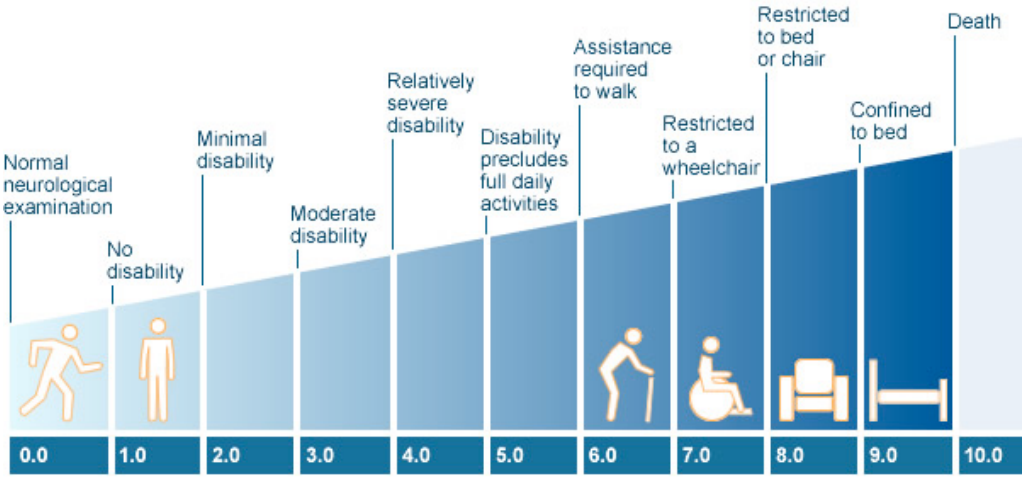
The presence of oligoclonal bands in protein electrophoresis of cerebral spinal fluids can also help the clinician diagnose this disease, although this is seen in only 90% of patients.

Due to the heterogeneous clinical presentation, a standardized method of clinical assessment has been formulated to help ensure an early diagnosis and treatment. The McDonald's criteria, establishes that two episodes must affect two separate sites within the CNS and have occurred at least 30 days apart [29]. One of these clinical presentations can be replaced with an MRI enhancing lesions but now this gadolinium-enhancing lesion must have occurred three months after the clinical event.

This diagnostics criterion is not error-free, as there are differential diseases such as systemic vasculitis (systemic disease complicated by CNS involvement), hereditary cerebellar ataxia (disease of the brain and spinal cord confined to a selected physiological system) and acute disseminated encephalomyelitis (monophasic disorder affecting many neuroanatomical sites) which must be excluded before diagnosis can be confirmed.

Assessment of disease progression and ensuing patient disability is measured using the Expanded Disability Status Scale (EDSS) [55] (Figure 3).

Figure 3: Expanded Disability Status Scale used in Evaluating Disability in MS Patients



Expanded Disability Status Scale (EDSS) -
Multiple Sclerosis Decision

PubMed Health

1.6.4: Current Treatments in MS

Current therapies in MS are not curative; in fact they only offer temporary improvement of disability, thus they are labeled as disease-modifying agents (DMA). The first line of treatment includes the use of corticosteroids for acute relapses and β-interferon or glatiramer acetate for long term treatment. For more aggressive forms of MS, mitoxantrone and natalizumab are prescribed [56].

During the period from 1980-90, trials with conventional immunotherapies such as cyclophosphamide and cyclosporine were largely unsuccessful. At that time, the patient cohorts largely included the progressive MS variant, which is now understood to be a disease largely driven by non-inflammatory mechanisms. During the 1970s, antiviral interferon beta (IFN-β) was studied as a possible therapy, since the pathogenesis of MS was considered to be an environmental trigger. Currently, IFNβ_{1a} (Avonex ®, Rebif ®) and IFNβ_{1b} (betaferon ®) are

believed to work by induction a Th2 cytokine shift through modulation of cytokine production from APCs [57]. IFNβ has a success rate of 35% but suffers from side effects such as local injection-site reactivity and flu-like symptoms with hyperthermia [58]. 5-30% of patients manifest the formation of neutralizing antibodies, whose presence is associated with a reduction in the treatment efficacy [58].

During attempts to promote EAE in mice, the Teitelbaum group developed Glatiramer Acetate (GA, Copaxone ®), a compound capable of suppressing both EAE, as well as MS symptoms. Although this drug is believed to induce tolerance in myelin-reactive lymphocytes by converting pro-inflammatory to anti-inflammatory T-cells [59],[60], new evidence indicates that GA may be activating regulatory CD4 (Tregs) and suppressor CD8 (Ts) T-cells [59],[61]-[63]. Evaluation of GA efficacy shows that although it may not stop disease progression, GA is capable of reducing disease relapse in 30% RRMS patients but has no effect on SPMS, PPMS or PRMS. Clinicians have studied the combined use of interferon β and GA in relapsing-remitting disease, but the studies only showed a reduction of 30% of new episodes in 2-3 years [64]-[66].

Natalizumab (Tysabri ®), a humanized monoclonal antibody is directed against the cell adhesion molecule $\alpha_4\beta_1$ integrin. The antibody works by blocking CNS infiltration by immune cells [67]. Unlike the first line of DMA, natalizumab has been shown to both reduce the number of relapses, as well as decrease progression of disability [56]. Although Tysabri has been shown to reduce relapse rate by 68% and MRI activity by 90% [68], it initially suffered a market withdrawal due to significant side effects [69]. By blocking immune cells surveillance of CNS tissues, natalizumab treatment was found to result in an increase in progressive multifocal leukoencephalopathy, a viral infection almost exclusively seen in severely immunocompromised

patients [70]. Because of this, natalizumab is mostly prescribed to patients whose disease is refractory to first line DMA and whose MS disease is progressing vigorously [70].

Mitoxantrone (Novantrone ®) is an anthracenedione antineoplastic drug that intercalates with DNA and inhibits the synthesis of both DNA and RNA. Mitoxantrone has been approved for RRMS, SPMS and RPMS [71]. Although it has been found to be more efficacious than interferons, with a reduction in relapse rate of 63-83% [71], its cardiotoxicity and acute leukemia incidence has delegated it to be prescribed in extreme worsening RRMS [71].

Fingolimod (Gilenya ®) was approved for MS in 2010, and has been shown to reduce the relapse rate of MS by half [72]. Fingolimod is a sphingosine-1-phosphate receptor modulator, which stops lymphocyte egress from secondary lymphoid tissues [73]. Fingolimod is associated with potentially fatal infections, bradycardia, skin cancer and hemorrhaging focal encephalitis. Similar to Mitoxantrone, Fingolimod is prescribed for excessively severe forms of MS.

Although there are other drugs currently under trial such as: anti-CD25, anti-CD20 (rituxumab), anti-CD52 (pan-lymphocyte depleting antibody) and bone marrow transplantation, all of these current trial therapies involve global immune suppression. Much like previous generation therapies, unless the protective immunity against foreign and altered-self pathogens is maintained, these trial drugs will suffer from side effects which plague current therapies.

1.6.5: Pathogenesis of MS

In healthy individuals, nerve-to-nerve communication requires proper saltatory conduction down the axon of a transmitting nerve. This is accomplished by insulating neuronal axons with a myelin sheath. Myelin is made by mature oligodendrocytes. Elongated oligodendrocyte processes make contact with a neuronal axon and at the point of contact, encircle the axon making the internodal myelinated segment. The myelinated segment is

composed of sodium (Na-1.2) channels which are segmented by Na-1.6 channels at the intervening nodes of Ranvier [74]. This alternative combination of sodium channels facilitates depolarization, generating an electrical current and thus allowing a proper saltatory conduction [74].

In MS, the first step in pathogenesis is believed to be a lymphocyte-driven inflammation. Autoreactive T-cells, most likely activated in the periphery, traffic through the blood brain barrier (BBB) and initiate a cascade of events, which potentiate autoimmunity [25],[67],[75]-[81],[81]-[83]. But the mere presence of autoreactive T-cells alone does not result in autoimmunity, as healthy individuals are known to harbor autoreactive T-cells [84],[85] and T-cells are known to perform an immuno-surveillance role in the brain [86], thus it seems that a conversion from protective to pathogenic role might be a necessary step towards CNS autoimmunity. Another possible mechanism leading to CNS autoimmunity might be a failure of immune regulation. Studies reveal a failure in both an extrinsic cellular (regulatory T-cells unable to suppress effector T-cells in MS patients) [87], as well as an intrinsic (autoreactive T-cells express β-arresting 1, an important promoter of T-cell survival) regulatory mechanisms leading to disease [88].

Previously, these encephalitogenic autoreactive T-cells were labeled as Th1 cells, indicating that these cells produced IFN-γ and thus were labeled pro-inflammatory. Recently, a new subset of T-cells called Th17 have been described which drive inflammation by secretion of interleukin 17 (IL17) under the control of IL23 [89]. In fact, Th17 T-cells are probably more potent encephalitogenic T-cells, as IL17 and IL22 have been shown to effectively disrupt the BBB [90],[91]. Although myelin antigen may be the most important inciting protein for Th1 and Th17 cells in MS, there are other possible candidate antigens. For instance, αB-crystallin, a

protein found in the lens of the eye, is known to prevent physiological suppression of inflammation [92]; anti-neurofascin antibodies, have been shown to mediate axonal injury in MS [93]; and 2',3'-cyclic nucleotide 3'-phosphodiesterase [94].

These pathogenic T-cells are believed to impede nervous system signal transduction in three ways; the production of soluble inflammatory mediators which can cause conduction block in intact axons [74], demyelination [74] or transection of neuronal axons [95].

Next, CNS resident antigen presenting cells (microglia) are activated and can now contribute to inflammation by contacting the oligodendrocyte-myelin unit that is opsonized with ligands for the Fc and complement receptors. This, in turn, initiates the production of TNF-α, another potent inflammatory cytokine. Interestingly, microglia may have a dual purpose as they have been shown to ameliorate disease by removal of myelin debris and promotion of remyelination [74].

Attempts at modulating the CNS tissue damage is performed by a balance between pro-inflammatory and CNS repair mechanisms. During the acute phase, there is active inflammation and demyelination, and quite interestingly there is a concurrent presence of remyelination, as evident by shadow plaques. Under chronic activation, oligodendrocytes adapt by redistributing ion channels [96]. For instance, in the peripheral immune system, gliomedin has been shown to trigger nodal-like clustering of Na channels, in an attempt to cue the formation of nodes of Ranvier [97]. A similar observation has been made in EAE and MS lesions and it is hypothesized that an oligodendrocyte factor is produced in an attempt to remyelinate [96]. Additionally, the CNS maintains a reservoir of undifferentiated oligodendrocytes, which upon lesion formation can encircle the tissue damage [98],[99], and be a potential source for remyelination of damaged axons [100]. This may be a reason, at least in RRMS, that patient disability is cyclical over time.

This repair mechanism seems to eventually become exhausted or unable to keep up with the ensuing damage. Continued chronic tissue injury eventually leads to the formation of scar-like plaques which build up around damaged axons [101]. Another hallmark of continued tissue damage is gliosis, which can act as a mechanical barrier to repair.

In summary, it is believed that MS is caused by the aberrant activation and infiltration of autoreactive T-cells into the CNS. By understanding the autoreactive T-cells we can further develop strategies that might enable us to control the encephalitogenicity of pathological T-cells. Thus, it becomes vitally important to dissect out which autoreactive T-cells are mediating or regulating CNS disease.

1.7: Experimental Autoimmune Encephalomyelitis

Initially described over 75 years ago, experimental autoimmune encephalomyelitis is an animal model of MS. The first incidence of EAE occurred in humans when the rabies virus was grown on rabbit spinal cords. Subsequent human vaccinations resulted in an inflammatory demyelinating CNS disease [102]. Later, Rivers and colleagues, using rhesus monkeys, determined that the CNS inflammation was not caused by the rabies virus itself but by the spinal cord contaminant in the vaccine [103]-[105]. Initially developed from observations in primates [103],[104], EAE is now inducible in mice, rats, guinea pigs and rabbits [106]. Today, immunizing susceptible animals with encephalitogenic myelin peptides emulsified in complete Freund's adjuvant induces CNS inflammation. Studies in EAE have been fruitful because they have allowed us to understand the pathological mechanisms in CNS autoimmunity, and have

provided an *in vivo* validation tool for studying the key immunologically important molecules in this autoimmune disease.

1.7.1: Induction of Murine EAE

EAE is inducible in C57BL/6, SJL and B10 mice [106]. Using CNS tissue or myelin peptides such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP) in combination with complete Freund's adjuvant (CFA), primary EAE can be induced [107]. EAE may also be induced by passive (adoptive) transfer of T-cells that are reactive to the previously mentioned myelin peptides (Figure 4) [108]. Adoptive EAE has been instrumental in establishing the key role of myelin-specific CD4 T-cells in disease pathogenesis [109]. In certain models, mice are co-injected with pertussis toxin (PTX), as a means to break down the blood-brain barrier and allow pathogenic immune cells to traffic into the CNS.

Depending on the strain and myelin antigen used, mice can develop a monophasic, relapsing-remitting or chronic form of EAE. The model used in this study, the B6 mouse immunized with MOG_{35-55} peptide presents initial symptoms two weeks after immunization and develops into a chronic, sustained form of EAE. Pathologically, this model of EAE is characterized by multifocal, confluent areas of mononuclear inflammatory infiltration and demyelination in the peripheral white matter of the spinal cord. The initial symptoms begin with a loss of tail tonicity with increasing paralysis that progresses to the hind and fore limbs. Somewhat similar to the EDSS chart, increasing EAE paralysis is quantified by an EAE clinical score chart (Figure 5) [110].

EAE disease can also be induced in SJL mice by using the myelin peptide $PLP_{139-151}$. A relapsing-remitting disease develops with T-cells reactivity spreading to new myelin peptide determinants (epitope spreading) [111]. In this model, lesions can be found in the optic nerve,

brainstem, spinal cord, cerebellum and cerebral cortex. Infiltration involves perivascular and meningeal tissues by lymphocytes and neutrophils [105].

Figure 4: Active and Primary EAE Induction Scheme

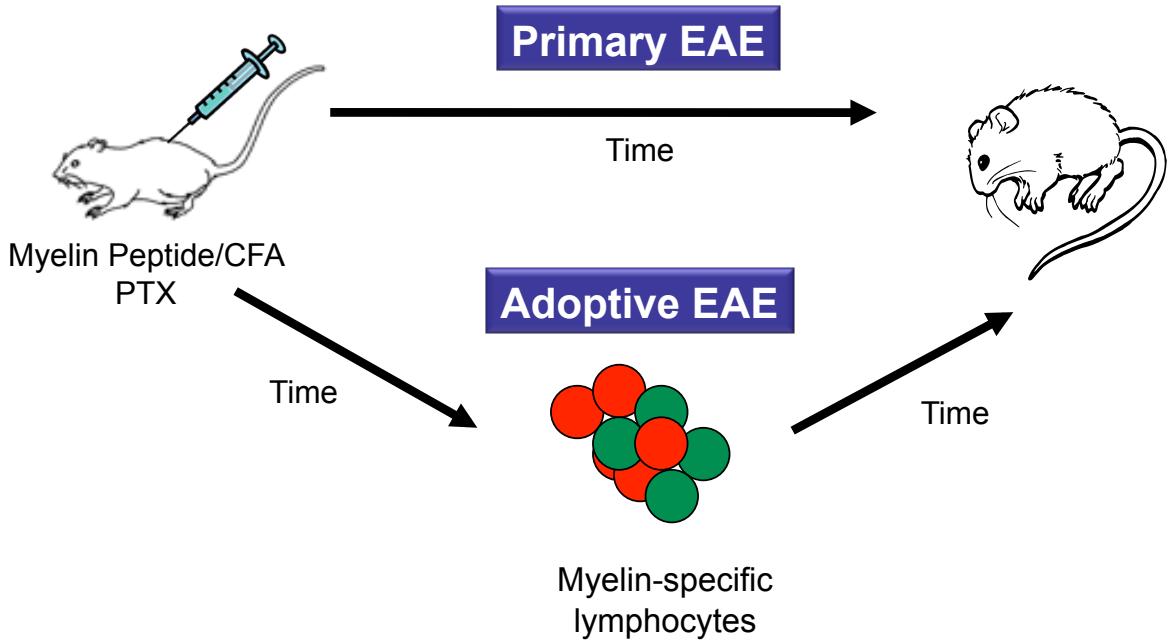
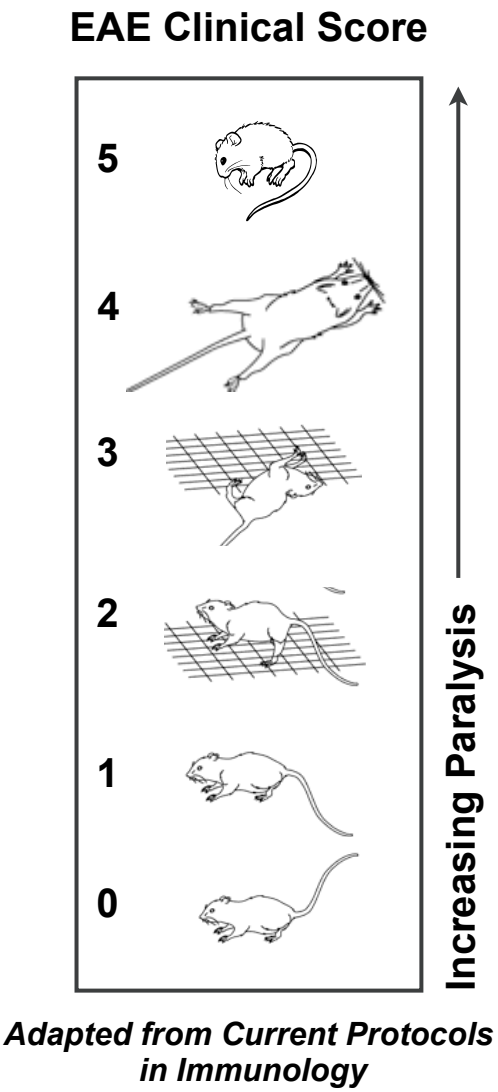


Figure 5: EAE Clinical Assessment Chart [110]



Adapted from Current Protocols in Immunology

1.7.2: Immunopathology of MS and EAE

EAE is an animal model of autoimmune inflammatory disease of the brain and spinal cord, and it resembles MS in many respects including immunologically, genetically and histopathologically [105].

EAE induction results in the generation of Th1 cells [112] which upon transfer are sufficient in inducing EAE [113]. But a new subset of T-cells, termed Th17 have been described to play a major role in EAE [114], although a transfer of pure Th17 (devoid of IFN-γ+ cells) cells does not induce disease [113], a combination of Th1:Th17 T-cell transfer can induce different forms of EAE, depending on the Th1/Th17 ratio [115], thus indicating that both subsets contribute to the initiation and progression of EAE. In fact, detectable levels of IL17 have been found in the CSF of MS patients, IFN-γ and IL17 producing T-cells have been associated with disease activity [91],[116],[117], and IL17 expression is found in brain MS lesions [118],[119], thus indicating that IL17 may be involved in the pathogenesis of MS.

CNS infiltration by Th1/Th17 has also been shown in EAE and MS [79]. The endothelial cell layers which surround CNS lesions have an increased expression of adhesion molecules in both MS and EAE studies [120]. Once infiltration has begun, CD4 T-cells are reactivated by microglia [81] presenting MHC-II associated peptides, and this stimulation allows them to produce pro-inflammatory cytokines, chemokines and MMP [82](51) which will break down the BBB and allow the recruitment of other immune cells such as: macrophages, B-cells and CD8 T-cells [18] (Figure 6). These new recruited cells are believed to augment the damage to CNS tissues through several different mechanisms [18]. The role of CD4 T-cells in CNS demyelinating disease is thought to be limited to recruitment of encephalitogenic immune cells [18] .

Macrophage infiltration has been shown to correlate with both EAE and MS disease severity. Upon activation, macrophages can produce neurotoxic agents such as MMPs, ROS or nitric oxide (NO), leading to CNS inflammation in MS and EAE [18].

CD8 T-cells, on the other hand, are believed to serve a role of tissue destruction. There have been reports of MHC-I allele association with disease [121], CD8 T-cells are enriched and expanded at the site of MS pathology [122], and adoptive transfer of CD8 T-cells has been shown to be sufficient in transferring EAE disease [18]. In fact, *in vitro* data have shown that CD8 T-cells are capable of cytotoxically targeting oligodendrocytes [123]. To the contrary, there have been reports that CD8 T-cells may be acting in a regulatory role, which is the focus of this study.

B-cells contribute to the CNS disease by producing autoantibodies, as well as acting as antigen presenting cells, which along with the production of NO from neutrophils can augment CNS damage [124]-[128].

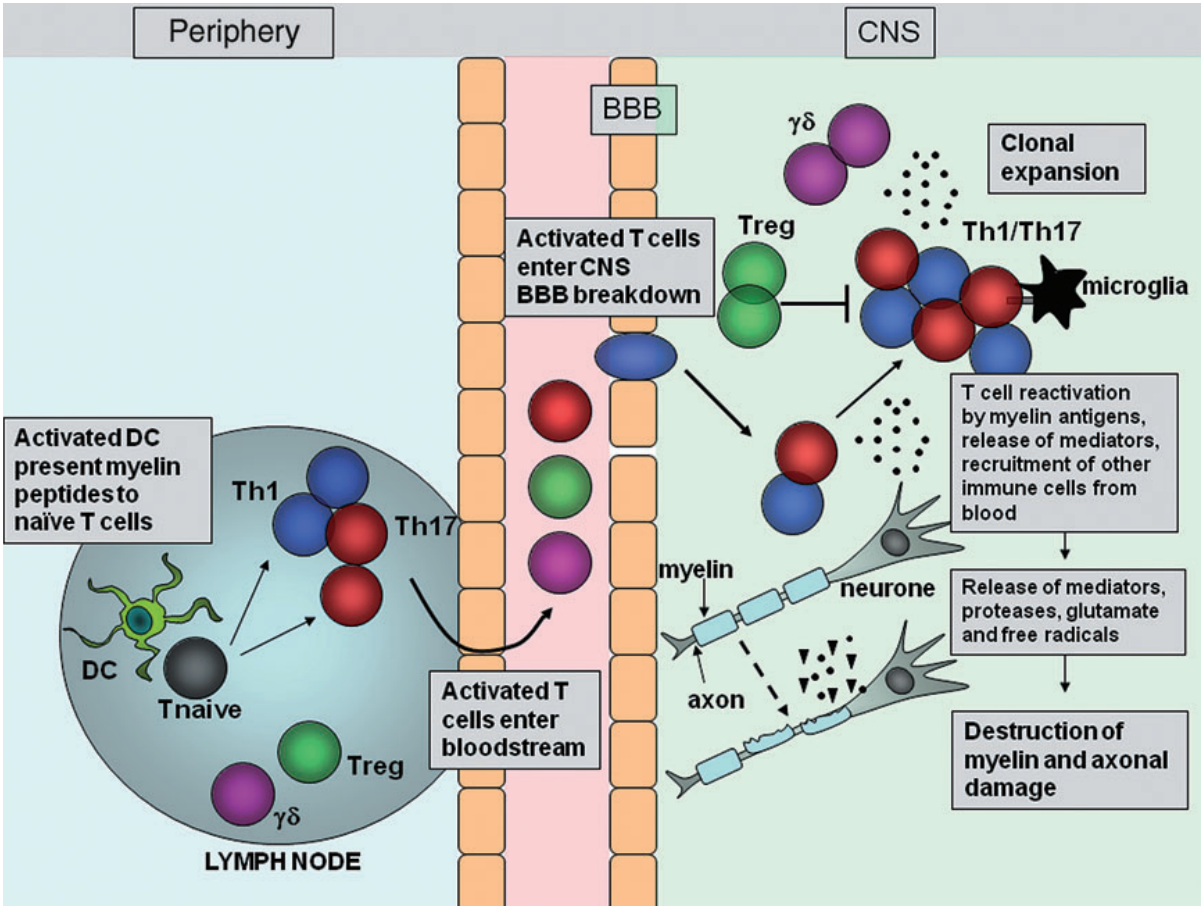
Although there are many immune cells that contribute to the CNS disease, CD4 T-cells have been implicated in the initiation as well as the maintenance of the disease. Adoptive transfer of CD4 T-cells specific to one myelin antigen has been shown to be sufficient for disease induction [108]. This is compounded by the phenomenon know as epitope spreading, where T-cells which were reactive to one myelin epitope, generate T-cells which can be reactive to other epitopes of the same antigen, or to other myelin proteins [129]. Furthermore, failure in the CD4 Tregs may also contribute to disease development. Loss of Foxp3 expression in mice leads to spontaneous disease [130] and similarly in human, it is believed that a defect in Foxp3 expression leads to a severe autoimmune disorder called immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). Furthermore, the adoptive transfer of CD25+ T-cells has been shown to be sufficient in reducing active EAE and in contrast, depletion of CD25+ cells leads to an exacerbation of disease [131].

EAE shares key pathological features with MS, such as a strong association with MHC-II allele and in certain strains of mice, females (B10.S, B10.PL and PL/J^b) are more susceptible than males [107]. The similarities also extend to environmental triggers where early infections or stimulation with superantigen trigger relapses. Clinically, depending on the immunizing antigen and strain of mouse, EAE can have the form of relapsing-remitting or progressive disease [112],[132].

The similarities continue into the histology, as EAE and MS are similar in inflammation, demyelination, axonal loss and gliosis of the CNS. There are similarities in white matter pathology with infiltration of Th1 T-cells, B-cells, CD4 and CD8 T-cells. Furthermore, there are antibodies to myelin in lesions [112] and there is a clonal expansion by both CD4 and CD8 T-cells to myelin components [112],[132].

These shared features have given investigators the opportunity to develop a hypothesis which is labeled the "three compartment hypothesis" in order to help explain the pathogenesis MS and EAE. This hypothesis proposes that autoreactive T-cells are activated in the peripheral immune compartment (first compartment) in EAE (immunization) or in MS (either by viral infection or trauma) [44],[80],[133]. The pro-pathogenic cells travel to the CNS, where they are reactivated by self-antigen and induce inflammation and demyelination (second compartment). Draining lymph nodes in the CNS clear the myelin debris, where new autoreactive T-cells with differing antigen specificity can be generated (third compartment). The new repertoire of autoreactive T-cells can now mitigate or exacerbate the on-going CNS disease [83],[105].

Figure 6: Migration and Effector T-cell Function in the CNS during EAE [77]



After immunization with myelin antigens, complete Freund's adjuvant (CFA) and pertussis toxin, dendritic cells (DC) are activated in the lymph nodes by Toll-like receptor (TLR) agonists within the mycobacterium tuberculosis component of CFA, and present myelin antigen to naive T cells. The activated myelin-specific T cells enter the bloodstream and traffic to and enter the CNS. Breakdown of the blood–brain barrier (BBB) occurs, allowing recruitment of other inflammatory cells into the CNS. T cells entering the CNS encounter their cognate myelin antigens and become reactivated by local APC. T cells expand and release inflammatory mediators which help recruit other immune cells to the site of inflammation. Activation of local microglial cells and infiltrating cells results in production of proteases, glutamate, reactive oxygen species and other cytotoxic agents which promote myelin breakdown. Damage to the myelin sheath surrounding axons is followed by axonal damage and neurological impairment. [77]

1.7.3: EAE and MS Treatment Success

In fact, due to similarities between MS and EAE, several first and second generation DMA were developed from observations in EAE. For instance, development of interferon-β stemmed from simultaneous MS and EAE studies. In 1982, IFN type 1 was found to reduce EAE

disease [134] while simultaneously intrathecal IFN-β was found effective in 5 out of 10 MS patients [135]. As previously discussed, GA was developed in 1971 as a putative encephalitogen but was found to inhibit EAE [136]-[138]. Development of natalizumab, stemmed from EAE research where blocking of the $\alpha_4\beta_1$ integrin inhibited lymphocyte trafficking into the CNS and thus EAE disease [139]. Although two initial studies revealed an exacerbation of MS [140],[141], natalizumab was licensed after successful disease amelioration in phase II and III trials [72],[142].

Furthermore, several other trial drugs have simultaneously shown disease amelioration in EAE and MS. Azathoprine [143]-[146] and mitoxantrone have shown considerable attenuation of disease [147]-[149]. Laquinimod and fingolimod have also translated well from EAE research and are awaiting approval.

1.7.4: Discrepancies between EAE and MS

There are cases where disease amelioration in EAE did not correspond with a reduction in MS. For instance, tolerance induction by oral administration of myelin antigen did show a reduction in EAE severity but studies in RRMS patients did not show a change in clinical disease, although immune modulation was evident [150]. IFN-γ treatment resulted in disease amelioration while IFN-$\gamma^{-/-}$ and signal transducer and activator of transcription-1 (STAT1) $^{-/-}$ mice exhibited a significant augmentation of clinical EAE disease [151]-[153]. The opposite was found in MS, where administration of IFN-γ increased patient relapse, and anti-IFN-γ antibody treatment suppressed MS [154]. Blockade of the p40 subunit (a subunit shared by IL12 and IL23 cytokines) with ustekinumab (human anti-p40 monoclonal antibody) resulted in no increase in clinical efficacy [155], although p40 knockout mice were found to be resistant to EAE induction.

Although this list of therapeutic failures is not complete, a possible source of discrepancy between model and disease may be attributable to methods of induction, type of EAE disease (primary vs. adoptive), clinical phase of disease (pre vs. established disease) and immunological system (murine vs. human).

1.8: CD8 T-cells in Autoimmunity

In 1969, Nishizuka and colleagues were the first to show that the immune system could not only cause autoimmunity, but it could also regulate it [156]. Shortly thereafter, in 1972, Gershon and colleagues published a seminal paper where for the first time they described a suppressor T-cell population that was able to "suppress" immune responses [157]. Later Cantor and colleagues revealed that suppressor CD8 T-cells target Qa-1 surface proteins [158]. Shortly thereafter, the suppressor T-cell research fell into disfavor as the technology at that time was not advanced enough to successfully isolate and phenotype the suppressor population. Eventually the spotlight was taken by the CD4+Foxp3+ Tregs described by Sakaguchi's ground-breaking work [159]. Now there is an increasing amount of evidence showing the role of suppressor CD8 T-cells, especially in several autoimmune diseases.

1.8.1: CD8 T-cells in Systemic Autoimmune Diseases

Rheumatoid arthritis (RA) is a systemic autoimmune reaction, which is primarily characterized by chronic inflammatory tissue destruction in the synovial lining of joints. The current hypothesis is that arthritogenic antigen(s) initiates a chronic immune response which contributes to uncontrolled expansion of the immune system and production of proinflammatory cytokines leading to inflammation in the synovium [160],[161]. Current treatments include the

use of monoclonal antibodies and engineered soluble receptor molecules whose role is to interfere with T-cell and B-cell function, as well as cytokine production. Recently, it has been shown that CD8+CD28-CD56+ T-cells, when transferred into a NOD-SCID chimera engrafted with human synovial tissues, were able to suppress inflammatory activity and decrease cytokine production within the synovial lesions [162]. Furthermore, tissue-derived IL16+CD8+ T-cells were successful in down-modulating the production of IL1β, IFN-γ and TNF-α [163]. In mice, Seo *et al.*, were able to generate CD8+CD11c+ T-suppressor cells by clonal expansion using anti-4-1BB antibody treatment. These cells were found to suppress CD4 T-cell responses to collagen type II-induced arthritis [164].

Systemic lupus erythematosus (SLE) is an autoimmune disease, which affects multiple systems, leading to pulmonary, cardiac, gastrointestinal and neurological involvement. Clinical presentation includes polyarthritis, Reynaud's phenomenon, renal disease, hematological manifestations and skin inflammation. Both the quality and length of life are severely affected in SLE. Current treatment involves the use of steroid and wide-spectrum immunosuppressants, with neither resulting in total amelioration of disease. Recently, Filaci *et al.,* demonstrated a loss of suppression by CD8 T-cells during patient relapse. Peripheral blood mononuclear cells were cultured with IL2 and GM-CSF. The suppressive ability of CD8 T-cells was tested by culturing with autologous PBMC, which were activated with anti-CD3 antibody. CD8 T-cells collected from relapsing patients were found to have a significant loss in suppressive ability as compared to stable or healthy controls [165]. In murine studies, Singh, Hahn, and colleagues used a synthetic peptide, called pConsensus (pCons), to tolerize lupus prone (NZB x NXW) F1 female (BWF1) mice [166],[167]. The tolerized mice showed an increase in the number of CD8+CD28- T-cells, which were able to inhibit CD4 T-cell and B-cell responses using TGF-β [168],[169]. In

fact, Kang *et al.,* were able to generate CD8 Ts cells by stimulating with nucleosomal histone peptide, $H_{471\text{-}94}$, a potential antigen target in SLE. Upon adoptive transfer, these CD8 Ts cells were able to suppress antibody production and nephritis [50].

Inflammatory bowel disease (IBD) includes ulcerative colitis and Crohn's disease, both major inflammatory autoimmune diseases affecting the gastrointestinal tract. In a mouse model of IBD, CD8+CD28- T-cells were found to reduce gut inflammation and this correlated with a reduction in IFN-γ producing CD4 T-cells and was dependent on IL10 [170]. In another study, Ho *et al.,* demonstrated that CD8+CD103+CD44lowCD62L+ T-cells were able to suppress disease as well as CD4 T-cell responses by a TGF-β dependent manner [171].

Diabetes mellitus type I (DMI) is characterized by the destruction of insulin producing β-cells in the pancreatic islets of Langerhans. Loss of tolerance to self, results in the production of antibodies and cellular mechanisms that lead to tissue destruction. Two important autoantigens described in the literature are glutamic acid decarboxylase (GAD), insulin and insulinoma-associated protein 2 (IA-2). James *et al.,* has reported that CD8+CD45RA+CD27- T-cells are capable of suppressing CD4 T-cell responses to GAD65 [172]. In another study by Herold *et al.,* DMI patients were treated with hOKT3y1 (ala-ala) and C-peptide. It was observed that even after a single treatment, insulin production improved over two years, [173]. In a later study, this beneficial effect correlated with an increase in CD8+CD25+Foxp3+CTLA-4+ Ts cells in the blood [174].

In summary, CD8 T-cells have been shown to have a regulatory role in several autoimmune diseases. More importantly, there is now evidence that disease initiating peptide induction of regulatory CD8 T-cells seems to provide an alternative treatment source for

autoimmune diseases, which offers the strength of suppressing only the pathogenic immune responses while maintaining antiviral and antitumor protective immune response.

1.8.2: CD8 T-cells in CNS Autoimmune Disease

A complete understanding of MS, an autoimmune-driven neurological disease necessitates an investigation into the role of those cells that are enriched at the site of pathology, the CD8 T-cells. This exploration is warranted by many factors: enrichment of cerebral spinal fluid (CSF) CD8 T-cells in MS patients [175], a profound infiltration of immune cells in the CNS parenchyma of MS patients and a predominance and oligoclonal expansion of CD8 T-cells in demyelinated lesions of MS patients [176],[177]. Murine studies have shown that CD8 T-cells may be mediators of disease, as shown in the C3H mouse strain using MBP-specific CD8 T-cell clones [178] and MOG-specific CD8 T-cells in C57BL/6 mice [179]. In contrast to those reports, there is an ever-increasing number of murine and human studies indicating that CD8 T-cells may also play a regulatory role. For instance, induction of MBP-mediated EAE in $CD8^{-/-}$ mice revealed a reduction in mortality but an increase in disease relapses, thus suggesting that CD8 T-cells could modulate disease remission [180],[181]. Similarly, mice that were unable to activate CD8 T-cells because they were classical and non-classical MHC-I deficient ($\beta 2m^{-/-}$) were found to have an increase in disease severity in both MOG_{35-55} and MBP-induced EAE [182]. Regulatory CD8 T-cells were found to modulate the phenotype of encephalitogenic CD4 T-cells in MBP-mediated EAE [183],[184] and the suppression was mediated by non-classical MHC-I (Qa-1) molecules presenting the Vβ8.2 chain of the TCR used by autoreactive CD4 T-cells. Selective deletion of the Qa-1 molecule, which was necessary for CD8 T-cell-mediated regulation resulted in an increase in disease as autoreactive CD4 T-cells were resistant to CD8 T-cell regulation [185]. Unspecified-antigen reactive regulatory CD8 T-cells were found to be

MHC Class Ib-restricted and could directly attenuate encephalitogenic CD4 T-cells [186]. Describing the phenotype of the regulatory CD8 T-cells has been a bit more challenging as there has been no consistent surface marker described. For instance, depletion of CD28+ cells in CD28-deficient mice lead to an increase in disease susceptibility [187]. In another study, induction of EAE results in a spontaneous induction of CD8+CD122+ regulatory T-cells, whose depletion resulted in the exacerbation of EAE symptoms, and adoptive transfer into recipient mice was found to suppress disease [188]. Human studies performed more than 20 years ago showed that in MS patients, CD8 T-cells were defective in their suppressor function, as compared to healthy controls [189],[190]. Subsequently, a single clone of CD8 T-cells was found to suppress autologous MBP-specific CD4 T-cells in a healthy donor [191]. Similar to murine studies, CD8 T-cells were found to target *in vitro* expanded myelin-specific CD4 T-cells by HLA-E (human homologue of Qa-1) presentation [192] and CD8+CD28- T-cells were detected in lower numbers in MS patients [193]. In fact, focusing on CD8 T-cells whose antigen specificity is targeted toward the site of pathology, the CNS-specific CD8 T-cells have only recently been shown to display regulatory properties. For instance, healthy and quiescent MS myelin-specific CD8 T-cells could suppress *in vitro* stimulated myelin-specific CD4 T-cells and this suppression was lacking in relapsing MS [194]. Furthermore, murine studies have revealed that wild type MOG_{35-55}-specific CD8 T-cells could significantly ameliorate EAE in B6 mice by modulating the antigen presenting cell (APCs) function and MOG_{35-55}-specific CD4 T-cell response [85].

Although autoreactive CD8 T-cells have been extensively studied as regulators of disease in murine Type 1 diabetes models [195] and rheumatoid arthritis [162],[163]; ***the mechanism used by neuroantigen-specific CD8 T-cells in the regulation of the autoimmune disease EAE,***

is still poorly understood. The importance of understanding these autoreactive CD8 T-cells is highlighted by our observations that both MS and healthy individuals harbor peripheral neuroantigen-specific CD8 T-cells [84] with homology to published TCR sequences from CNS-infiltrating T-cells in MS lesions [196] and relapsing MS patients exhibit a decrease in the suppressive ability of autoreactive CD8 T-cells [194]. Since these cells are already present at the site of pathology, are disease suppressive and are cytotoxic towards their target cell, these cells show characteristics, which would make them promising therapeutics. A more complete understanding of immune regulation by autoreactive CD8 T-cells becomes an important goal due to the significant therapeutic implications.

1.9: Objectives

Although our initial goal had been to explore the pathogenic role of these CD8 T-cells, our previous observations showed that these T-cells, when isolated from non-TCR manipulated mice, were capable of potently attenuating EAE. In this study, using permutations of myelin peptides and mouse strains, we further confirm the existence of disease-suppressing autoreactive CD8 T-cells. Using a combination of *in vivo* (knockout and transgenic mice), cellular and molecular assays, we now describe a novel and unexpected mechanism involved in the suppression of autoimmune demyelination via cytotoxic autoreactive regulatory CD8 T-cells. These studies are aimed at understanding the enigmatic autoreactive immune-regulatory mechanisms, which is essential in the design of effective immuno-therapies in MS, by addressing the following aims. The **first aim** will determine the overall role of myelin-specific CD8 T-cells by using several models of EAE, including $CD8^{-/-}$, $H\text{-}2^{b}$ (B6) and $H\text{-}2^{S}$ (SJL) mice (Chapter 3). The **second aim**, will focus on the $MOG_{35\text{-}55}$/B6 model and elucidate the cellular targets of autoregulatory T-cells (Chapter 4). Finally, the **third aim**, will incorporate a clinical focus by

evaluating the generation of autoregulatory CD8 T-cells, determining if these cells are clinically effective and explore possible means of augmenting clinical efficacy (Chapter5).

CHAPTER 2: MATERIALS AND METHODS

2.1: Mice

2.1.1: Maintenance and Ethics Approval

All experiments used female, six-eight week old mice which were housed in climate-controlled pathogen-free facilities under the supervision of certified veterinarians, maintained on a twelve-hour lights on/off cycle, and allowed food and water *ad libitum* at the UT Southwestern Medical Center Animal Resource Center and used according to approved IACUC protocols.

2.1.2: Mouse Strains

B6.129 $CD8^{-/-}$, B6.129 $\beta 2m^{-/-}$, C57BL/6-Tg(Tcra2D2,Tcrb2D2) and C57BL/6 $Prf^{-/-}$ were purchased from Jackson Laboratory (Bar Harbor, ME). B6.129 IFN-$\gamma^{-/-}$ were purchased from Jackson Laboratory and kindly provided by Dr. Jerry Niederkorn (UT Southwestern Medical Center, Dallas, TX). B6.129 $Tap^{-/-}$ mice were kindly provided by Dr. James Forman, (UT Southwestern Medical Center, Dallas TX). C57BL/$K^bD^{b-/-}$ mice were purchased from Taconic (Hudson, NY). Wild type (WT) C57BL/6 (B6) mice were purchased from Taconic and the UT Southwestern Mouse Breeding Core Facility (Dallas, TX). B6 Ly5.2/Cr and SJL/J (SJL) mice were purchased from National Cancer Institute (Bethesda, MD).

2.2: Buffers and Solutions

2.2.1: Buffers for Cell Purification

AutoMacs running buffer and MACS buffer
Bovine Serum Albumin
EDTA
In Dulbecco's phosphate buffered saline, sterilized with steriflip filter unit (Millipore), stored at 4°C

AutoMacs rinsing buffer
EDTA
In Dulbecco's phosphate buffered saline, sterilized with steriflip filter unit (Millipore), stored at 4°C

2.2.2: Buffers for Flow Cytometry

FACS Buffer
Sodium azide
Bovine serum albumin
EDTA
in Dulbecco's phosphate buffered saline, stored at 4°C

Fixing Buffer
Paraformaldehyde
EDTA
In Dulbecco's phosphate buffered saline, stored at 4°C

2.3: Proteins and Peptides

2.3.1: Neuroantigen and Control Peptides

Myelin oligodendrocyte glycoprotein 35-55-peptide (MOG_{35-55}), proteolipid protein 178-191-peptide ($PLP_{178-191}$), proteolipid protein 139-151-peptide ($PLP_{139-151}$), myelin oligodendrocyte glycoprotein 37-46-peptide (MOG_{37-46}), myelin oligodendrocyte glycoprotein 40-49-peptide (MOG_{40-49}), myelin oligodendrocyte glycoprotein 44-54-peptide (MOG_{44-54}), myelin oligodendrocyte glycoprotein 37-50-peptide (MOG_{37-50}) and ovalbumin 323-339 peptide were synthesized by UT Southwestern Protein Chemistry Technology Center according to

sequence listed on (Table 1). Purity of each peptide was between 70-95% as measured by MS and HPLC. All myelin peptides were dissolved in PBS and stored at -20°C until use in *in vivo* or *in vitro* assays.

Table 1: Amino Acid Sequence of Neuroantigen and Control Peptides

Name	Sequence	Purity
MOG_{35-55}	MEVGWYRSPFSRVVHLYRNGK	≤70%
$PLP_{178-191}$	NTWTTCQSIAFPSK	≤90%
$PLP_{139-151}$	HSLGKWLGHPDKF	≤70%
MOG_{37-46}	VGWYRSPFSR	≤90%
MOG_{40-49}	YRSPFSRVVH	≤90%
MOG_{44-54}	FSRVVHLYRNG	≤90%
MOG_{37-50}	VGWYRSPFSRVVHL	≤90%
$OVA_{323-339}$	ISQAVHAAHAEINEAGR	≤90%

2.3.2: Proliferation Peptide

Concanavalin A (conA, SIGMA) , Streptococcocus Enterotoxin B (SEB, SIGMA) and murine anti-CD3 (aCD3, BD Biosciences) were used as a proliferation controls in certain assays. Peptides were dissolved in PBS and stored at -20°C until use in *in vivo* or *in vitro* assays.

2.4: EAE induction

2.4.1: Active EAE and Evaluation

Primary B6-EAE was induced using MOG_{35-55} or $PLP_{178-191}$. On day of immunization, (Day 0), B6 mice were subcutaneously immunized with 200 µg of MOG_{35-55} (in later experiments, 100 µg of peptide was used due to disease severity) or 100 µg of $PLP_{178-191}$ in complete Freund's adjuvant (CFA) supplemented with 4 mg/ml mycobacterium tuberculosis (MTB, H37Ra, Difco, Detroit, MI). The components were emulsified using one homogenization

cycle of 100 seconds. An additional 30 second cycle was performed if solution was not of proper consistency. The mice received 50 µl of emulsion subcutaneously in each hind limb flank, for a total of 100 µl volume per mouse. Additionally, at day 0 and 2, mice were administered 250 ng of pertussis toxin (PTX, List Biological Laboratories, Campbell, CA) via intraperitoneal (i.p.) injection.

Primary SJL-EAE was induced by either subcutaneous immunization of SJL/J mice with 100 µg of $PLP_{139-151}$, or $PLP_{178-191}$ at day 0. Only $PLP_{178-191}$ induced EAE received administration of 250 ng of pertussis toxin at days 0 and 2.

Clinical EAE disease was assessed using the following criteria; 0, no paralysis; 1, loss of tone in the tail; 2, mild hind limb weakness; 3, significant hind limb paralysis; 4, complete hind limb paralysis; 5, hind limb paralysis and forelimb weakness/moribund/death (Figure 5). When appropriate, each experimental condition was represented across multiple cages and evaluator was blinded to experimental condition, i.e. 2-way blinded EAE scoring.

2.4.2: Adoptive EAE

Lymph node cells from day 10 post- MOG_{35-55} immunized B6 mice were harvested and incubated for 72 hours at 37 °C in EAE culture media (RPMI medium supplemented with 10% fetal calf serum (FSC), L-glutamine, penicillin, streptomycin, HEPES buffer, non-essential amino acids, sodium pyruvate and β-mercaptoethanol) containing 20 µg MOG_{35-55} and murine rIL-12 (10 ng/mL). CD4+ T cells were obtained using anti-CD4 (L3T4) microbeads (Miltenyi Biotech, Germany) and a total of $5x10^6$ live CD4+ T-cells were injected i.p. into naive, wild type B6 mice at day 0. Pertussis toxin was administered on day 0 and 2 and EAE disease monitored daily.

2.5: Tissue Harvest and Processing

2.5.1: Preparation of Cells from Spleen and Lymph Nodes

Mice were euthanized by carbon dioxide asphyxiation. For preparation of single cell suspension, spleen and lymph node were processed through a 70 µm nylon cell strainer (BD Bioscience) using the back end of a 3 ml syringe (BD Biosciences). Lymph node cells were washed twice by resuspending cells in EAE culture media and centrifuged at 1500 rpm for 5 mins at 27°C. Similarly, single cell suspension from spleen tissues were washed twice, overlaid onto 20 ml of Lympholyte-M (Cedarlane Laboratories Ltd,. Burlington, NC), in order to remove dead/unwanted cells. Suspension was centrifuged at 1500 x g. for 20 mins at room temperature (rt). Afterwards, mononuclear cells were collected form the interface layer and placed in a new 50 ml conical tube (BD Bioscience) and washed twice with EAE culture media.

For counting the cells, both lymph node cells and splenocytes were combined, and a 20 µl sample of the cell suspension was mixed with 180 µl of 0.25% trypan blue counting solution (Invitrogen). Using a hemocytometer (HyClone), visibly round and clear cells were counted under 100x magnification with an Olympus inverted microscope (Olympus).

2.5.2: Preparation of Cells from Blood

Blood was collected from mice either by tail vein or submandibular bleed. For tail vein bleed, mice were placed under a heat source for 4 minutes to allow dilation of the lateral vein. The distal 1-2mm section of the tail was removed using a sterile blade and 4-5 drops of blood were collected into FACS tubes containing 200 µl of acid citrate dextrose (ACD) solution. The tubes were maintained at rt until further use.

For collecting blood by submandibular bleed, mice were laid on their side and a lancet was used to perforate the submandibular vein located at the back of the jaw of the mouse, behind the hinge of the jawbone. Using a FACS tube, 4-5 drops of blood were collected into FACS tubes containing 200 µl of ACD solution. The tubes were maintained at rt until further use.

2.5.3: Preparation of Cells from CNS

For isolation of mononuclear cells from CNS, brain and spinal cord tissues were processed with 70 µm nylon cell strainer into a single cell suspension and resuspended in 20 mL of sterile PBS. Suspension was centrifuge for 15 mins at 2120 x g at rt. Cell pellet was resuspended in 5 ml of 30% Percoll and centrifuge for 15 mins at 2120 x g at rt with centrifugal brakes off. Lipid layer was suctioned and cell pellet and about 1 ml of PBS was left intact. Cells were resuspended and transferred to a new sterile 50 ml conical tube. Tube was filled with PBS to quantity sufficient (QS) and centrifuged for 5 mins at 1500 rpm.

2.6: CD8 T-cell Adoptive Transfer

Lymph nodes and splenocytes from myelin peptide-immunized mice were stimulated with cognate antigen and murine rIL-2 (10 ρg/ml) for 72 hours at 37 °C in a culture flask at 7.5 x 10^6 / ml concentration. Highly purified (TRCβ+CD4-CD8+) CD8 T-cells (Figure 15) were obtained using anti-CD8 (Ly-2) microbeads (Miltenyi Biotech, Germany) and a total of $5x10^6$ live CD8 T-cells were injected via tail vein intravenous injection. After 24 hours, primary or adoptive EAE was induced and clinical disease evaluated.

2.7: Proliferation Assays

2.7.1: CFSE-based Proliferation

Antigen specific responses were evaluated using the CFSE-based dilution assay using bulk splenocyte and lymph node cells from myelin peptide immunized mice. Bulk cells were suspended at a 1 x 10^6/ml concentration in PBS and incubated for 7 mins with 0.25 μM CFSE. Next, these cells were washed twice with serum-containing media and resuspended at 2x10^6/ml concentration in EAE culture media. Cells were activated with cognate antigen (MOG_{35-55}, MOG_{37-44}, $PLP_{139-151}$, $PLP_{178-191}$ or $OVA_{323-339}$) at 20 μg/ml and mIL-2 at 10 ρg/ml at 37ºC in 5% CO_2 for 5 days. Subsequently, cells were washed with FACS staining buffer, incubated for 5 mins at 4°C with mouse FcR blocking reagent (Miltineyi Biotec) and labeled with anti-CD8, anti-CXCR3, anti-CTLA-4), anti-CD4, anti-CD62L, anti-CCR7, anti-CD122, anti-CD44, anti-CD25, anti-PD-1, anti-TCRβ, anti-CD90.2, anti-CD11c, anti-NKG2D, anti-CD94 and anti-CD103 fluorescent antibodies (Table 3). After a 45 mins incubation at 4 °C, cells were washed with staining buffer and fixed in 1% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA.) Flow cytometric data were acquired using a BD LSR II flow cytometer using FACS Diva 5.0 software. FlowJo 9.0 (Tree Star, Ashland OR) software was used to gate on Live Gate+ TCRβ+ CD4-CD8+ or Live Gate TCRβ+ CD8- CD4+ T-cell subsets and analyzed cognate antigen specific responses within the CFSE low population.

2.7.2: ^{3}H Thymidine Proliferation Assay

Lymph nodes or spleens were harvested and processed into single cell suspensions. CD8 or CD4 T-cells were magnetically purified using the positive selection protocol (Miltenyi Biotec). APCs were isolated by T-cell depleting splenocytes and irradiated with 3500 rads. Bulk or

purified T-cells were incubated in 96 well plates for 72 hours at a concentration of $2x10^5$ with APCs. The cells were then pulsed with 0.5 μCi/well with [3H]-methyl-thymidine for 16 hours. Cells were then washed and harvested onto a glass fiber mat and allowed to dry for 24 hours overnight. [3]-methyl-thymidine incorporation was detected using a Betaplate counter (Wallac).

2.8: Flow Cytometry

2.8.1: Intracellular Cytokine Staining

Following *in vitro* stimulation with cognate antigen, cells were re-stimulated with 25 ng/ml of phorbol myristate acetate (PMA, Sigma), 1μg/ml of Ionomycin (IO, Sigma) and 10 μg/ml of brefeldin-A (BFA, Sigma) at 37ºC in 5% CO_2 for 5 hours. Next, cells were permeabilized and fixed using murine Foxp3 staining buffer set (Miltenyi Biotec) as per manufacture's instructions. Cells were then stained for IFN-γ, TNF-α, IL17, IL10, IL4, GM-CSF, perforin and Foxp3 with fluorescent antibodies (Table 2), fixed with 1% paraformaldehyde and flow cytometric data were acquired within 24 hours.

Table 2: Intracellular Staining Panel

Antibody	Clone	Manufacturer
PE-Cy7-anti-IFN-γ	XMG1.2	BD Bioscience
PE-Cy7-anti-TNF-α	MP6-XT22	BD Bioscience
PE-anti-IL17A	TC11-18H10	BD Bioscience
APC-anti-IL10	JES5-16E3	BD Bioscience
APC-anti-IL4	11B11	BD Bioscience
PE-anti-GM-CSF	MP1-22E9	BD Bioscience
PE-anti-perforin	eBioOMAK-D	eBioscience
APC-anti-Foxp3	FJK-16s	eBioscience

2.8.2: Surface Marker Staining

Following *ex vivo* cell harvest or post *in vitro* stimulation with cognate antigen, cells were washed with FACS buffer and centrifuged at 1500 rpm for 5 mins at 4°C. Nonspecific staining was blocked by incubating cells with mouse FcR blocker (Miltenyi Biotec) for 5 mins. at 4°C. Cells were then stained with anti-TCRβ, CD4 and CD8 for subsetting and anti-CD122, CCR7, CXCR3, CTLA-4, PD-1, CD11c, CD62L, CD44 and CD25 antibodies (Table 3) for 30 mins at 4°C in the dark. Cells were then washed and fixed with 1% paraformaldehyde and flow cytometric data were acquired within 24 hours.

Table 3: Surface Marker Staining Panel for Murine Cells

Antibody	Clone	Manufacturer
PE-Cy5-anti-TCRβ	H57-597	BD Biosciences
APC-Cy7-anti-CD4	GK1.5	BD Biosciences
eFluor-605NC-anti-CD8	53-6.7	eBioscience
PE-anti-CD122	5H4	BD Biosciences
Percp-Cy5.5-anti-CCR7	4B12	BD Biosciences
Percp-Cy5.5-anti-CXCR3	CXCR3-173	eBioscience
PE-anti-CD94	18d3	BioLegend
APC-anti-CTLA-4	UC10-4B9	eBioscience
PE-anti-PD-1	J43	BD Biosciences
PacBlue-anti-CD11c	N418	BioLegend
V450-anti-CD62L	MEL-14	BD Biosciences
APC-anti-CD44	IM7	BD Biosciences
PE-Cy7-anti-CD25	PC61	BD Biosciences
Alexa700-anti-CD90.2	30-H12	BioLegend
APC-anti-NKG2D	CX5	BioLegend
PacBlue-anti-CD103	2E7	BioLegend

2.8.3: Instrument and Analysis

A customized BD Bioscience LSR II high parametric desktop flow cytometer was used with FACS Diva 6.0 software for data acquisition. The LSR II was equipped with four fixed-

alignment, air-cooled lasers. Photon acquisition was performed by a collection of optics including two octagon and 2 trigon optical arrays and 17 photo multiplier tubes (PMT). Flow cytometric data were analyzed using Flow Jo Software V9.0 (Treestar).

Table 4: Optics Setup for the BD LSR II Flow Cytometer

Laser	Flourophore	Filter
488 (Blue)	Percp, PerCp-5.5	675/40
	FITC, CFSE	515/20
	SSC	488/10
405 (Violet)	Qdot 705	710/50
	Qdot 655	660/40
	Qdot 605, eNC605	605/40
	Qdot 585	585/42
	Qdot 560	580/40
	Am-Cyan	515/20
	PacBlue, V450	450/50
532 (Green)	PE-Cy7	780/60
	PE-Cy5.5	685/35
	PE-Cy5	660/20
	PE-Texas Red	610/20
	PE	575/26
635 (Red)	APC-Cy7	780/60
	Alexa 700	730/45
	APC	660/20

2.9: *In vivo* CD4 T-cell Suppression Assay

For CD8 donor cells, congenic Ly5.2+ (CD45.1+) B6 mice were immunized with MOG_{35-55} emulsion and PTX, as previously described. At day 20, draining lymph nodes and spleens were harvested and single-cell suspensions prepared. These cells were then placed into culture for 3 days in EAE culture media with stimulating antigen (MOG_{35-55} or OVA323-339) at 20 ug/ml and murine rIL-2 (10 pg/ml). Post *in vitro* stimulation, dead cells were removed using

Dead Cell removal kit (Miltenyi Biotec) and CD8+ T-cells isolated using CD8 (Ly-2) microbeads (Miltenyi Biotec) as per manufacturer's protocol. The purity of CD8 T-cells was consistently higher than 95%. A total of 5 x 10^6 CD8 T-cells were injected intravenously into naive, wild-type B6 female mice at day -1.

For CD4 donor cells, congenic Ly5.2+ (CD45.1+) B6 mice were immunized with MOG_{35-55} emulsion and PTX, as previously described. At day 10, draining lymph nodes were harvested and single-cell suspensions prepared. These cells were then placed into culture for 3 days in EAE culture media with stimulating antigen (MOG_{35-55}) at 20 ug/ml and murine rIL-12 (10 pg/ml). Post *in vitro* stimulation, dead cells were removed using Dead Cell removal kit (Miltenyi Biotec) and CD4+ T-cells isolated using CD4 (L3T4) microbeads (Miltenyi Biotec). The purity of T cells was consistently higher than 95%. A total of 5 x 10^6 CFSE stained CD4 T-cells were injected i.p. into naive, wild-type B6 female mice at day 0 (in some later experiments WT (CD45.2+) T-cells were transferred into congenic Ly5.2 (CD45.1+) host). Subsequently, at day 10 and 20, mice were anesthetized with 100 µl of 1.5% Avertin, perfused with 20 ml of cold PBS via left ventricle puncture. Brain and spinal cord tissues were harvested and processed via 30% Percoll gradient. Cervical and inguinal lymph nodes and spleens were harvested and processed using EAE washing media, followed by RBC lysis buffer and subsequent wash. Once all tissues where processed into single-cell suspension, cells where washed with FACS buffer and incubated for 5 minutes at 4°C with mouse FcR blocking reagent (Miltneyi Biotec). Cells were then stained with APC-anti-45.1, Percp5.5-anti-CD45.2, PacBlue-anti-CD4, PE-Cy7-anti-CD8 (BD Biosciences), incubated for 45 mins. in 4°C, washed with staining buffer and fixed with 1% PFA.

2.10: *In vivo* CNS CD8 T-cell Trafficking Assay

Congenic Ly5.2+ (CD45.1+) B6 mice were immunized with MOG_{35-55} emulsion and PTX, as previously described. At day 20, draining lymph nodes and spleens were harvested and single-cell suspensions prepared. These cells were then placed into culture for 3 days in EAE culture media with stimulating antigen (MOG_{35-55} or OVA323-339) at 20 ug/ml and murine rIL-2 (10 pg/ml). Post *in vitro* stimulation, dead cells were removed using Dead Cell removal kit (Miltenyi Biotec) and CD8+ T-cells isolated using CD8 (Ly-2) microbeads (Miltenyi Biotec) as per manufacturer's protocol. The purity of CD8 T-cells was consistently higher than 95%. A total of 5 x 10^6 CD8 T-cells were injected intravenously into naive, wild-type B6 female mice at day 0 (in some later experiments WT (CD45.2+) T-cells were transferred into congenic Ly5.2 (CD45.1+) host). Subsequently, at day 10 and 20, mice were anesthetized with 100 µl of 1.5% Avertin, perfused with 20 ml of cold PBS via left ventricle puncture. Brain and spinal cord tissue were harvested and processed via 30% Percoll gradient. Cervical and inguinal lymph nodes and spleens were harvested and processed using EAE washing media, followed by RBC lysis buffer and subsequent wash. Once all tissues where processed into single-cell suspensions, cells where washed with FACS buffer and incubated for 5 minutes at 4°C with mouse FcR blocking reagent (Miltneyi Biotec). Cells were then stained with APC-anti-45.1, PerCp5.5-anti-CD45.2, PacBlue-anti-CD4, PE-Cy7-anti-CD8 (BD Biosciences), incubated for 45 mins in 4°C, washed with staining buffer and fixed with 1% PFA.

2.11: *In vitro* Killing Assay

As described previously [61] but adapted for murine cells, cytotoxic MOG-specific CD8 T cells were obtained using splenocytes from day 12-immunized mice, which were *in vitro*

activated and expanded for 7 days in MOG_{35-55} at 20 µg/mL and purified using negative selection CD8 T-cell Isolation Kit (Miltenyi Biotec, Germany). Target splenocytes were harvested from naive WT mice at day 18 and incubated with MOG_{35-55} (20 µg/mL) and concanavalin A (ConA) at 0.5 µg/ml in 37ºC and 5% CO_2 and the following day CFSE stained. CFSE stained (targets) cells were resuspended in a 96-well plate at 5000 cells/well. Effector cells were suspended with targets at 0:1; 1:1; 4:1; 16:1; 64:1 ratio in 200 µl of EAE culture media. Following 24 hour incubation at 37ºC and 5% CO_2, wells were seeded with a constant number of fluorescent allophycocyanin beads, as an external counting control (BD Biosciences) and data immediately collected on a BD FACS Calibur flow cytometer. For control purposes, a redirected cell lysis using a mastocytoma cell line (P815) was incubated with murine anti-CD3 (1 µg/mL) and effector CD8 T-cells at indicated ratios. Percent killing was calculated as previously described [61].

2.12: Histology

Mice were anesthetized using 1.5% Avertin and efficacy of anesthesia asserted by hind limb pinch test. Similar to CNS isolation method, systemic perfusion was performed by left ventricular puncture using 20 ml of cold 4% PFA. Vertebral column was dissected from about c7 to sacrum and allowed to set in 4% PFA for 24 hours in the dark. Spinal cord was dissected by performing a laminectomy and gently removing the intact cord and placing in cold PBS. Sectioning, luxol fast blue (LFB) and hematoxylin and eosin (H&E) staining were performed by the histology core lab.

2.13: Statistical Analysis

Statistical analyses between groups were performed using GraphPad Prism 5.0c. Difference in disease severity, peak and onset were evaluated using two-tailed Student's t-test. A p value ≤ 0.06 was considered borderline significant (labeled *), while a p value ≤ 0.05 was considered statically significant (labeled **).

CHAPTER 3: CHARACTERIZING AUTOREACTIVE CD8 T-CELLS IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS, AN MS MODEL

3.1: Introduction

3.1.1: CD8 T-cells in Autoimmunity

MS and EAE are considered to be diseases primarily driven by T-cells. Indeed there is some literature supporting the hypothesis that autoreactive CD8 T-cells contribute to CNS damage in EAE [178],[179],[197]. However, there is increasing evidence that CD8 T-cells can have a disease suppressing role in other immune diseases [162],[169],[195],[198],[199], as well as in EAE and MS [85],[188],[192],[194],[200]. Although this role may be counter intuitive to current immunological thinking, these recent observations warrant an investigation into these enigmatic autoreactive, yet disease ameliorating CD8 T-cells.

3.1.2: Objectives

Autoreactive CD8 T-cells, contrary to current immunological understanding, are increasingly being described as suppressors of autoimmune disease [17],[85],[201]. However, it is unclear how autoregulatory CD8 T-cells are mediating disease suppression. The primary aim of the experimental work presented in this chapter is to characterize the EAE ameliorating autoregulatory CD8 T-cells. The second objective is to elucidate mechanism(s) used for EAE suppression. The characterization and understanding of the mechanism(s) of suppression will further the development of improved therapeutics.

3.2: Results

3.2.1: $CD8^{-/-}$ B6 Mice Exhibit Increased Disease Severity in MOG_{35-55}-induced EAE

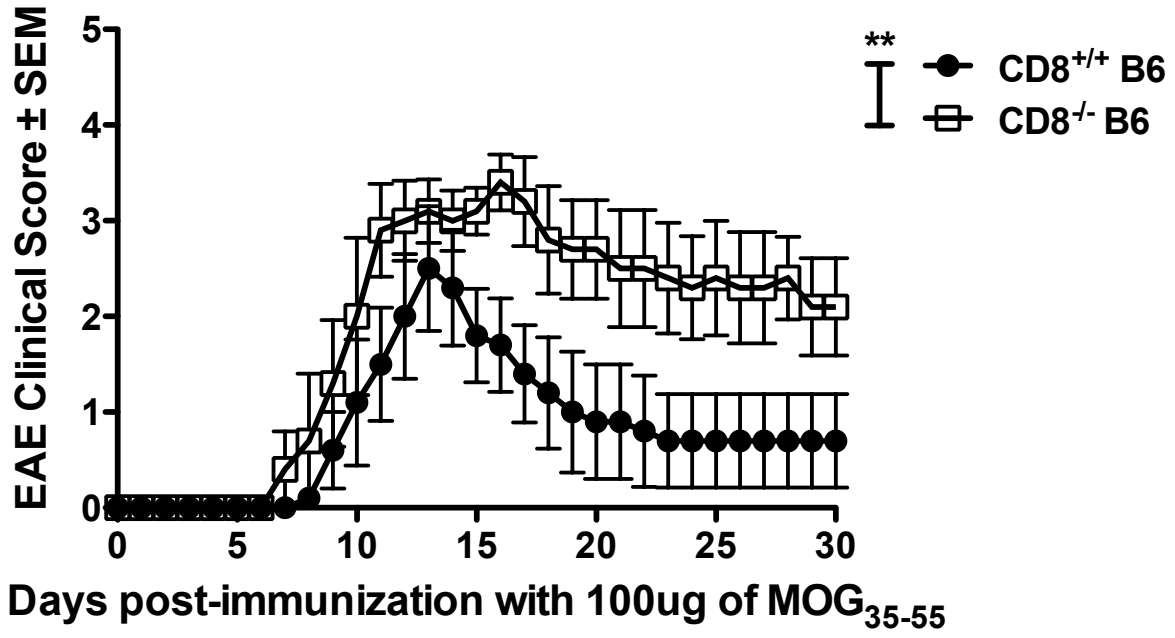
Induction of primary MBP-mediated EAE in $CD8^{-/-}$ mice has been shown to increase disease relapses [181]. Thus, I wanted to ascertain if the B6/MOG_{35-55} EAE model exhibited a similar alteration of disease severity. First, I confirmed $CD8^{-/-}$ EAE susceptibility in the B6 model by inducing EAE using a wide range of immunizing antigen dosage. Using $CD8^{-/-}$ and $CD8^{+/+}$ (littermates) B6 mice, I induced primary EAE using the standard dosage of 200 μg (1X), 100 μg (0.5X) and 50 μg (0.25X) of MOG_{35-55} peptide per mouse on day 0. All mice received equal concentrations of pertussis toxin (PTX) at days 0 and 2. Significant clinical differences were observed between littermate controls and $CD8^{-/-}$ mice and these differences were found to increase as neuroantigen dosage decreased. At mid-optimal concentration, the recovery (day 15-20 post immunization) and chronic (day 20+) phases of EAE were decreased in severity in CD8 competent mice as compared to $CD8^{-/-}$ mice (Figure 7). In CD8 competent mice, as peptide concentration decreased, incidence and peak disease severity decreased (100% vs. 80% and 4.3 ± 0.2 SEM vs. 3.05 ± 0.6 SEM), while day of onset increased (9.5 ± 0.2 SEM vs. 15.3 ± 2.8 SEM). Conversely, in the absence of CD8 T-cells, although immunizing antigen dosage was decreased to 25% of optimal concentration, incidence (100% vs. 100%), mean maximum score (3.6 ± 0.3 SEM vs. 4.35 ± 0.2 SEM) and mean day of onset (9.4 ± 0.3 SEM vs. 9.5 ± 0.5 SEM) were maintained at comparable levels (Figure 8). These observations were confirmed by antibody-mediated depletion of CD8 T-cells in WT mice. Peripheral blood sampling confirmed CD8 depletion, which was maintained up to day 10 (Figure 9). Compared to IgG controls, CD8 T-cell depleted mice were found to exhibit an earlier day of onset, as well as an increase in disease severity at the acute and chronic phase of EAE (Figure 10). Specifically, in CD8-depleted mice

mean maximum score decreased slightly (3.4 ± 0.1 SEM vs. 2 ± 0.02 SEM) while in IgG treated mice the mean maximum score decreased robustly (4.1 ± 0.2 SEM vs. 0.4 ± 0.06 SEM), as antigen concentration decreased. Mean day of onset in CD8 depleted mice was maintained at comparable levels (9.4 ± 0.2 SEM vs. 15.4 ± 0.3 SEM), while in IgG treated mice the day of onset increased markedly (9.4 ± 0.2 SEM vs. 26.2 ± 2.8 SEM) as antigen concentration decreased (Figure 11). These data confirm that CD8 T-cells play a regulatory role in autoimmune demyelinating disease and mice deficient in CD8 T-cells become highly susceptible to induction of paralysis, even at suboptimal antigen concentrations.

In order to confirm that loss of CD8 T-cells did indeed alter EAE susceptibility, I initially performed a rescue experiment, were I tried to reconstitute $CD8^{-/-}$ mice with naïve CD8 T-cells by adoptive transfer. Although transfer of naïve CD8 T-cells was successful, engraftment of transferred tissue was unsuccessful, possibly due to missing endogenous factor(s), which stabilize the CD8 T-cell population. Next, the experiment was adjusted by reconstituting $CD8^{-/-}$ B6 mice with autoregulatory CD8 T-cells, inducing primary EAE and comparing clinical disease relative to control recipients. Analysis of peripheral blood confirmed successful transfer and maintenance of CD8 T-cells (Figure 12). In $CD8^{-/-}$ mice, clinical disease in MOG_{35-55}-specific CD8 T-cell recipients was found to be significantly suppressed compared to the $OVA_{323-339}$-specific CD8 T-cell recipients (Figure 13).

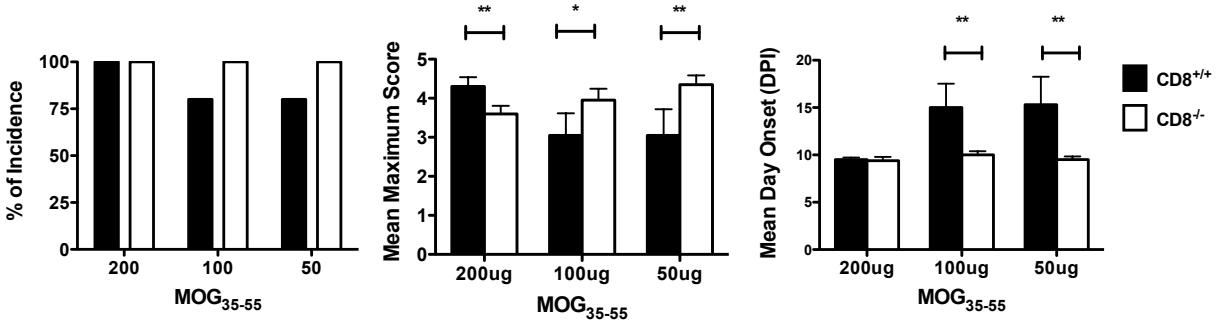
Taken together, these data indicate that in the absence of endogenous CD8 T-cells, the induction of EAE was significantly augmented, thus suggesting that CD8 T-cells are acting in a regulatory role. This increase in disease severity was not due to some altered immunological adaptation caused by genetic modification, as autoregulatory CD8 T-cells were found to be sufficient in reversing EAE disease severity in $CD8^{-/-}$ mice.

Figure 7: Increase in Primary EAE Severity in $CD8^{-/-}$ Mice.



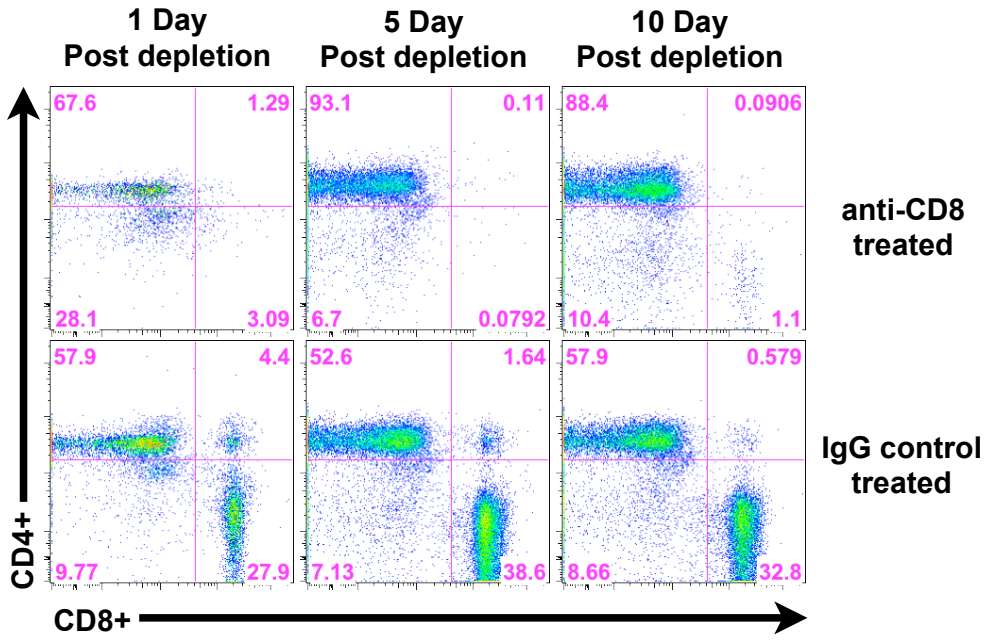
Primary EAE severity was compared between $CD8^{+/+}$ and $CD8^{-/-}$ mice. Naïve $CD8^{-/-}$ or $CD8^{+/+}$ mice were immunized with 100 µg of MOG_{35-55} peptide containing emulsion subcutaneously. All mice received 250 ng of PTX on days 0 and 2. Clinical disease was assessed for 30 days using our 2-way blinded scheme. Data are representative of four independent experiments, 5-7 mice per condition. The mean EAE score is shown with error bars representing ± standard error of the mean (SEM). **=$p \leq 0.05$.

Figure 8: Clinical Disease Differences Increase as Immunizing Antigen Concentration Decreases in CD8$^{-/-}$ versus WT Mice



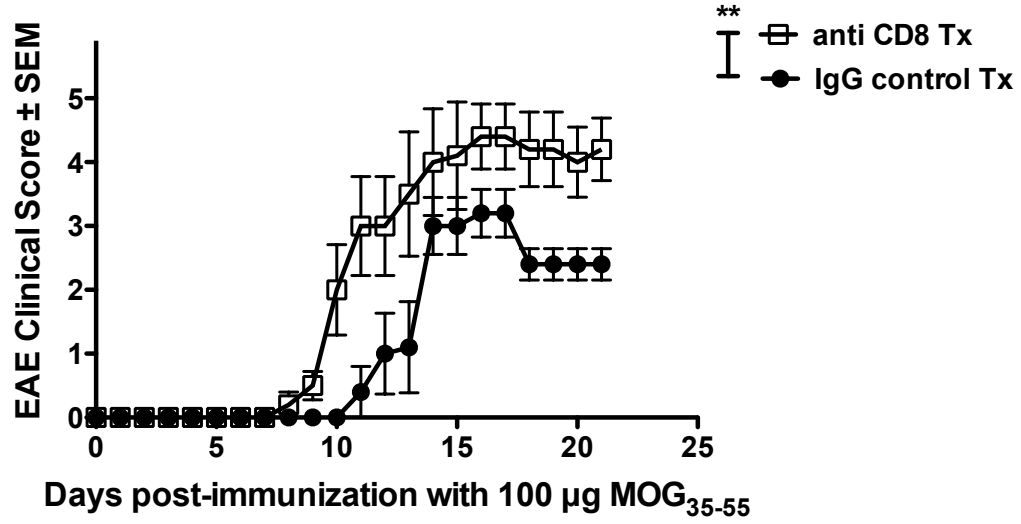
Clinical disease differences were compared between WT and CD8$^{-/-}$ mice. Data were obtained from Figure 1 experiments. % of incidence is the percentage of mice which developed EAE paralysis irrespective of disease severity. Mean maximum score is the average of the highest EAE score each mouse attained. Mean day onset is an average of the day in which each mouse first developed EAE symptoms. Data are representative of two independent experiments, 5-7 mice per condition. DPI=days post immunization, *=p<0.06, **=p≤0.05.

Figure 9: Removal of CD8 T-cells by anti-CD8 Depleting Antibody



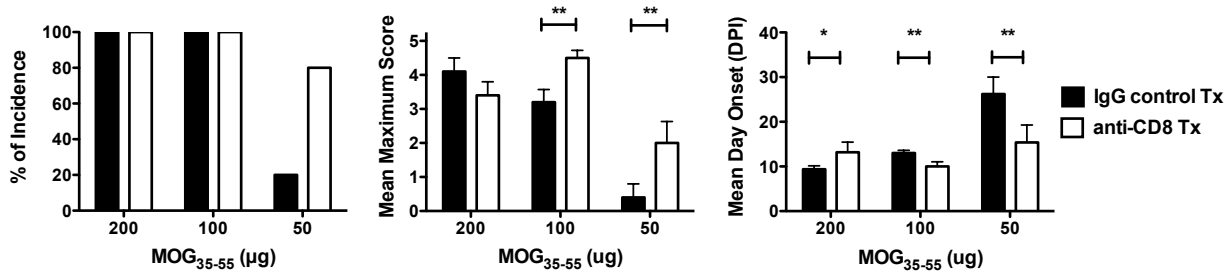
Successful CD8 T-cell depletion was assessed by sampling peripheral blood. Naïve B6 mice were treated with 200 µg of CD8 depleting antibody or isotype control i.p. Assessment of *in vivo* CD8 depletion was assessed one day post-treatment by submandibular bleed. A blood sample of approximately 100µl was collected in acid citrate dextrose containing tubes. Red blood cells were lysed for 4 minutes with lysis buffer and washed twice with FACS buffer. Cells were then stained with anti-TCRβ, CD4 and CD8 antibodies and fixed. Dots plots show cells that have been gated for TCRβ expression. Data are representative of three independent experiments, 5-7 mice per condition.

Figure 10: *In vivo* Depletion of CD8 T-cells Augments EAE Severity



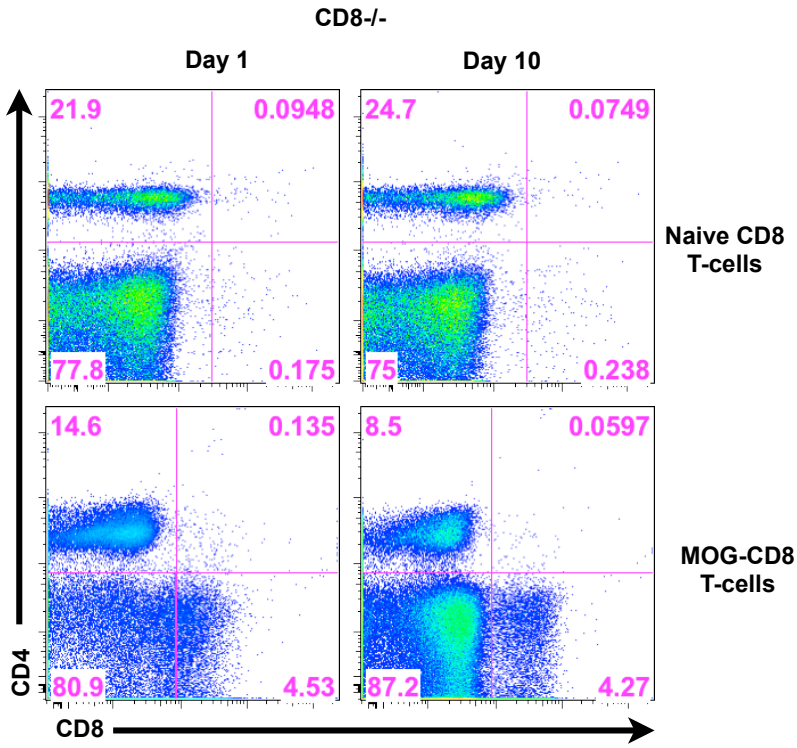
EAE severity was compared between CD8 depleted and control mice. Naïve B6 mice were treated with 200 µg of CD8 depleting antibody (2.43) or isotype control i.p. Primary EAE was induced the following day with antigen dosage at 100 µg per mouse. PTX treatment was administered on days 0 and 2. Clinical disease was evaluated using our 2-way blinded scheme. **=p≤0.05. Error bars represent means ± standard error of the mean (SEM).

Figure 11: EAE Severity in Antibody Mediated CD8 Depletion



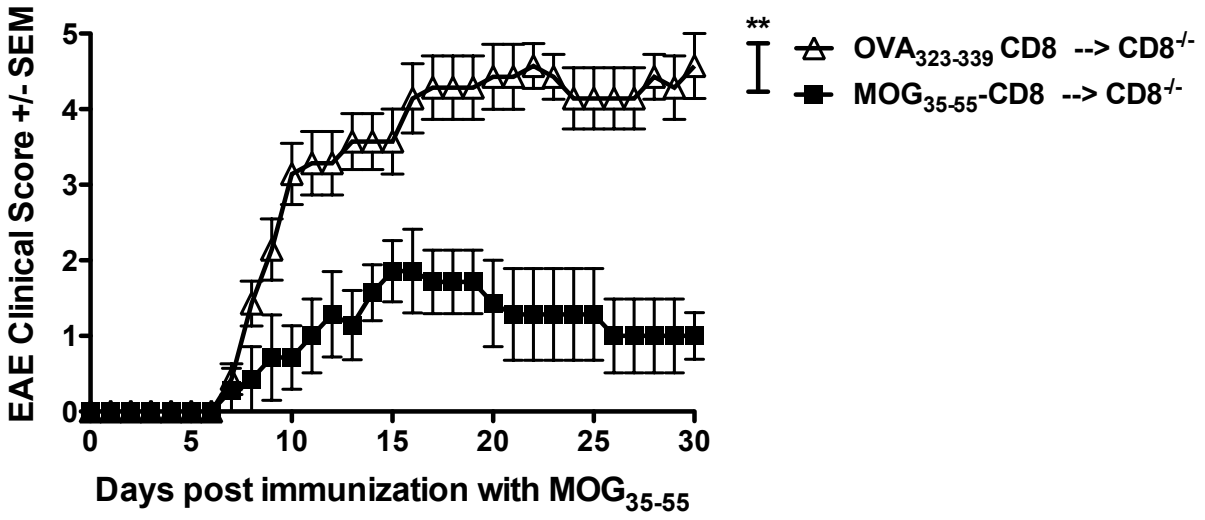
Clinical disease differences were compared between CD8 depleted and control mice. Data were obtained from Figure 4 experiments. % of incidence is the percentage of mice which developed EAE paralysis irrespective of disease severity. Mean maximum score is an average of the highest EAE score each mice attained. Mean day of onset is an average of the day in which each mouse first developed EAE symptoms. Data are representative of two independent experiments, 5-7 mice per condition. *=p≤0.06, **=p≤0.05.

Figure 12: Reconstitution of CD8$^{-/-}$ Mice with Autoregulatory CD8 T-cells



Successful transfer of CD8 T-cells was assessed by peripheral blood examination. Naïve CD8$^{-/-}$ mice were infused with naïve CD8 (top row) or MOG_{35-55}-specific CD8 T-cells (bottom row). Engraftment of tissue was examined on day 1 post transfer (first column) or at day 10 (2nd column) by submandibular bleed. Dot plots are showing events, which are gated on TCRβ+ cells.

Figure 13: Reconstitutions of Autoregulatory CD8 T-cells is Sufficient for Reversing $CD8^{-/-}$ EAE Susceptibility



Modulation of EAE disease was assessed in autoregulatory CD8 T-cell recipients. MOG_{35-55}- or $OVA_{323-339}$-specific CD8 T-cells were transferred into $CD8^{-/-}$ mice at day -1 via tail vein i.v. All mice were immunized with MOG_{35-55} peptide on day 0. PTX was administered i.p. at days 0 and 2. Clinical disease was evaluated using our 2-way blinded scheme. Error bars represent mean clinical disease ± standard error of the mean (SEM). Data are representative of three independent experiments, 5-7 mice per condition. . **=$p \leq 0.05$.

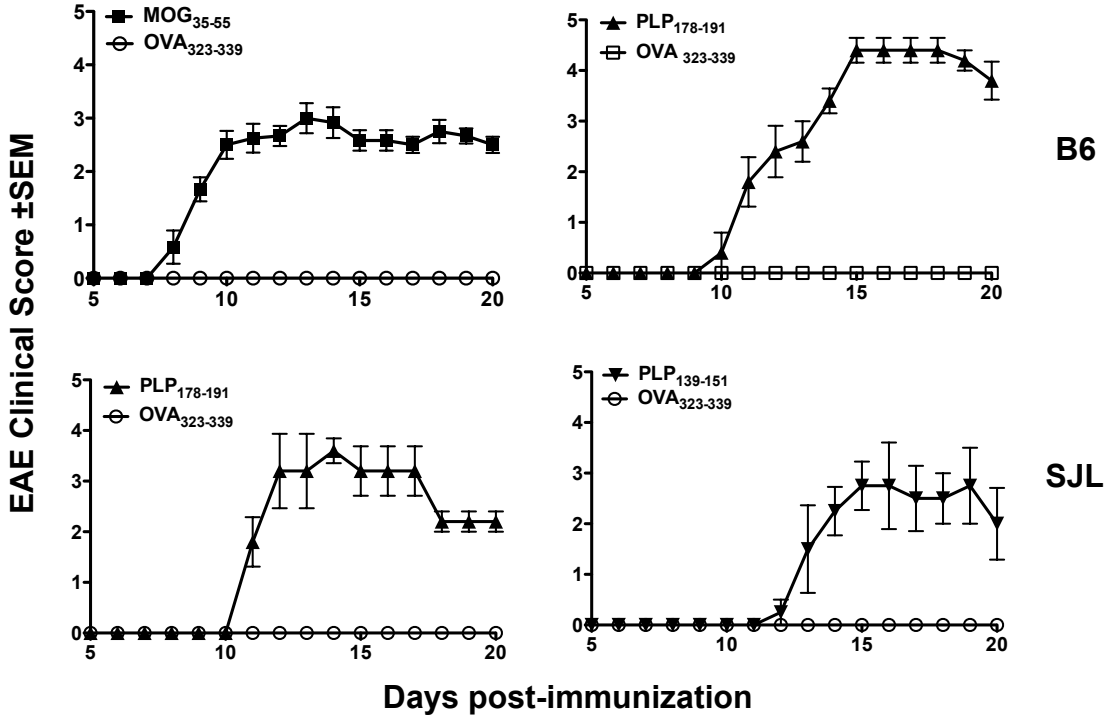
3.2.2: Role of Autoreactive CD8 T-cells in Various EAE Models

In this study, I wanted to extend our knowledge by evaluating the role of autoreactive CD8 T-cells in other murine models of EAE. Thus, using established protocols [107], I induced EAE using $PLP_{178-191}$ (alternative peptide) in B6 mice, $PLP_{178-191}$ in SJL mice (alternate strain) and $PLP_{139-151}$ in SJL mice (alternate peptide and alternate strain). All permutations of EAE induction resulted in characteristic clinical presentation (Figure 14). At day 20, similar to adoptive transfer experiments previously described [85], we isolated draining lymph node and spleen cells, cultured for 3 days with cognate antigen (*in vitro* expansion phase) and adoptively transferred highly purified (>95% TCRβ+CD8+CD4-, Figure 15) CD8 T-cells into naïve wildtype (WT) mice via intravenous (i.v.) tail vein injection. Prior to *in vitro* culture, an aliquot of cells were used in a CFSE-based recall response assay in order to confirm antigen-specific response. All EAE induction methods resulted in a CD8 T-cell response (Figure 16).

Following the adoptive transfer, EAE was induced in recipient mice using cognate antigen of the transferred CD8 T-cells and clinical disease evaluated for 30 days using 2-way blinded evaluation scheme. Similar to disease amelioration observed in MOG_{35-55}-specific CD8-treated mice (Figure 17), $PLP_{178-191}$-specific CD8 T-cells were found to be capable of suppressing $PLP_{178-191}$-based EAE in B6 mice (Figure 18). In fact, $PLP_{178-191}$-specific CD8 T-cells in the SJL strain were also capable of suppressing $PLP_{178-191}$-induced EAE (Figure 19). Interestingly, CD8 T-cells isolated from $PLP_{139-151}$ immunized mice were not capable of suppressing $PLP_{139-151}$-induced EAE in SJL mice (Figure 20), nor did they enhance disease, an observation that will be further evaluated in future studies. These observations further support

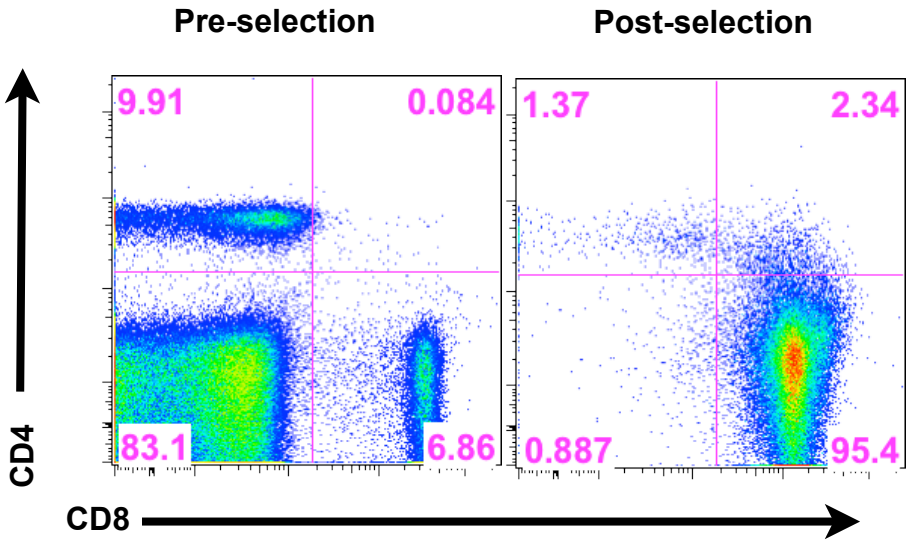
the existence of autoreactive CD8 T-cells that exhibit a protective regulatory role by limiting autoimmune disease.

Figure 14: Primary Clinical Disease in Various EAE Models



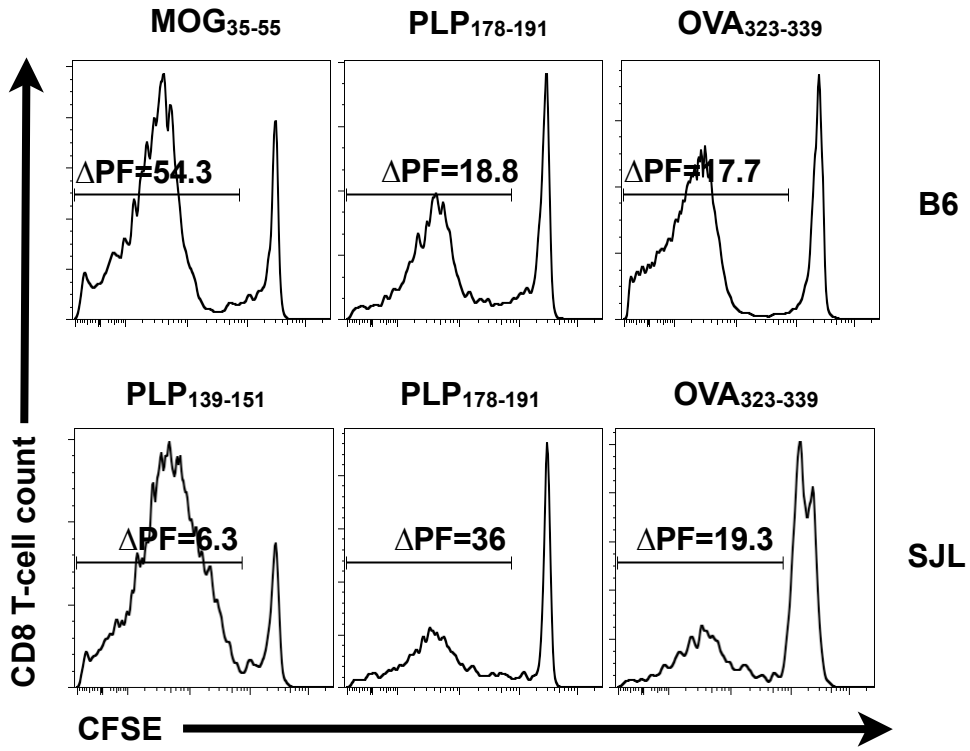
Representative primary EAE is shown. Naïve B6 mice were immunized with myelin or control ($OVA_{323-339}$) antigen (200 µg/mouse) at day 0 subcutaneously. PTX treatment was administered on days 0 and 2 by i.p. injection. EAE clinical disease was evaluated daily until day 20 and tissues were harvested for adoptive transfer experiments. Data are representative of two-five independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM).

Figure 15: Highly Purified Adoptive Transfer of Myelin-specific CD8 T-cells



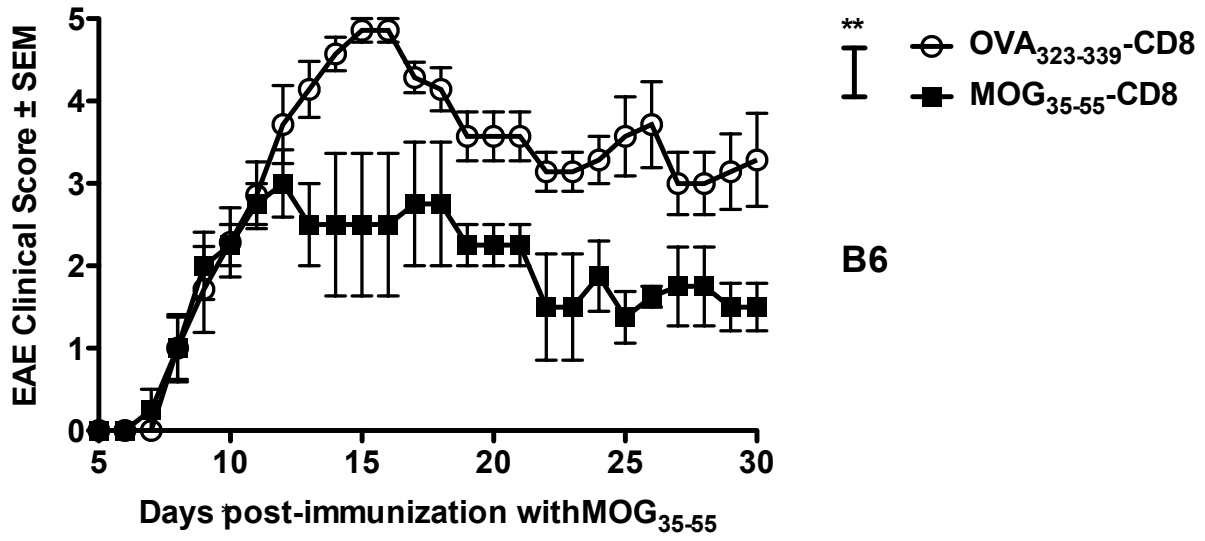
Assessment of CD8 T-cell selection and purity. Following CD8 T-cell selection using anti-CD8 magnetic beads, cells were FACS stained using anti-TCRβ, CD4 and CD8 fluorescent antibody. Dot plots are gated on live gate and +100,000 events are shown.

Figure 16: Antigen Response by CD8 T-cells in Various EAE Models



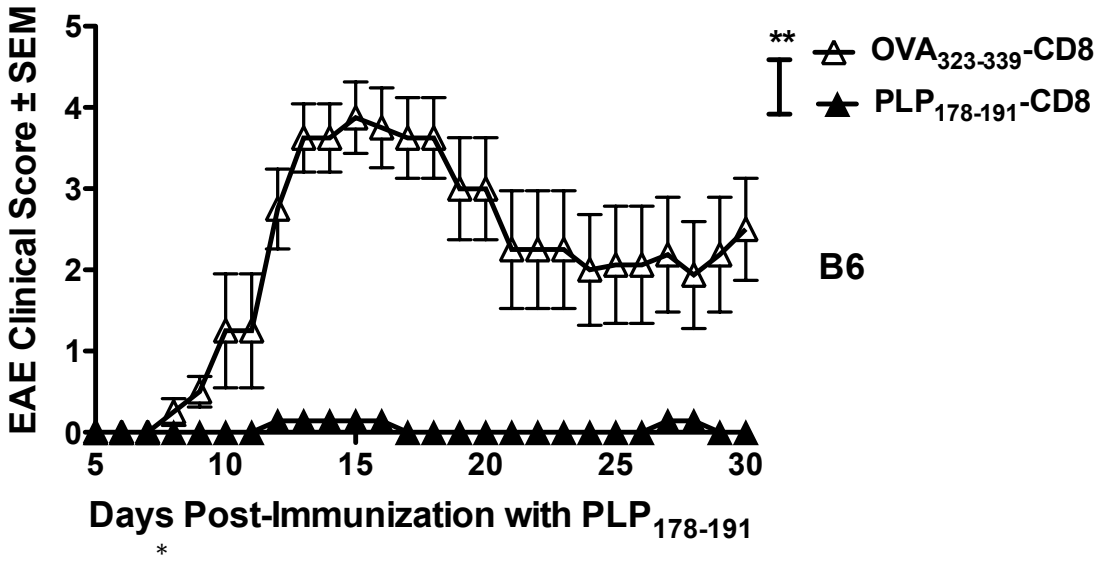
CD8 T-cell recall response to cognate antigen was evaluated using CFSE dilution assay. Naïve B6 mice were immunized with myelin or control antigen (200 µg/mouse) at day 0 subcutaneously. PTX treatment was administered on days 0 and 2 i.p. EAE clinical disease was evaluated daily until day 20, lymph nodes and spleens were harvested and tissues processed into single-cell suspension. Cells were CFSE stained and cultured for five days with cognate antigen. Cells were washed and stained with TCRβ, CD4 and CD8 antibodies and CFSE dilution assayed by flow cytometry. Histograms show events gated on live gate+TCRβ+CD8+CD4- cells. Interval indicate the percentage of CFSE low cells within the CD8 T-cell population. Delta proliferation fraction (ΔPF) was calculated by subtracting the background (no antigen condition) from the cognate antigen stimulated condition (n=5 per condition).

Figure 17: MOG_{35-55}-specific CD8 T-cells can Suppress MOG_{35-55}-induced EAE in B6 Mice



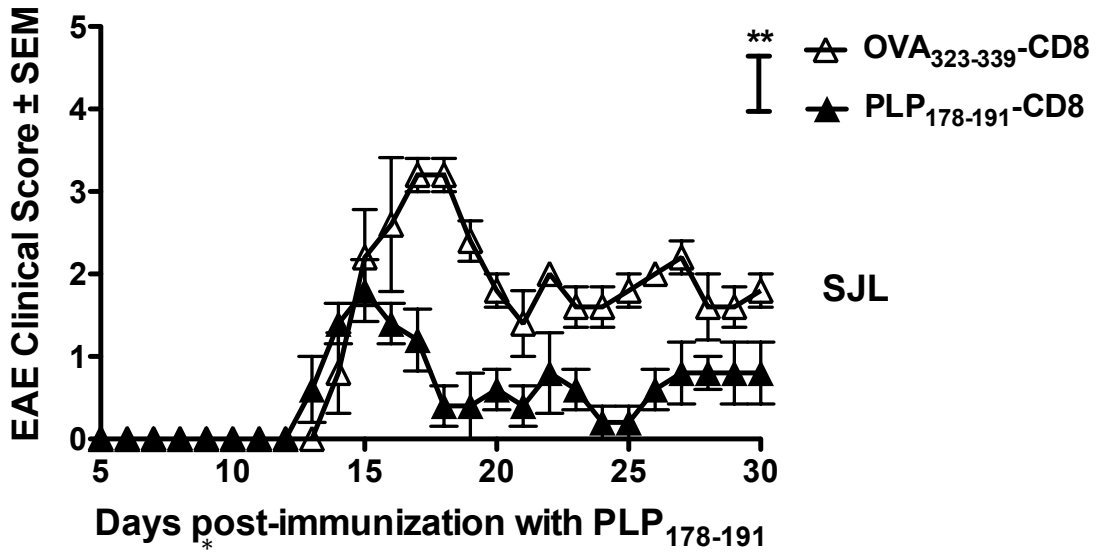
Disease regulating role of autoreactive myelin-specific CD8 T-cells in EAE. MOG_{35-55} or $OVA_{323-339}$ CD8 T-cells were adoptively transferred i.v. into naïve B6 mice at day -1. Next, all mice were immunized with 200 µg of MOG_{35-55} in CFA emulsion. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of five independent experiments, 7-10 mice per condition. Error bars represent means ± standard error of the mean (SEM). **=$p \leq 0.05$.

Figure 18: $PLP_{178-191}$-specific CD8 T-cells are Capable of Suppressing $PLP_{178-191}$-induced EAE in B6 Mice



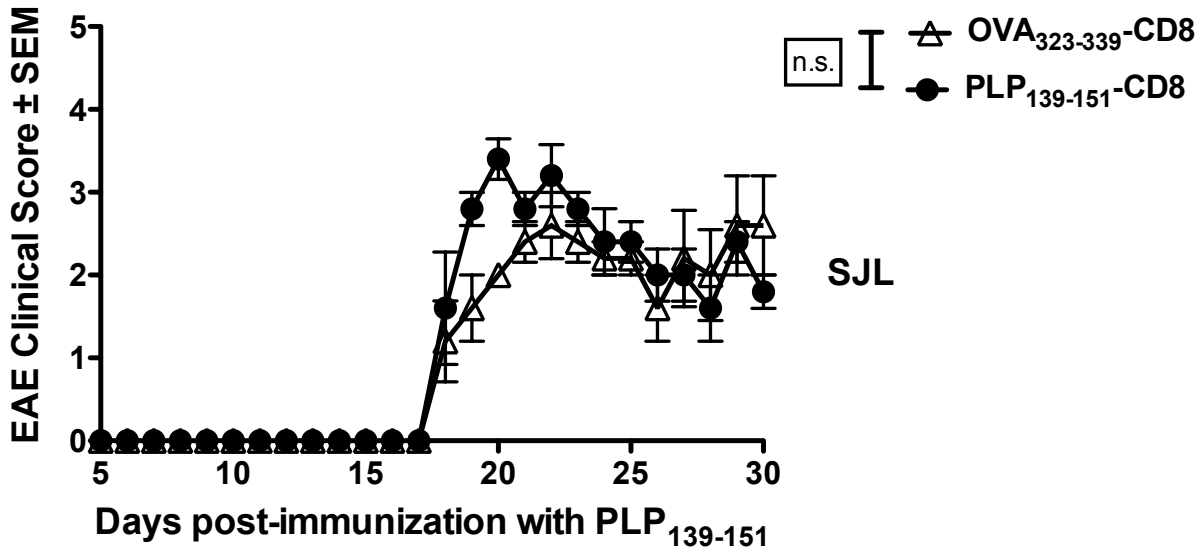
Disease regulating role of autoreactive myelin-specific CD8 T-cells in EAE. $PLP_{178-191}$ or $OVA_{323-339}$ CD8 T-cells were adoptively transferred i.v. into naïve B6 mice at day -1. Next, all mice were immunized with 100 µg of $PLP_{178-191}$ in CFA emulsion. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of three independent experiments, 7-10 mice per condition. Error bars represent means ± standard error of mean (SEM). **=$p \leq 0.05$.

Figure 19: $PLP_{178-191}$-specific CD8 T-cells are Capable of Suppressing $PLP_{178-191}$-induced EAE in SJL Mice



Disease regulating role of autoreactive myelin-specific CD8 T-cells in EAE. $PLP_{178-191}$ or $OVA_{323-339}$ CD8 T-cells were adoptively transferred i.v. into naïve SJL mice at day -1. Next, all mice were immunized with 100 µg of $PLP_{178-191}$ in CFA emulsion. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of two independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p \leq 0.05$.

Figure 20: $PLP_{139-151}$-specific CD8 T-cells are not Capable of Suppressing $PLP_{139-151}$-induced EAE in SJL Mice

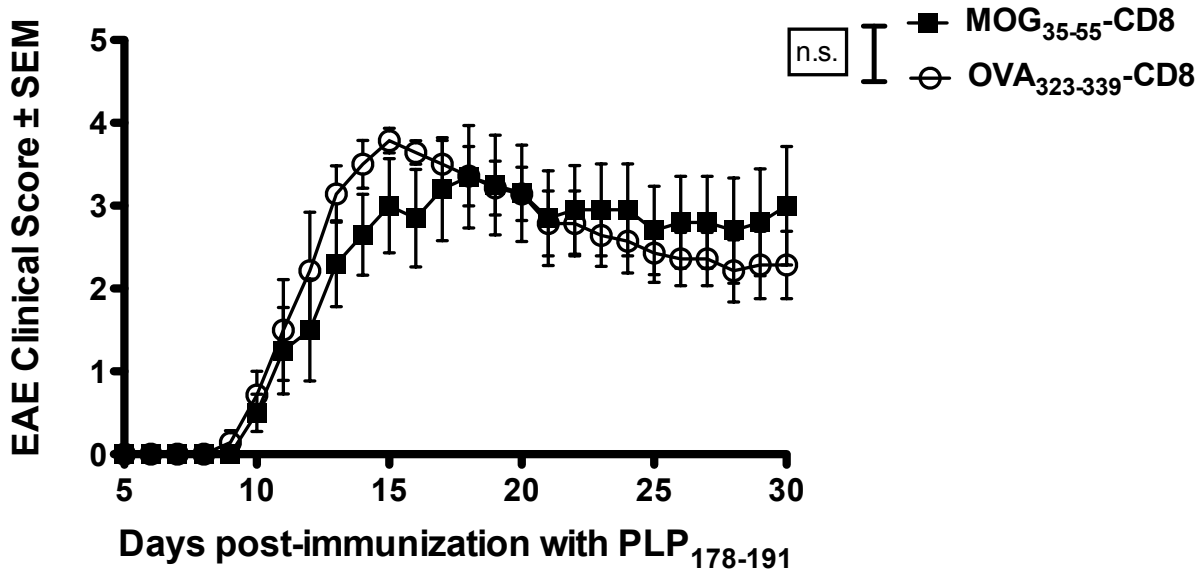


Some autoreactive CD8 T-cells do not exhibit a regulatory (or pathogenic role) in EAE. $PLP_{139-151}$ or $OVA_{323-339}$ CD8 T-cells were adoptively transferred i.v. into naïve SJL mice at day -1. Next, all mice were immunized with 100 µg of $PLP_{139-151}$ in CFA emulsion. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of three independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). n.s. = not significant

3.2.3: Antigen Specificity of Autoregulatory CD8 T-cells

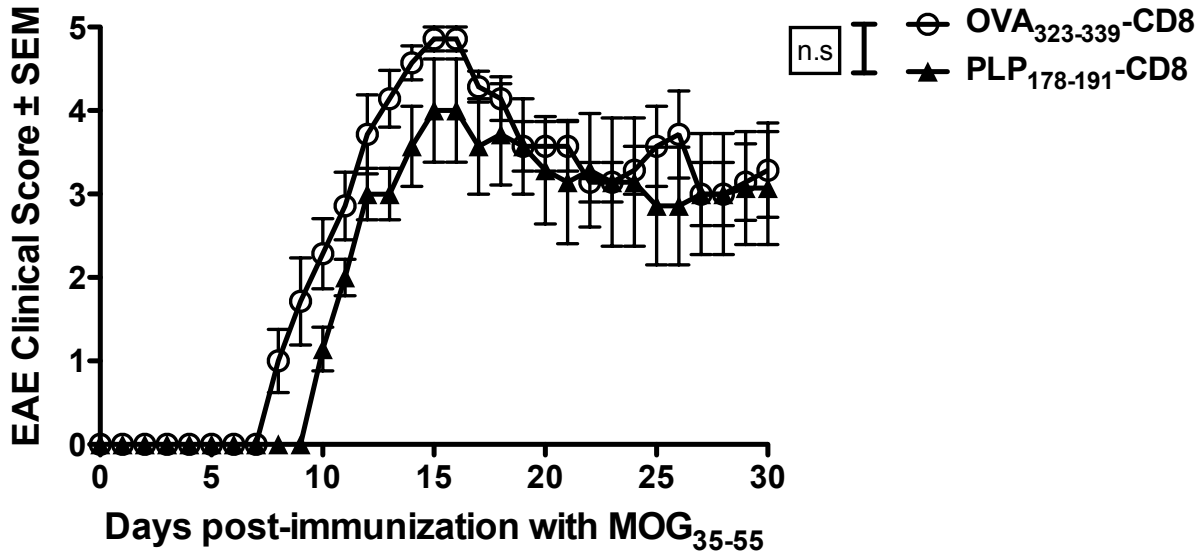
Having observed two distinct regulatory populations that are generated in the same mouse strain but differ in their antigen specificity (Figure 17 and Figure 18), I evaluated the disease suppressing ability of myelin-specific CD8 T-cells when EAE was induced using an alternative myelin peptide, i.e. antigen specificity requirements during the effector phase. First, an experiment was set up to evaluate if MOG_{35-55}-specific CD8 T-cells were capable of suppressing $PLP_{178-191}$-induced EAE. I obtained donor cells by immunizing B6 mice with MOG_{35-55} peptide, isolated the ln and spl at 20 days-post immunization and transferred purified MOG_{35-55}-specific CD8 T-cells i.v. into naïve WT mice. Next, we induced EAE using $PLP_{178-191}$ peptide and evaluated clinical disease for 30 days. MOG_{35-55}-specific CD8 T-cells were unable to protect mice from $PLP_{178-191}$ induced EAE disease (Figure 21). Conversely, $PLP_{178-191}$-specific CD8 T-cells were unable to modulate MOG_{35-55}-induced EAE (Figure 22), unless CD8-cognated antigen ($PLP_{178-191}$) was present in the recipient's immunizing emulsion, as observed in the MOG_{35-55} /$PLP_{178-191}$ co-immunized recipient mice (Figure 23). These data reveal that disease suppression by neuroantigen-specific CD8 T-cells is antigen-specific.

Figure 21: Ineffective Suppression of $PLP_{178-191}$ Disease by MOG_{35-55}-specific CD8 T-cells



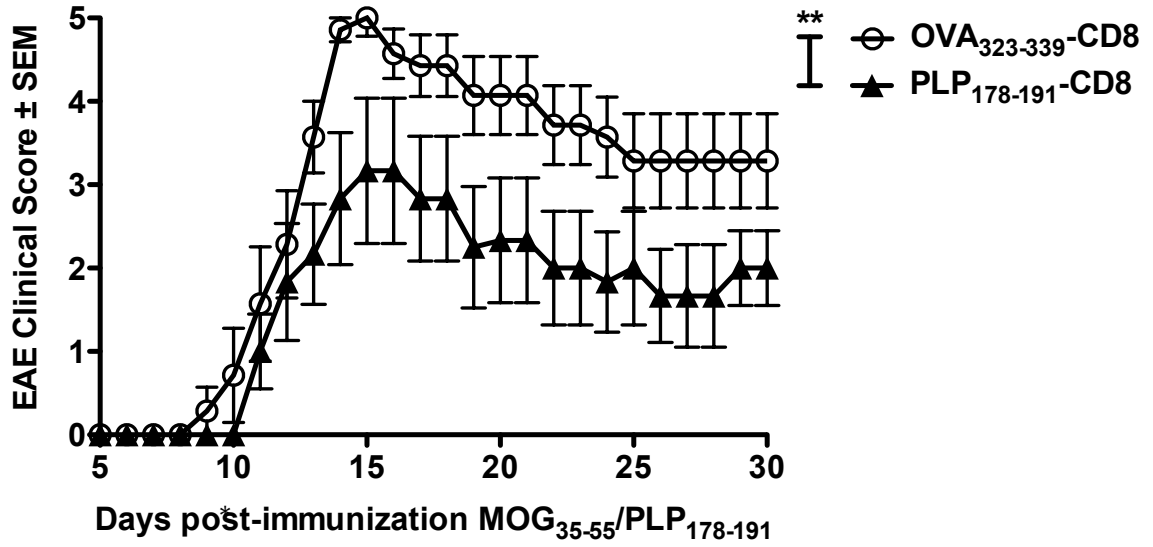
Antigen specificity requirements were evaluated by transferring neuroantigen-reactive CD8 T-cells into mice, followed by EAE induction with non-cognate antigen immunization. MOG_{35-55}- or $OVA_{323-339}$-specific CD8 T-cells were transferred into naïve B6 mice at day -1. All mice were immunized with $PLP_{178-191}$ on day 0. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Representative of two independent clinical experiments (n=5-7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). n.s.= non significant.

Figure 22: Ineffective Suppression of MOG_{35-55} Disease by $PLP_{178-191}$-specific CD8 T-cells



Antigen specificity requirements were evaluated by transferring neuroantigen-reactive CD8 T-cells into mice, followed by EAE induction with non-cognate antigen immunization. $PLP_{178-191}$- or $OVA_{323-339}$-specific CD8 T-cells were transferred into naïve B6 mice at day -1. All mice were immunized with MOG_{35-55} on day 0. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Representative of two independent clinical experiments (n=5-7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). n.s.= non significant.

Figure 23: $PLP_{178-191}$-specific CD8 T-cell Suppression of EAE is Dependent on Cognate Antigen Presentation



Antigen specificity requirements were evaluated by transferring neuroantigen-reactive CD8 T-cells into mice, followed by EAE induction with cognate/noncognate antigen immunization. $PLP_{178-191}$- or $OVA_{323-339}$-specific CD8 T-cells were transferred into naïve B6 mice at day -1. All mice were co-immunized with MOG_{35-55} and $PLP_{178-191}$ on day 0. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Representative of two independent clinical experiments (n=5-7 per condition). Error bars represent means ± standard error of the mean (SEM). n.s.= non significant.

3.2.4: MHC Class I Requirements for Disease Amelioration by Autoregulatory CD8 T-cells

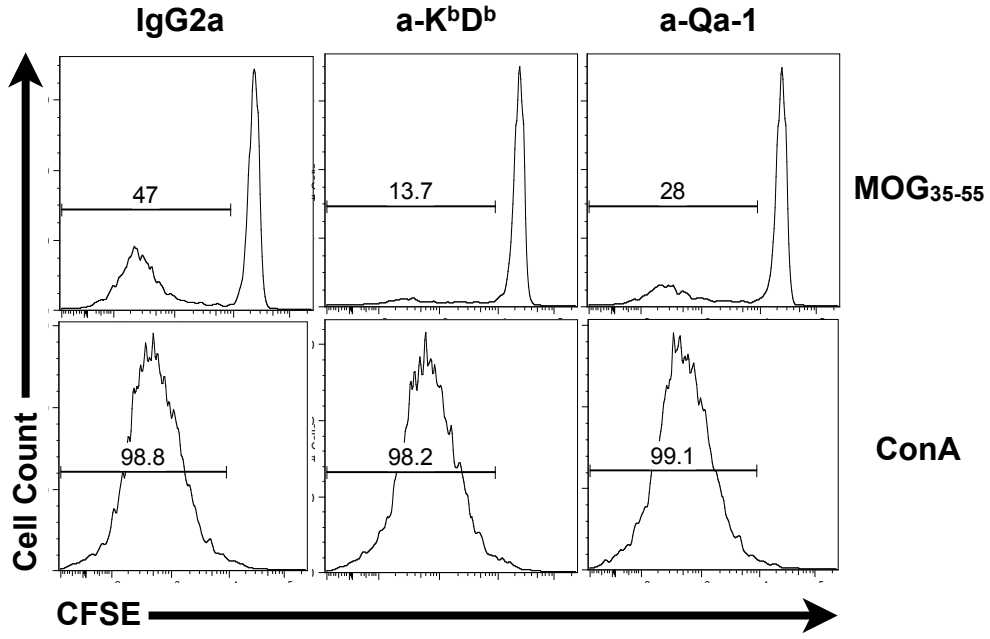
Next, I evaluated the necessity for MHC Class I based antigen presentation in disease suppression. First, I determined if MHC-I molecules mediated CD8 T-cell responses to MOG35-55. *In vitro* blocking assays were performed using CFSE-stained bulk spleen cells from MOG-immunized mice. These cells were cultured in EAE media with IgG isotype control, anti-K^bD^b (classical MHC-I in B6) antibody or anti-Qa-1^b (non-classical MHC-I) antibody for one hour. Subsequently, these cells were stimulated with either MOG_{35-55} or concanavalin A (ConA) for 5 days, after which the cells were stained with fluorescent anti-CD3, anti-CD4 and anti-CD8 antibodies and CFSE dilution measured by flow cytometry. Relative to the isotype control, both K^bD^b and Qa-1^b blockade showed a reduction in MOG_{35-55}-CD8 T-cell responses, although most of the response was decreased when K^bD^b molecules were blocked (Figure 24). Thus, these data suggest that MOG peptide is being presented to CD8 T-cells for activation by MHC Class I molecules.

Understanding that MOG_{35-55} peptide was indeed being presented by MHC Class I molecules, I next evaluated the necessity of *in vivo* MHC Class I based antigen presentation during disease regulation. WT B6 MOG_{35-55}-specific CD8 T-cells were transferred into $\beta2m^{-/-}$ or WT mice, followed by MOG_{35-55} EAE induction. Compared to the protection seen when WT MOG_{35-55}-specific CD8 T-cells were transferred into WT recipient mice, the transfer of WT MOG_{35-55}-specific CD8 T-cells into $\beta2m^{-/-}$ recipients mice resulted in a profound loss of disease suppression (Figure 25). Similarly, WT-MOG_{35-55}-specific CD8 T-cells were incapable of suppressing disease when transferred into $Tap^{-/-}$ (MHC Class I deficient) mice (Figure 26). To elucidate whether this MHC requirement was classical or non-classical MHC-I dependent, I transferred MOG_{35-55}-specific CD8 T-cells into $K^bD^{b-/-}$ (mice which do not express MHC-Ia but

still maintain MHC-Ib expression) mice and observed that autoregulatory CD8 T-cells were ineffective in disease amelioration (Figure 27). Hence, I concluded that autoregulatory CD8 T-cells require *in vivo* cognate antigen presentation by MHC Class Ia molecules.

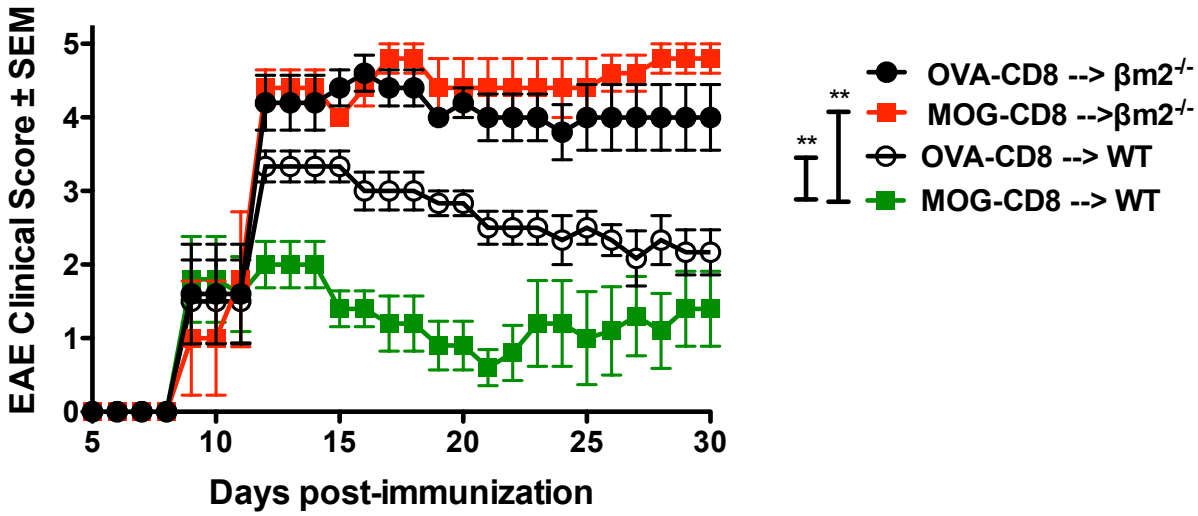
Taken together these data show that MOG peptide is presented to CD8 T-cells by MHC-I molecules. This in turn activates CD8 T-cells and allows them to perform their regulatory function.

Figure 24: *In vitro* Response to Cognate Antigen by MOG_{35-55}-specific CD8 T-cells is MHC Class I Dependent



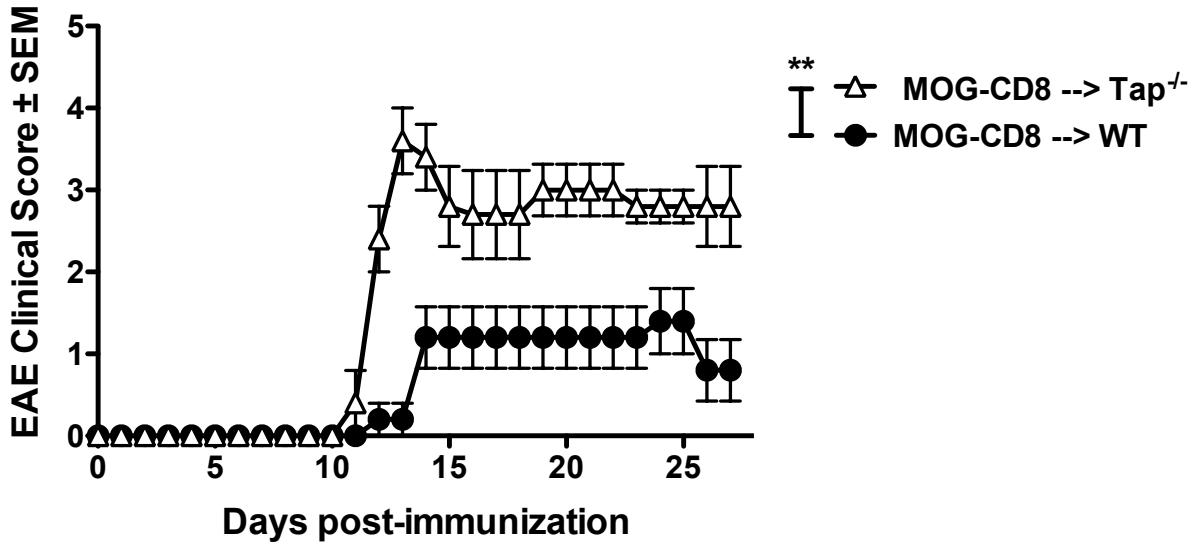
In vitro blocking experiments using antibodies to classical and non-classical MHC Class I molecules were performed in order to evaluate the role of MHC Class I in MOG peptide mediated activation of CD8 T-cells. CFSE dilution was used to measure the CD8 T-cell response to MOG_{35-55} peptide. Histogram shows events gated on CD3+CD4-CD8+ T-cells. Interval indicates percentage of CFSE low T-cells in the CD8 T-cell population. Compared to IgG control, both anti-K^bD^b and anti-Qa-1^b antibody were able to inhibit CFSE dilution in CD8 T-cells.

Figure 25: EAE Amelioration by Autoregulatory CD8 T-cells is MHC Class I-dependent



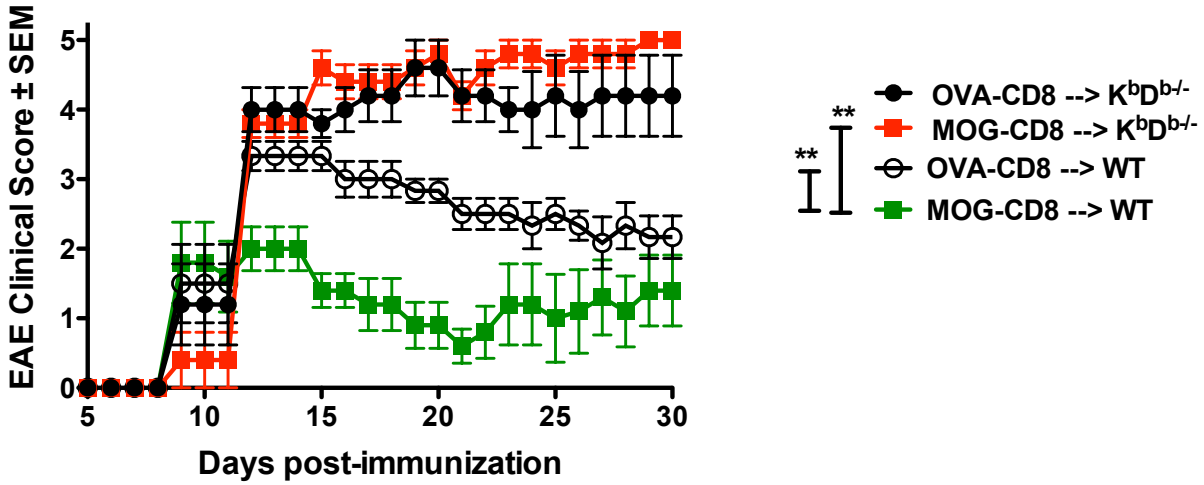
In vivo MHC-I requirements were evaluated by transferring MOG_{35-55}-specific CD8 T-cells (or $OVA_{323-339}$-specific CD8 T-cells as control) into $\beta 2m^{-/-}$ mice. MOG_{35-55} EAE was induced the following day and clinical disease evaluated for 30 days. Disease curves for $OVA_{323-339}$-CD8 or MOG_{35-55}-CD8 T-cell treated (day -1) MHC Class I deficient ($\beta 2m^{-/-}$ and WT recipient mice are shown. Representative of two independent experiments (n=10 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM).**=$p<0.05$

Figure 26: Autoregulatory CD8 T-cells are Ineffective at Ameliorating EAE in Tap$^{-/-}$ Host



Autoreactive regulatory CD8 T-cells are ineffective in Tap$^{-/-}$ mice. Disease curve for MHC Class I deficient (Tap$^{-/-}$) versus control group (WT) which received WT MOG_{35-55}-specific CD8 T-cells at day -1 and immunized with MOG_{35-55}/CFA at day 0. Representative of two independent experiments (10 mice per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM).**=$p<0.05$

Figure 27: Autoregulatory CD8 T-cell Suppression of EAE is MHC Class Ia-dependent



Autoreactive CD8 T-cells require MHC Class Ia *in vivo* for disease amelioration Disease curves for $OVA_{323-339}$-CD8 or MOG_{35-55}-CD8 T-cell treated (day -1) MHC Class Ia deficient ($K^bD^{b-/-}$) and WT recipient mice are shown. Representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$

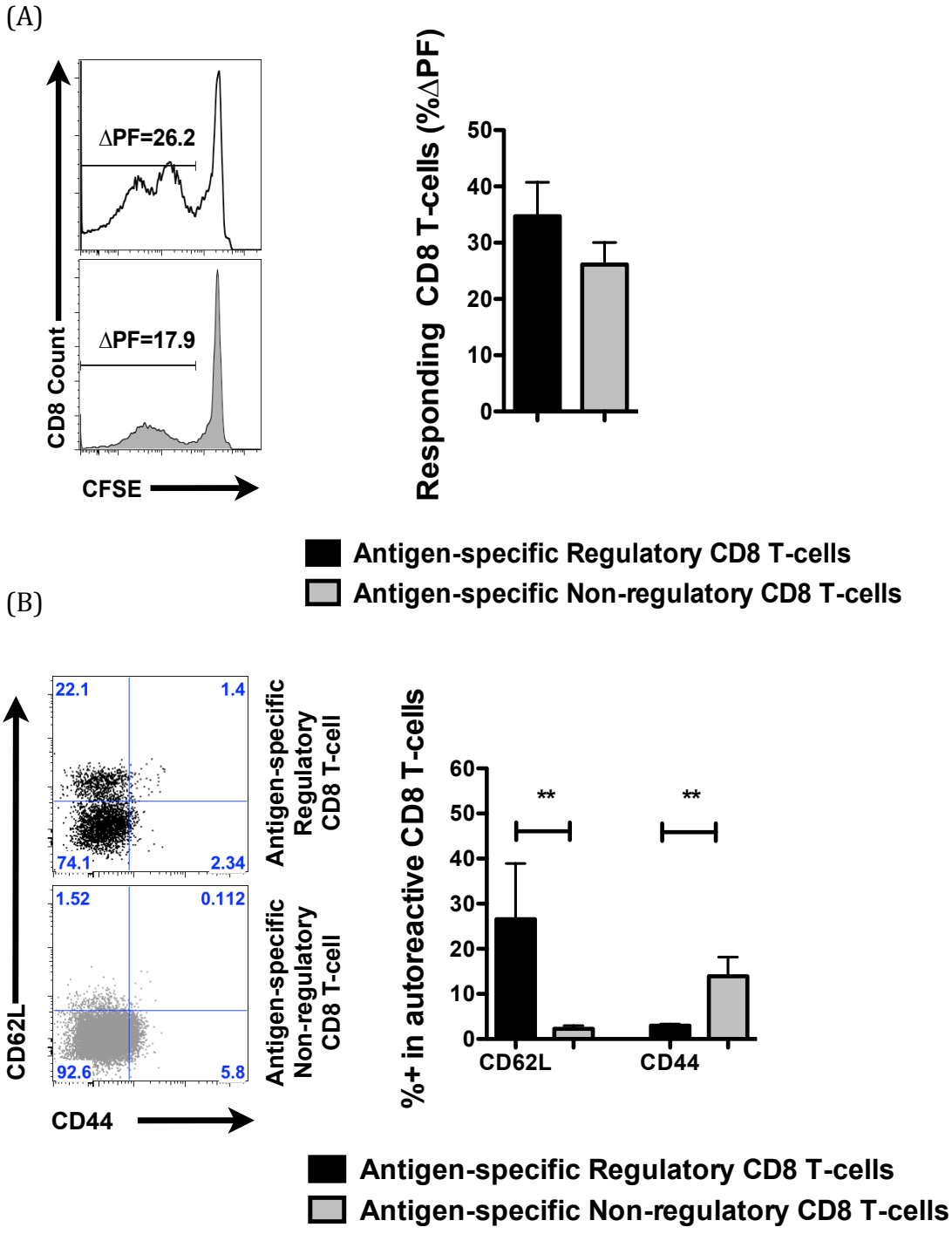
3.2.5: Central Memory Phenotype of Autoregulatory CD8 T-cells

The phenotypic characteristics of autoregulatory CD8 T-cells were examined by combining the CFSE dilution assay with extracellular surface (ECS) staining and evaluating the expression of previously described suppressor T-cell markers between disease ameliorating regulatory CD8 and non-regulatory CD8 T-cells. Using our neuroantigen peptide panel, I immunized B6 mice with MOG_{35-55} (which produces regulatory CD8 T-cells), $PLP_{178-191}$ (regulatory), MOG_{37-46} (non-regulatory), $OVA_{323-339}$ (non-regulatory) and SJL mice with $PLP_{178-191}$ (regulatory) and $PLP_{139-151}$ (non-regulatory) peptides and 20 days later, harvested draining lymph nodes and spleens. Cells were then CFSE stained and stimulated *in vitro* for 5 days with cognate antigen. Next, the cells were stained with anti-TCRβ, CD4 and CD8 fluorescent antibodies for T-cell subsetting and anti-CD122, CCR7, CXCR3, CTLA-4, PD-1, CD11c, CD62L, CD44 and CD25 for evaluation of suppressor T-cell markers. Analysis of the proliferative response between regulatory and non-regulatory cells revealed no significant difference, but the differentiation status between regulatory versus non-regulatory CD8 T-cells revealed a larger proportion of antigen-specific cells expressing CD62L and less CD44 in autoregulatory than in non-regulatory CD8 T-cells (Figure 28). Further analysis of regulatory surface markers revealed a higher expression of CCR7 in autoantigen-responding regulatory, as compared to the non-regulatory CD8 T-cells (Figure 29).

Subsequently, we evaluated the cytokine profile of the autoregulatory CD8 T-cell by combining our CFSE dilution assay with intracellular cytokine staining. Cells were stimulated with cognate antigen for 5 days, restimulated with PMA for 5 hours, then stained with fluorescent antibodies to IFN-γ, IL17, IL10, TNF-α, perforin, IL4 and Foxp3. Autoregulatory CD8 T-cells were found to produce robust amounts of IFN-γ, and TNF-α but negligible levels of

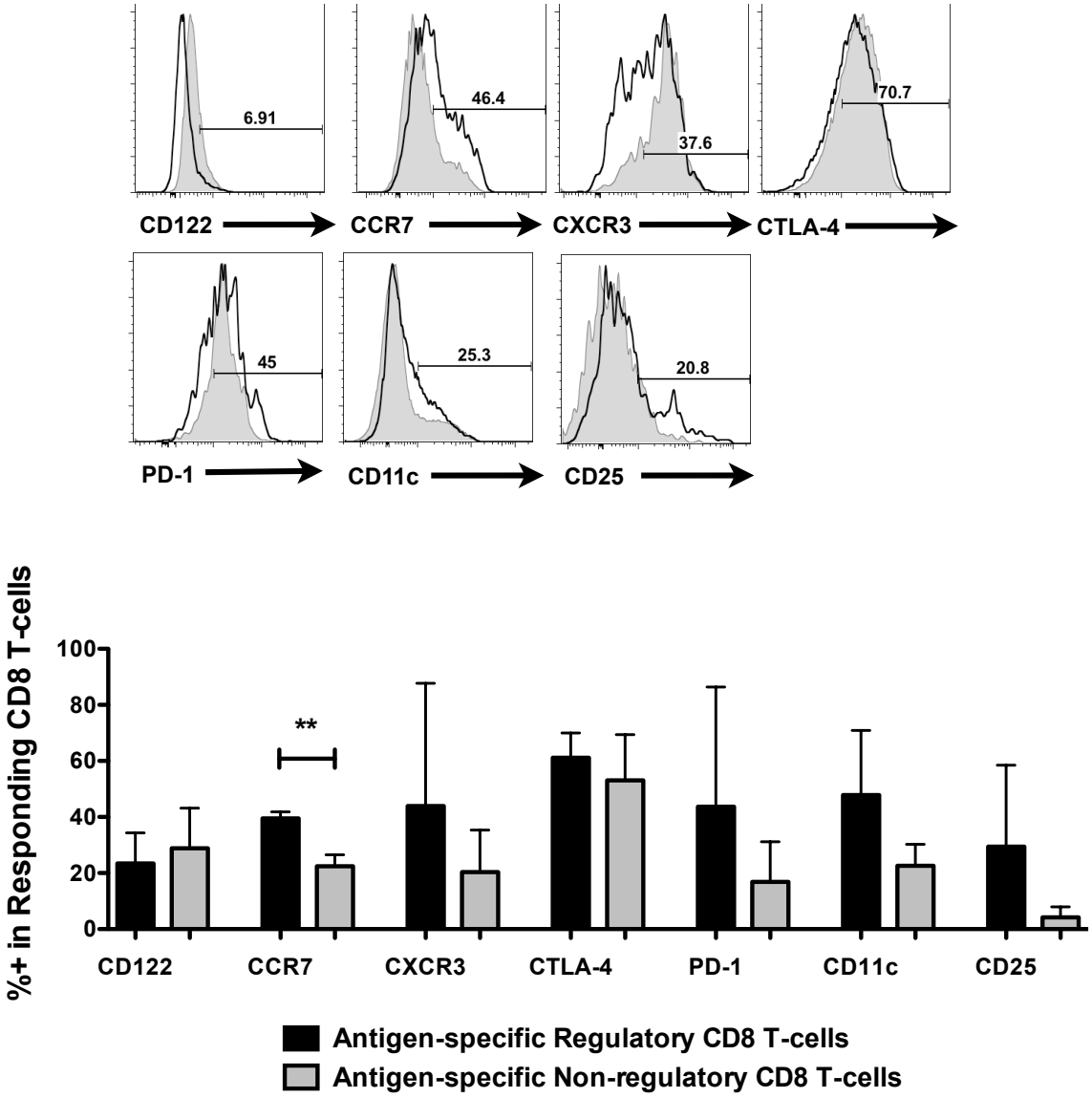
IL10, IL17 or Foxp3 protein (Figure 30). Comparison between regulatory and non-regulatory CD8 T-cells revealed only a slight significant difference in perforin production (data not shown).

Figure 28: Autoregulatory CD8 T-cells Express a Central Memory Phenotype



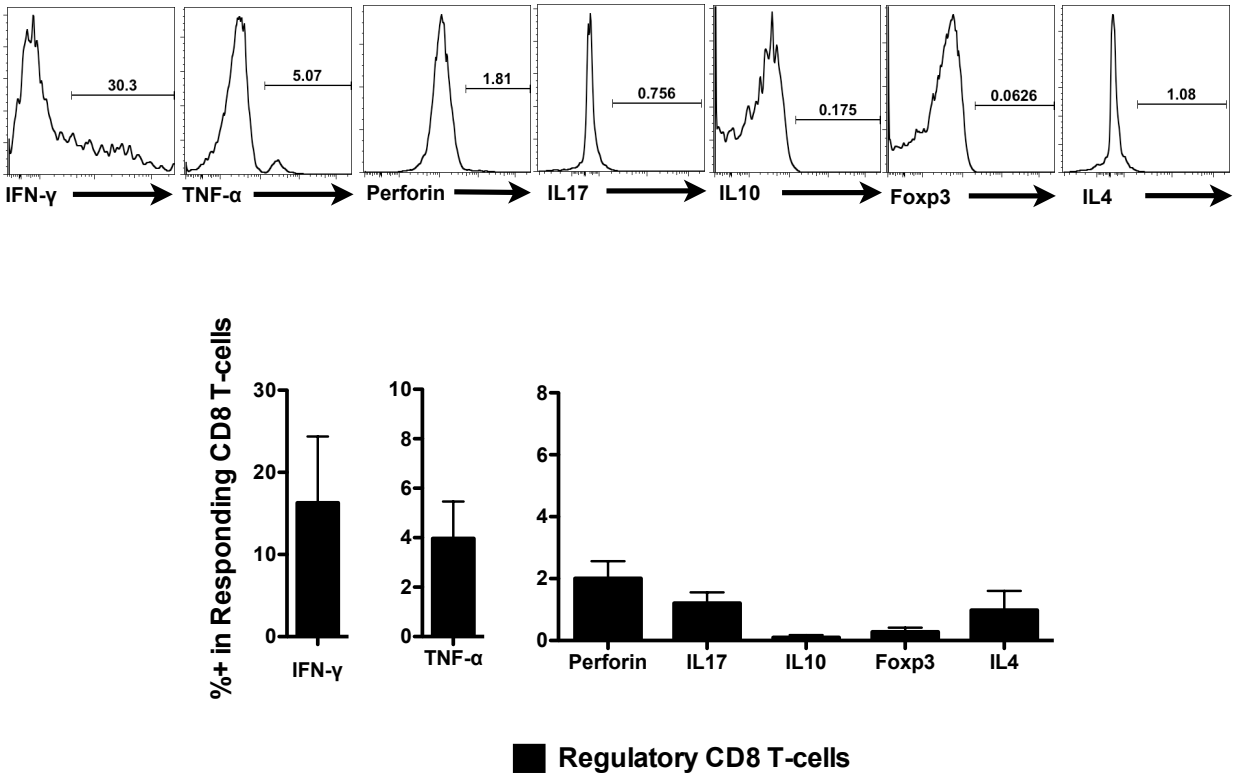
Autoreactive CD8 T-cells exhibit a unique regulatory phenotype. (A) Histogram shows representative proliferation of TCRβ+CD4-CD8+ T-cells isolated from MOG_{35-55} and $PLP_{178-191}$ (regulatory) versus $OVA_{323-339}$ or MOG_{37-46} (non-regulatory) immunized aged-matched WT B6 mice. (B) Dot plot shows representative data of CD44 versus CD62L phenotype of proliferating CD8 T-cells isolated from MOG_{35-55} or $OVA_{323-339}$ immunized mice. Cumulative data show the average percentage of CD8 T-cells expressing indicated protein ± standard error of the mean (SEM). **=$p<0.05$

Figure 29: Differential Expression of CCR7 between Regulatory and Non-regulatory CD8 T-cells



Flow cytometry profile of regulatory markers on autoreactive or control-specific CD8 T-cells isolated from immunized WT B6 mice. Histograms gated on TCRβ+CD8+CD4- CFSE low T-cells. Representative of 5 independent experiments (n=3 mice per condition). Cumulative data show the average percentage of CD8 T-cells expressing indicated protein ± standard error of the mean (SEM). **=$p<0.05$

Figure 30: Functional Profile of Autoregulatory CD8 T-cells



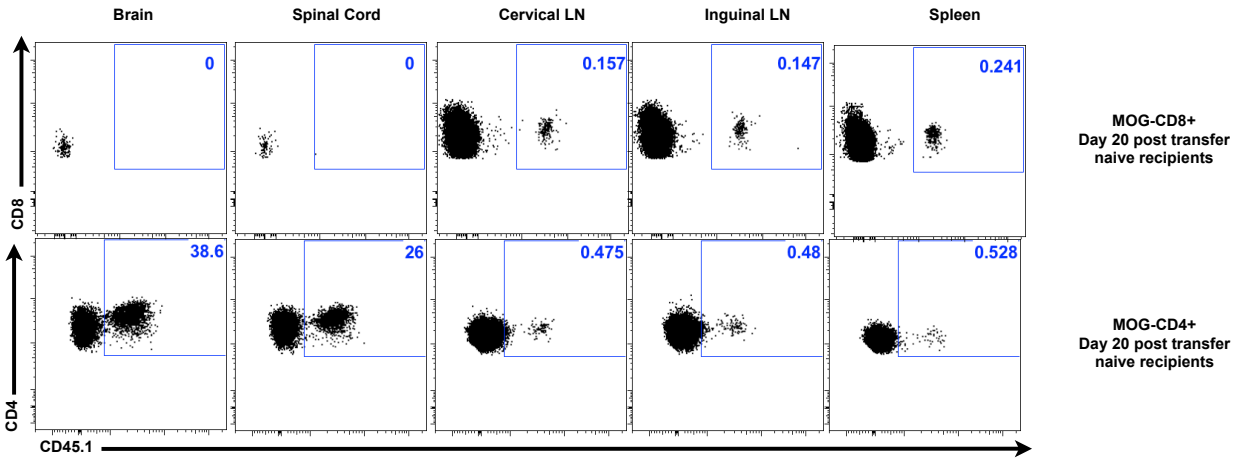
Intracellular cytokine stain of proliferating (CFSE Low) TCRβ+CD8+CD4 T-cells from MOG_{35-55} immunized WT B6 mice. Histograms show representative data of CD4-CD8+ CFSE low cells. Interval indicates the percentage of cytokine expressing cells within the antigen responding (CFSE Low) population. Cumulative data show the average percentage of CD8 T-cells expressing indicated protein ± standard error of the mean (SEM).

3.2.6: CNS Infiltration by Autoregulatory CD8 T-cells

Next, we wanted to ascertain the location of the autoimmune disease regulation. First, we asked whether the autoregulatory CD8 T-cells were capable of trafficking into the CNS, independent of autoimmune disease. Thus, we performed an *in vivo* trafficking experiment, where we transferred CD45.1+ MOG_{35-55}-specific CD8 T-cells into naïve WT (CD45.2+) mice and analyzed their tissue location at days 5, 10 and 20 post transfer. No infiltration of adoptively transferred MOG_{35-55}-specific CD8 T-cells into the brain or spinal cord of naïve WT mice could be observed at days 5 and 10 (data not shown) and 20, although they could be consistently detected in the cervical, inguinal lymph nodes and spleen (Figure 31). We did observe a minor but distinct endogenous CD8 T-cell population in the CNS. As a control, we transferred CD45.1+ MOG_{35-55}-specific CD4 T-cells and true to their purported role, they were detected in the CNS as well as the peripheral immune compartment. In fact, their increase in percentages alluded to a possible expansion of the transferred population. Next, we evaluated the tissue infiltration of these autoregulatory CD8 T-cells during active CNS inflammation. We transferred CD45.1+ MOG_{35-55}-specific CD8 T-cells and 24 hours later induced MOG_{35-55} EAE disease and assessed cellular infiltration. During active disease, MOG_{35-55}-specific CD8 T-cells were detectable in the CNS at days 10 and 20 (Figure 32).

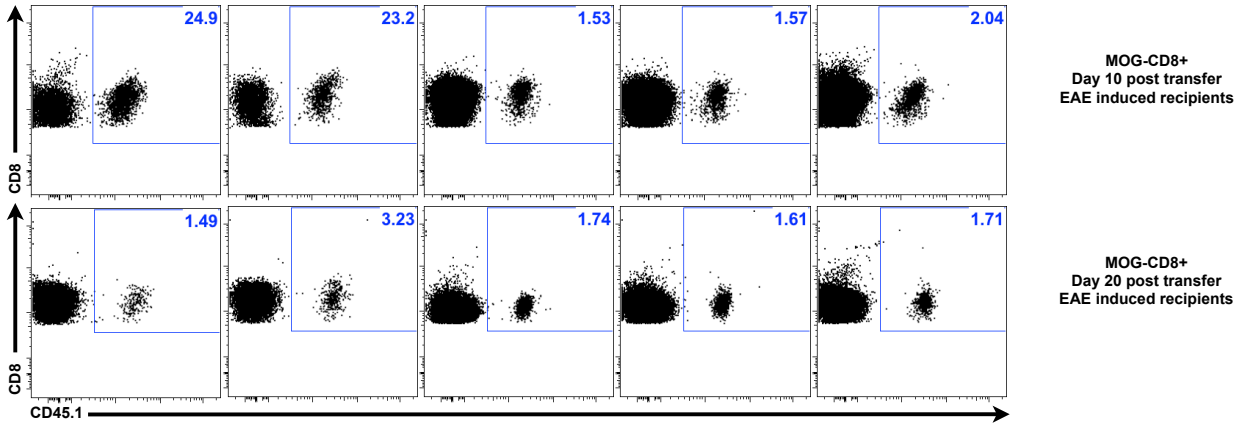
Since we had only seen CD8 T-cell infiltration when EAE was induced, we concluded that CNS inflammation was a requirement for infiltration and possible expansion within the CNS. We did not observe a focusing of cellular infiltration to any unique location; hence we concluded that autoregulatory CD8 T-cells invoked both a peripheral immune compartment, as well as a CNS mechanism of action for disease suppression.

Figure 31: Autoregulatory CD8 T-cells do not Infiltrate and/or Expand in the CNS of Naive Mice



In vivo trafficking assay was performed using MOG-specific CD45.1+CD8-CD4+ or CD45.1+CD4-CD8+ T-cells that were transferred into naive (CD45.2+) mice and indicated tissues harvested. Dots plots are gated on live gate+ CD90+CD8-CD4+ or live gate+CD90+CD4-CD8+ evens and show representative experiment of two independent experiments evaluating days 5 and 10 (not shown) and 20 post-transfer (5 mice per condition).

Figure 32: CNS Infiltration of Autoregulatory CD8 T-cells During CNS Inflammation



Autoregulatory CD8 T-cells can access the CNS during ongoing inflammation. Mice were treated similarly as in (Figure 31) except that recipient mice were MOG_{35-55} immunized 1 day post transfer. Dots plots are gated on live gate+CD90+CD8-CD4+ or live gate+CD90+CD4-CD8+ evens and show Representative experiment of two independent experiments evaluating days 10 and 20 post transfer (5 mice per condition).

.

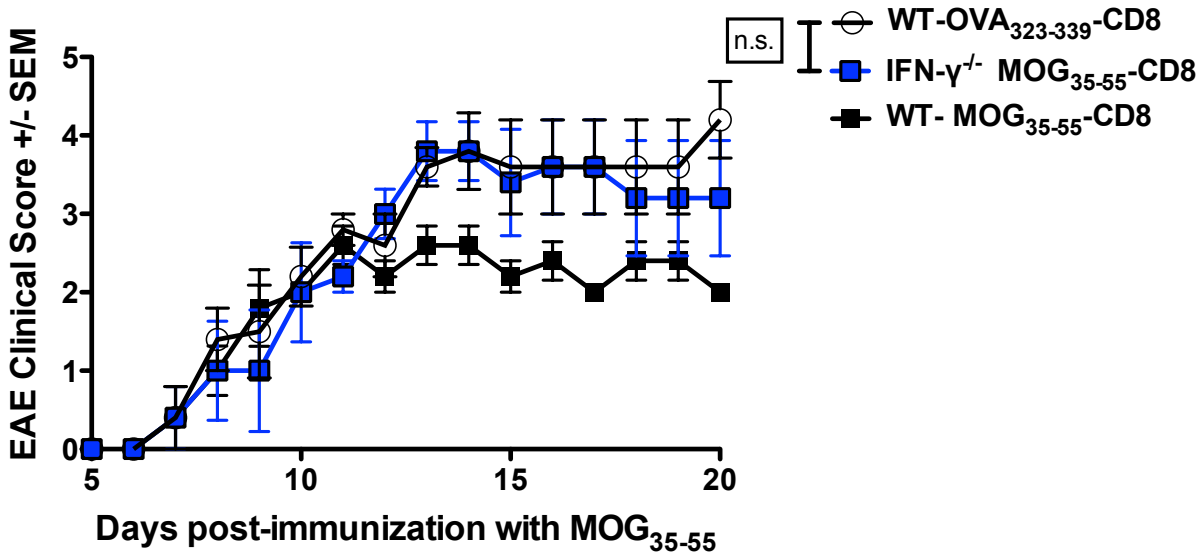
3.2.7: Autoregulatory CD8 T-cells are IFN-γ and Perforin-dependent

As our FACS analysis had shown that these autoregulatory CD8 T-cells were capable of producing effector cytokines (Figure 30), we next evaluated which of these cytokines were relevant to disease suppression. It has been previously shown that undefined-antigen-specific IFN-γ producing CD8 T-cells may act in a suppressive manner [186],[202],[203], thus we evaluated the role of IFN-γ in autoregulatory CD8 T-cell mediated disease inhibition. We obtained IFN-γ deficient MOG_{35-55} -specific CD8 T-cells by immunizing IFN-$\gamma^{-/-}$ B6 mice with MOG_{35-55} peptide. After 20 days, lymph node and spleen cells were harvested and expanded *in vitro* for 3 days. We subsequently transferred purified CD8 T-cells into WT naive recipient mice at day -1. For control purposes we also transferred MOG_{35-55} and $OVA_{323-339}$-specific CD8 T-cells from WT mice. At day 0, EAE was induced and clinical disease blindly evaluated for 30 days. IFN-γ incompetent MOG_{35-55}-specific CD8 T-cells recipients were found to have a more severe disease at the acute and chronic phase of EAE, as compared to the WT MOG_{35-55}-specific CD8 T-cell recipients (Figure 33).

Similarly, we evaluated the mechanistic role of the cytotoxic molecule perforin using MOG_{35-55}-specific CD8 T-cells from perforin$^{-/-}$ donor mice. Again, as compared to WT-MOG_{35-55}-specific CD8 T-cell recipients (protected mice), perforin$^{-/-}$ MOG_{35-55}-specific CD8 T-cells were found to have a diminished capacity to inhibit EAE disease (Figure 34), The observation that perforin is needed for disease regulation helps correlate and explain the observation of *in vitro* (Figure 44) and *in vivo* killing of pathogenic cells [85]. In contrast, autoregulatory CD8 T-cells obtained from IFN-γ-R$^{-/-}$ (Figure 35), IL4$^{-/-}$ (Figure 36) and IL10$^{-/-}$ (Figure 37) mice were still capable of suppressing EAE.

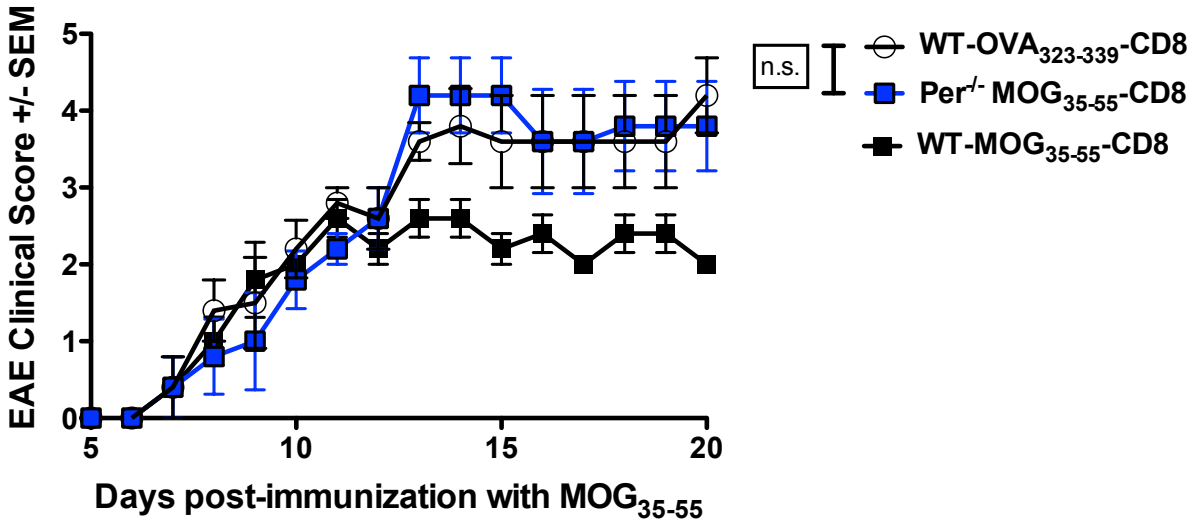
Thus, I concluded that autoregulatory CD8 T-cells use IFN-γ as an effector molecule for disease amelioration. These cells were also found dependent on perforin, which corroborated with our *in vitro* and *in vivo* killing data previously described.

Figure 33: Amelioration of EAE by MOG_{35-55}-specific CD8 T-cells is IFN-γ-dependent



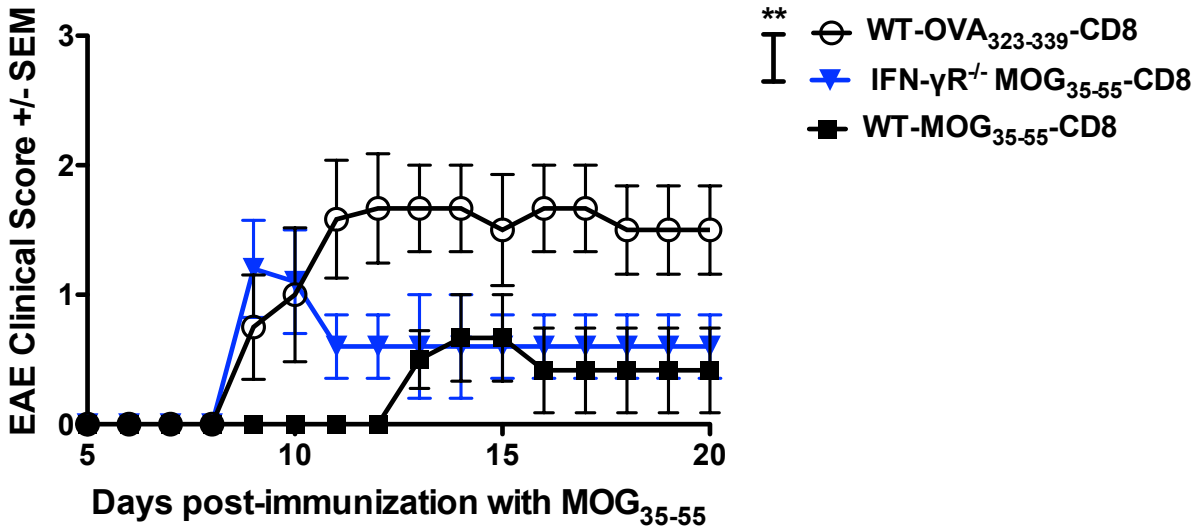
Autoregulatory CD8 T-cells ameliorate EAE disease via IFN-γ. IFN-γ incompetent CD8 T-cells were obtained by immunizing IFN-$\gamma^{-/-}$ B6 mice with 100 µg of MOG_{35-55} at day 0. PTX was given at days 0 and 2. Next WT-$OVA_{323-339}$, WT-, or IFN-$\gamma^{-/-}$-MOG_{35-55}-specific CD8 T-cells were transferred into WT mice (day -1), followed by MOG_{35-55}-immunization. Disease suppression was not transferrable with autoregulatory CD8 T-cells isolated from IFN-γ deficient mice. Data are representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). n.s.= not significant.

Figure 34: Amelioration of EAE by MOG_{35-55}-specific CD8 T-cells is Perforin-dependent



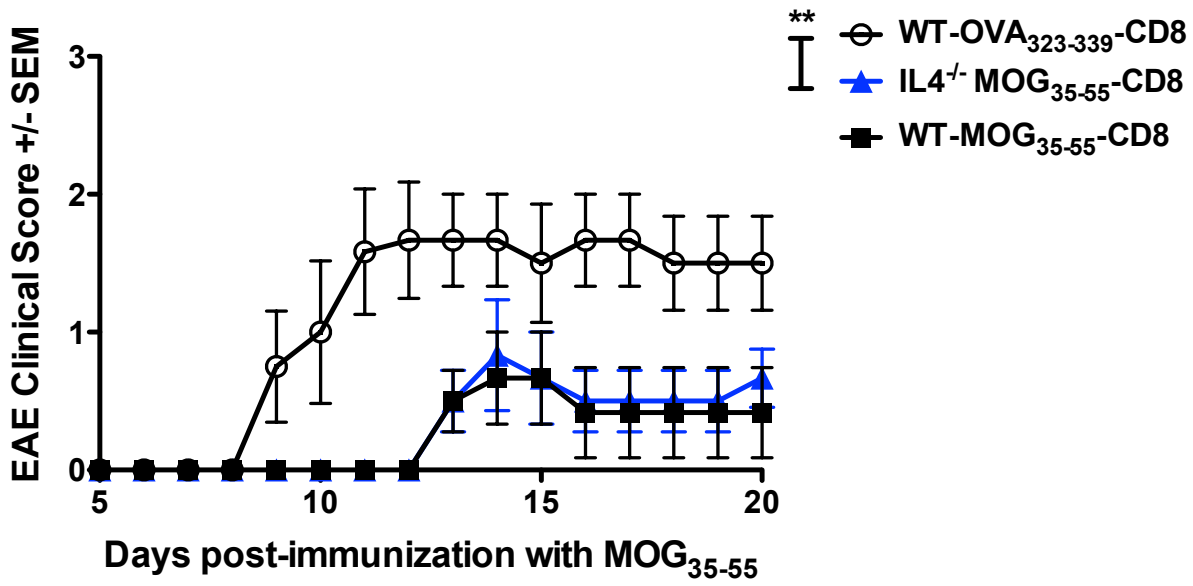
Autoregulatory CD8 T-cells ameliorate EAE disease via perforin (Per). Perforin incompetent CD8 T-cells were obtained by immunizing $Per^{-/-}$ B6 mice with 100 µg of MOG_{35-55} at day 0. PTX was given at days 0 and 2. Next WT-$OVA_{323-339}$, WT-, or $Per^{-/-}$-MOG_{35-55}-specific CD8 T-cells were transferred into WT mice (day -1), followed by MOG_{35-55}-immunization. Disease suppression was not transferrable with autoregulatory CD8 T-cells isolated from perforin deficient mice. Data are representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). n.s.= not significant.

Figure 35: Amelioration of EAE by MOG_{35-55}-specific CD8 T-cells is IFN-γR Independent



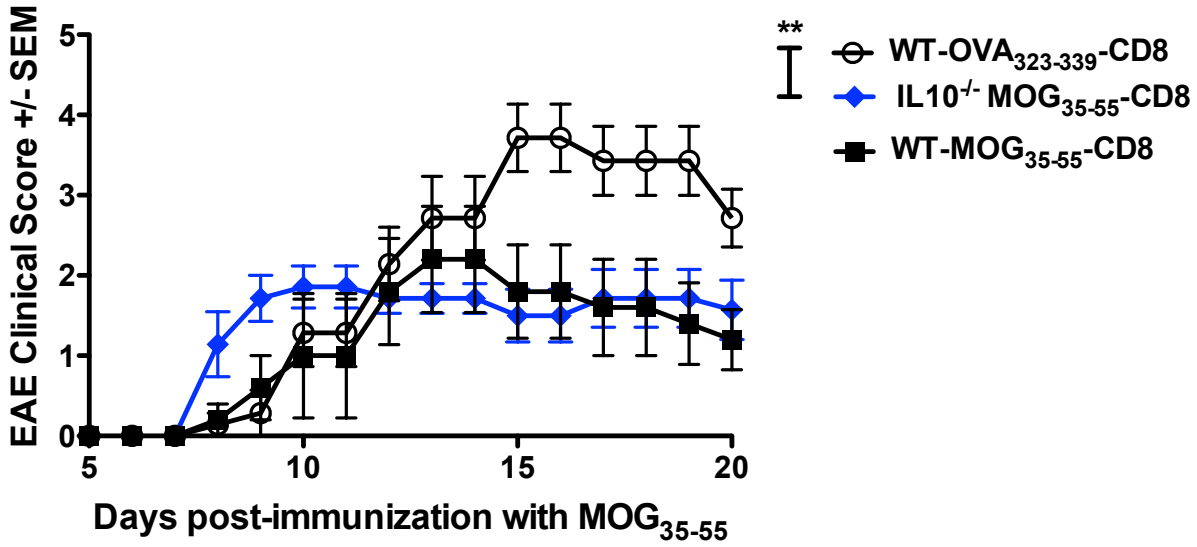
Autoregulatory CD8 T-cells ameliorate EAE disease via IFN-γR independent mechanism. IFN-γR incompetent CD8 T-cells were obtained by immunizing IFN-$\gamma R^{-/-}$ B6 mice with 100 µg of MOG_{35-55} at day 0. PTX was given at days 0 and 2. Next WT-$OVA_{323-339}$, WT-, and IFN-$\gamma R^{-/-}$-MOG_{35-55}-specific CD8 T-cells were transferred into WT mice (day -1), followed by MOG_{35-55}-immunization. Disease suppression was transferrable with autoregulatory CD8 T-cells isolated from IFN-γR deficient mice. Data are representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$.

Figure 36: Amelioration of EAE by MOG_{35-55}-specific CD8 T-cells is IL4 Independent



Autoregulatory CD8 T-cells ameliorate EAE disease via IL4 independent mechanism. IL4 incompetent CD8 T-cells were obtained by immunizing $IL4^{-/-}$ B6 mice with 100 µg of MOG_{35-55} at day 0. PTX was given at days 0 and 2. Next, WT-$OVA_{323-339}$, WT-, and $IL4^{-/-}$-MOG_{35-55}-specific CD8 T-cells were transferred into WT mice (day -1), followed by MOG_{35-55}-immunization. Disease suppression was transferrable with autoregulatory CD8 T-cells isolated from IL4 deficient mice. Data are representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$.

Figure 37: Amelioration of EAE by MOG_{35-55}-specific CD8 T-cells is IL10 Independent



Autoregulatory CD8 T-cells ameliorate EAE disease via IL10 independent mechanism. IL10 incompetent CD8 T-cells were obtained by immunizing $IL10^{-/-}$ B6 mice with 100 µg of MOG_{35-55} at day 0. PTX was given at days 0 and 2. Next, WT-$OVA_{323-339}$, WT-, and $IL10^{-/-}$-MOG_{35-55}-specific CD8 T-cells were transferred into WT mice (day -1), followed by MOG_{35-55}-immunization. Disease suppression was transferrable with autoregulatory CD8 T-cells isolated from IL10 deficient mice. Data are representative of two independent experiments (n=7 per condition). Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$.

3.3: Summary

Thus, I now provide strong evidence confirming the regulatory role of CD8 T-cells in an autoimmune disease by evaluating several models and elucidating the mechanisms used in disease suppression. It is now appreciated that indeed autoreactive regulatory CD8 T-cells can act as suppressors of CNS disease. This suppression requires cognate antigen presentation by MHC-Ia *in vivo* for disease amelioration. Much like their CD4 Treg counter part, autoregulatory CD8 T-cells express a central memory phenotype, along with lymph node homing markers. Finally, autoreactive CD8 T-cells do not traffic or expand within a naïve CNS, suggesting that these cells cannot function in a pathogenic role. In fact, autoregulatory CD8 T-cells are only able to traffic into the CNS during CNS inflammation thereby indicated that their presence in the CNS is a result of tissue destruction and not as mediators of CNS disease.

CHAPTER 4: TARGETING ENCEPHALITOGENIC CD4 T-CELLS TO TREAT AUTOIMMUNE INFLAMMATION

4.1: Introduction

4.1.1: CD4 T-cells in Autoimmunity

Myelin-specific CD4 T-cells are the putative encephalitogenic population in EAE. In fact, a significant amount of data show that this may also be the case in human MS. Hence, if indeed autoregulatory CD8 T-cells are ameliorating EAE disease, this amelioration might be explained by a modulation of the putative encephalitogenic population.

4.1.2: Objectives

The primary objective in this chapter is to determine if autoregulatory CD8 T-cells could modulate and/or target CD4 T-cells, thus explaining their disease ameliorating capacity.

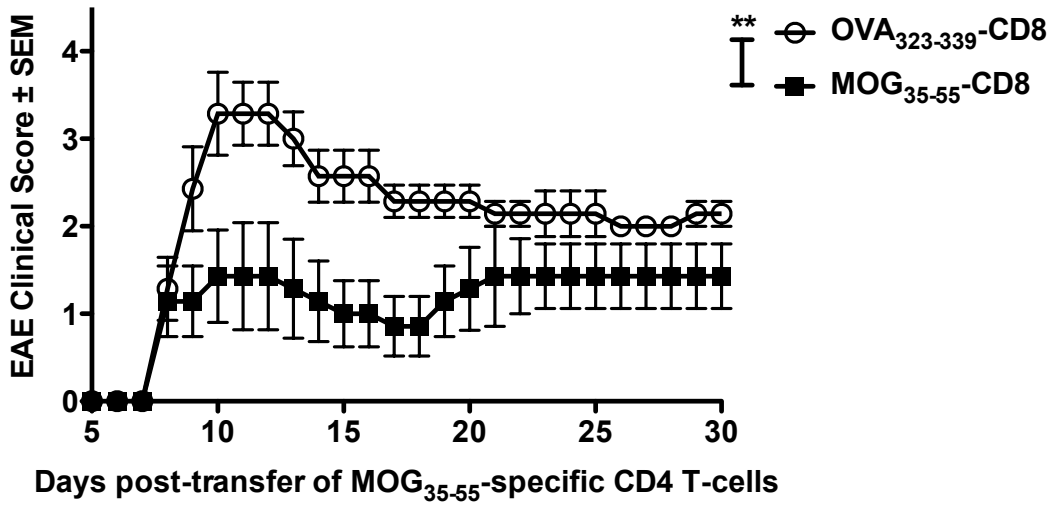
4.2: Results

4.2.1: Autoregulatory CD8 T-cells Suppress CD4 T-cell Mediated Autoimmune Disease

Having an understanding that autoregulatory CD8 T-cells could modulate primary EAE; we next wanted to extend our knowledge by evaluating the relationship between the disease ameliorating T-cells and the purported encephalitogenic myelin-specific CD4 T-cells. Interaction between both cell types is warranted by the observations that both CD8 and CD4 T-cells are present within the site of pathology and other subtypes of regulatory CD8 T-cells have been described capable of modulating CD4 T-cell phenotype [183]. First, I began by determining

whether autoregulatory CD8 T-cells could suppress EAE that was mediated by myelin-specific CD4 T-cells, i.e. are autoregulatory CD8 T-cells capable of suppressing adoptive EAE. B6 mice were immunized with MOG_{35-55} peptide in order to produce the encephalitogenic CD4 T-cells. These cells were transferred into recipient mice, which had received MOG_{35-55}-specific CD8 T-cells one-day earlier, and clinical disease evaluated relative to control recipients ($OVA_{323-339}$-specific CD8 T-cells) for 30 days. Similar to primary disease suppression, autoregulatory CD8 T-cells were found to suppress adoptively transferred (AT-EAE) EAE disease (Figure 38). These observations suggest that autoregulatory CD8 T-cells could be acting directly on pathogenic CD4 T-cells.

Figure 38: Autoregulatory CD8 T-cells are Capable of Suppressing CD4 T-cell Mediated EAE



Assessment of autoregulatory CD8 T-cell inhibition of CD4 T-cell mediated EAE. Naïve B6 mice were immunized with MOG_{35-55} peptide and at day 10, inguinal lymph node harvested and processed into a single cell suspension. Cells were cultured for 3 days with cognate antigen and IL12 (10 ng/ml). CD4 T-cells were purified and transferred into mice, which received either MOG_{35-55}-specific CD8 T-cells or control $OVA_{323-339}$-specific CD8 T-cells. Recipient mice also received PTX on day 0 and 2 post adoptive transfer. Data are representative of five independent experiments, 5-7 mice per condition. Error bars represent mean clinical score ± standard error of the mean (SEM). **=$p<0.05$

4.2.2: CD8$^{-/-}$ mice Exhibit an Augmented CD4 Autoreactivity, which can be reversed with Autoregulatory CD8 T-cells

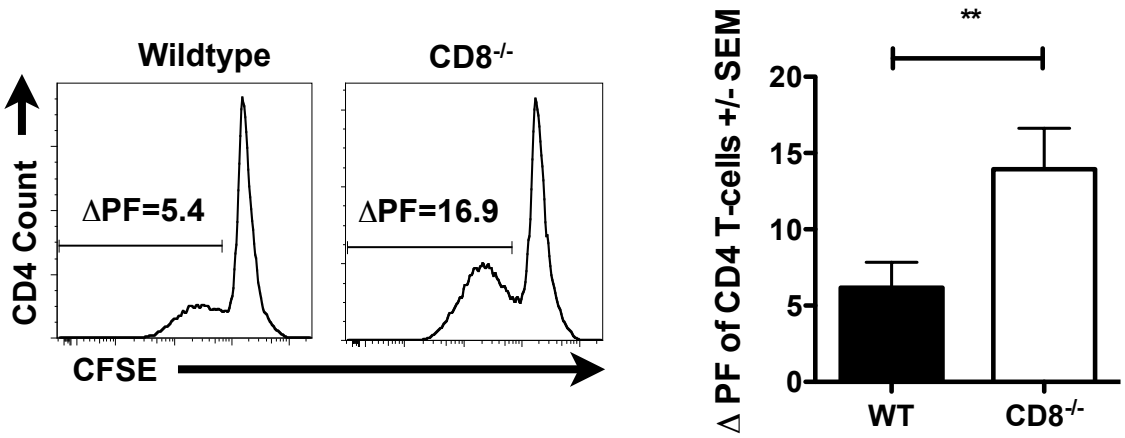
In order to confirm that indeed autoregulatory CD8 T-cells were modulating pathogenic CD4 T-cells, I returned to the CD8$^{-/-}$ B6 model, where we had previously seen that EAE disease was augmented (Figure 7) and compared MOG_{35-55}-specific CD4 T-cells autoreactivity between CD8$^{-/-}$ and WT mice using the CFSE dilution assay. Briefly, WT and CD8$^{-/-}$ mice were immunized with MOG_{35-55} and twenty days later draining ln and spl cells harvested. Next, the cells were CFSE labeled and cultured for five days with cognate antigen. Subsequently cells were stained with fluorophore conjugated anti-TCRβ, CD4 and CD8 antibodies. CFSE dilution was measured within the TCRβ+CD4+CD8- population. Peripheral and CNS CD4 T-cells from CD8$^{-/-}$ mice were found to have a significantly higher recall response to MOG_{35-55} peptide as compared to CD4 T-cells from WT mice (Figure 39 and Figure 40). In fact, evaluation of intracellular cytokine production revealed an increase in IL17, IFN-γ and TNF-α producing MOG_{35-55}-specific CD4 T-cells in CD8$^{-/-}$ mice as compared to WT (Figure 41). Foxp3+ and IL10+ MOG_{35-55}-specific CD4 T-cells were found to be comparable between CD8$^{-/-}$ and WT cohorts.

Next, I wanted to determine if infusion of autoregulatory CD8 T-cells was sufficient in reversing the modulation of pathogenic CD4 T-cells. Thus, I performed a rescue experiment, where I reconstituted the CD8$^{-/-}$ mice with autoregulatory CD8 T-cells, induced primary EAE and compared autoreactivity, as well as inflammatory cytokine production of CD4 T-cells between CD8$^{-/-}$ and WT mice. In CD8$^{-/-}$ mice, recall response analysis of autoreactive CD4 T-cells from autoregulatory CD8-treated CD8$^{-/-}$ mice versus control CD8 T-cell treated CD8$^{-/-}$ mice revealed a decrease in CD25, TNF-α, IFN-γ and GM-CSF producing myelin-specific CD4 T-cells (Figure 42). Confirming and explaining the role of autoregulatory CD8 T-cells, treatment of

EAE-induced $CD8^{-/-}$ mice with MOG_{35-55}-specific CD8 T-cells was found to be sufficient for reversing the increased disease susceptibility (Figure 13).

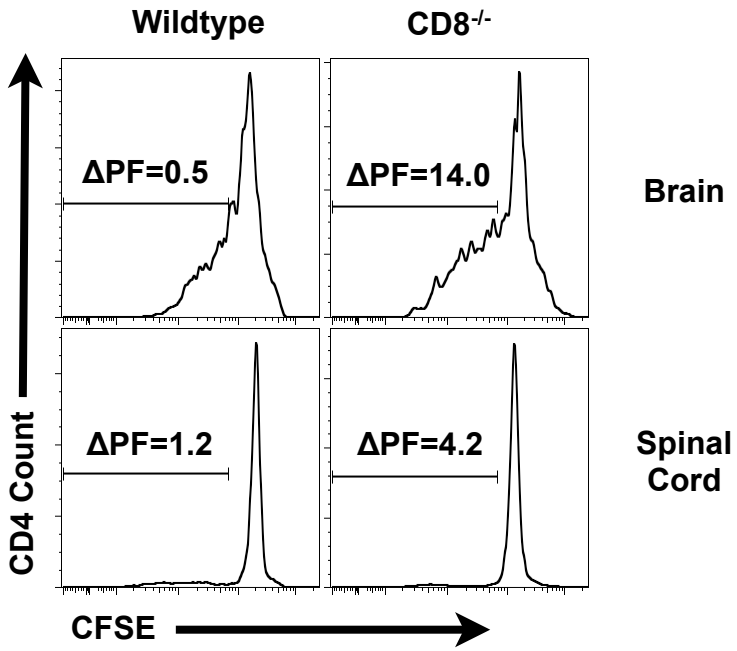
Collectively, these data explain how that CD8 T-cells play a regulatory role in autoimmune demyelinating disease. In the absence of CD8 T-cells, encephalitogenic CD4 T-cells have an increased capacity to respond to MOG_{35-55} peptide and this response is characterized by an increase in prototypic inflammatory cytokines, thus explaining the increase in disease severity of $CD8^{-/-}$ mice. Finally, autoregulatory CD8 T-cells were found to be sufficient in reversing the increased disease severity and this correlated with a modulation of both CD4 autoreactivity and functional profile.

Figure 39: Increase in Peripheral CD4 Autoreactivity in $CD8^{-/-}$ Mice



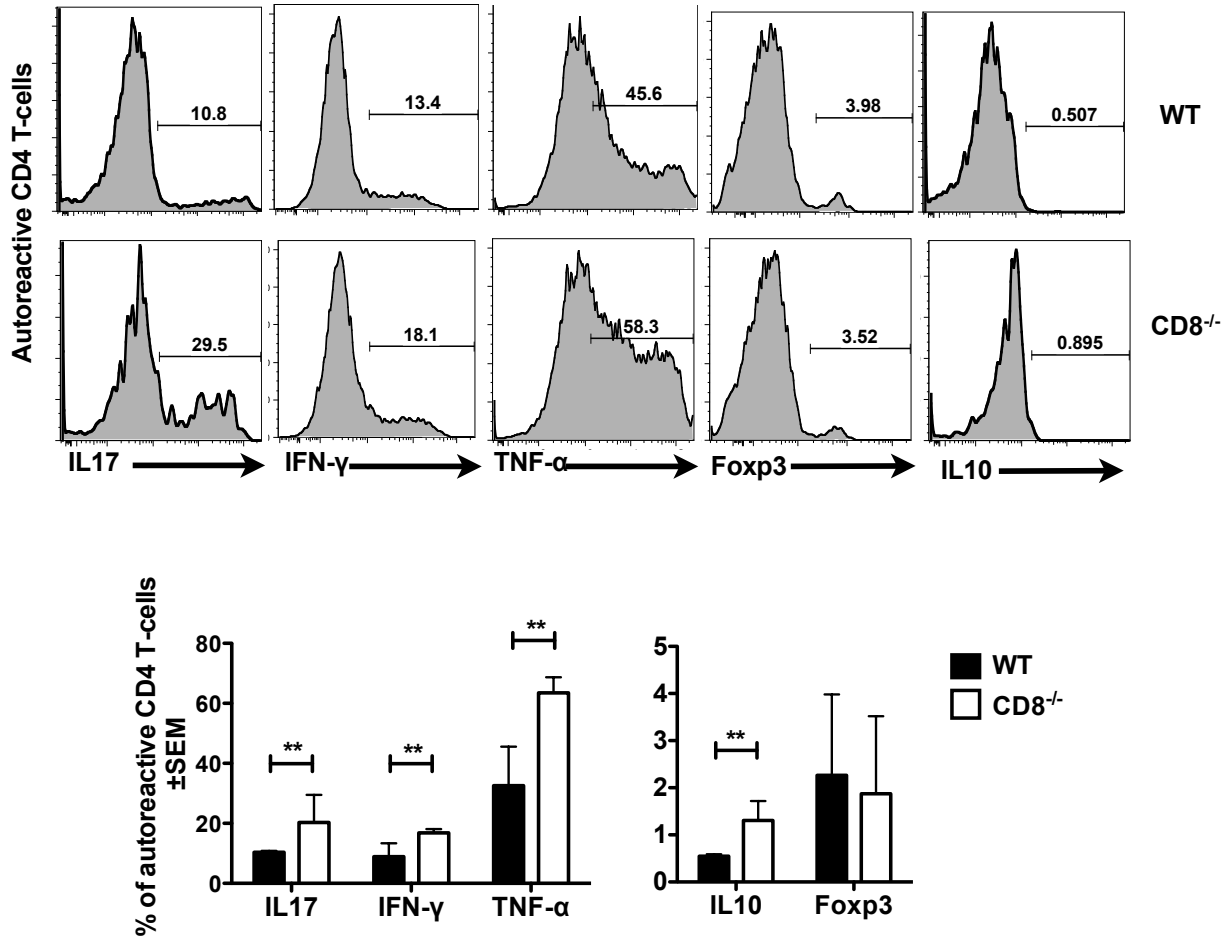
Peripheral CD4 autoreactivity was compared between WT and $CD8^{-/-}$ mice using the CFSE dilution assay. Lymph nodes cells were harvested and processed into single cell suspensions from either WT or $CD8^{-/-}$ mice. Cells were CFSE stained and cultured with cognate antigen and IL2 for five days. Cells were then washed and stained for TCRβ, CD4 and CD8, fixed and CFSE dilution measured using flow cytometry. Histograms show events that have been gated for TCRβ+CD4+CD8- cells and interval indicates the percentage of CFSE low cells. ΔPF is the delta proliferation value (percentage of CFSE low cells in cognate antigen stimulated condition minus no stimulation condition). Data are representative of three independent experiments, 3-5 mice per condition. Error bars represent the mean clinical score ± standard error of the mean (SEM). **=$p<0.05$

Figure 40: Increase in CNS CD4 Autoreactivity in CD8$^{-/-}$ Mice



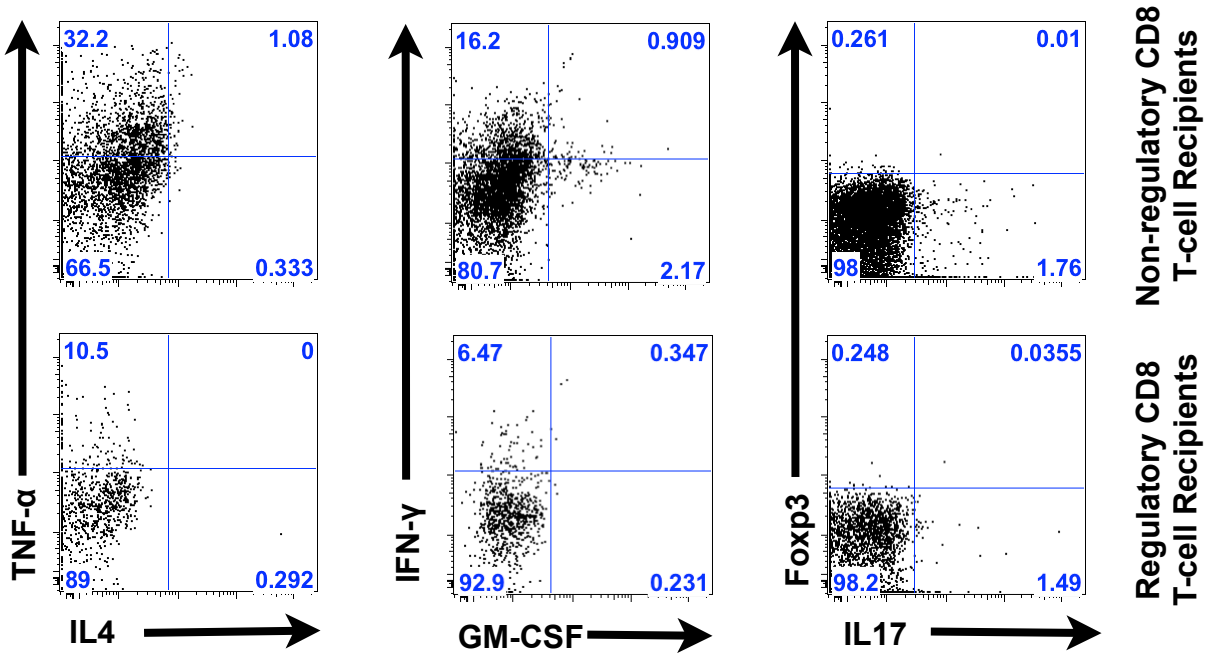
Autoreactivity of CD4 T-cells isolated from the CNS of WT and CD8$^{-/-}$ mice were compared using the CFSE dilution assay. CNS tissues were harvested and processed into single cell suspensions from either WT or CD8$^{-/-}$ mice. Myelin debris and lipids were removed by using a 30% Percoll gradient. Cells were CFSE stained and cultured with cognate antigen and IL2 for five days. Cells were then washed and stained for TCRβ, CD4 and CD8, fixed and CFSE dilution measured using a flow cytometer. Histograms show events, which have been gated for TCRβ+CD4+CD8- cells, and intervals indicate the percentage of CFSE low cells. ΔPF is the delta proliferation value (percentage of CFSE low cells in cognate antigen stimulated condition minus no stimulation condition). Data are representative of three independent experiments, 3-5 mice per condition.

Figure 41: Increase in Inflammatory Autoreactive CD4 T-cells in CD8$^{-/-}$ Mice



Lymph node cells were harvested and processed into single cell suspensions from either WT or CD8$^{-/-}$ mice. Cells were CFSE stained and cultured with cognate antigen and IL2 for five days. Cells were then restimulated with PMA/IO/BFA for 5 hours. Cells were then washed, permeabilized and stained for TCRβ, CD4, CD8, IL17, IFN-γ, TNF-α, Foxp3 and IL10, fixed and CFSE dilution measured using a flow cytometer. Histogram shows events that have been gated for TCRβ+CD4+CD8-. CFSE low cells and interval indicates the percentage of cytokine producing cells that are CFSE low. Data are representative of three independent experiments, 3-5 mice per condition. Error bars represent means ± standard error of the mean (SEM). **=p<0.05

Figure 42: Treatment with Autoregulatory CD8 T-cells modulates Inflammatory Autoreactive CD4 T-cells in CD8$^{-/-}$ Mice



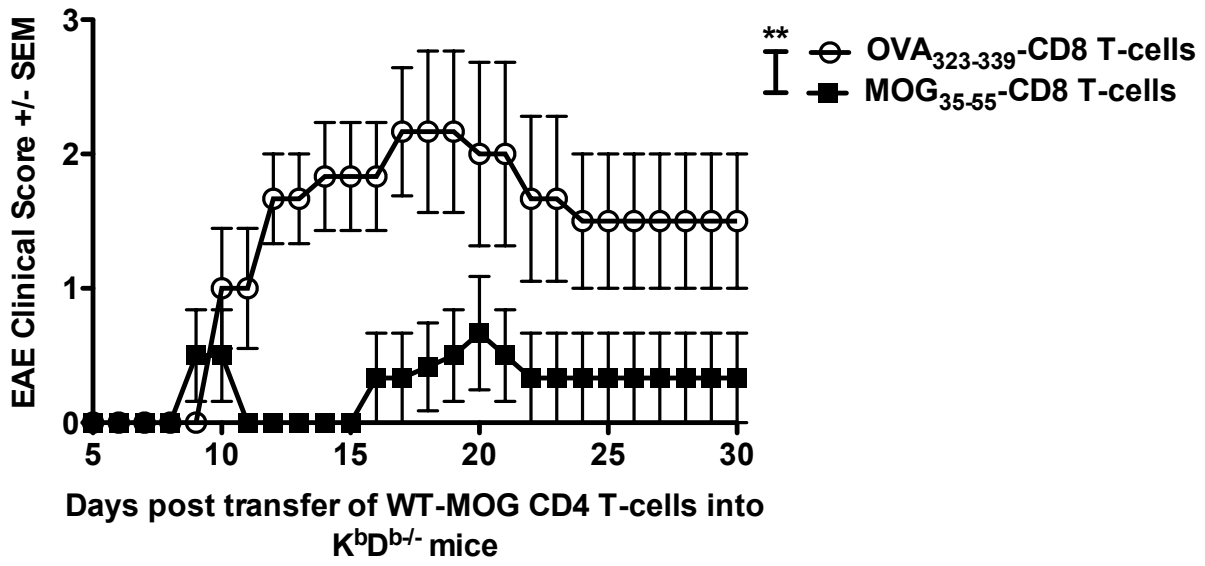
Autoregulatory CD8 (Regulatory CD8 Recipient) or control CD8 (Non-regulatory CD8 Recipient) T-cells were transferred i.v. into CD8$^{-/-}$ mice and primary EAE induced the following day. Twenty days post-immunization, ln were harvested and processed into single cell suspensions. Lymphocytes were CFSE stained and cultured for 5 days with cognate antigen. Cells were restimulated with PMA/IO/BFA for 5 hours. Cells were washed, permeabilized and stained for TCRβ, CD4, CD8, TNF-α, IL4, IFN-γ, GM-CSF, Foxp3 and IL17. Dots plots indicate events that have been gated for TCRβ+CD4+CD8-CFSE low events. Data is representative of two independent experiments, 3-5 mice per condition.

4.2.3: Autoregulatory CD8 T-cells target Pathogenic CD4 T-cells by MHC Class Ia Molecules

Our observations that autoregulatory CD8 T-cells could suppress CD4 T-cell-mediated disease, and given that MHC Class Ia was required for EAE suppression, I investigated the possibility that autoregulatory CD8 T-cell targeting of encephalitogenic CD4 T-cells might be sufficient for EAE amelioration. Thus, I evaluated if autoregulatory CD8 T-cells could effectively suppress AT-EAE when only the encephalitogenic CD4 T-cell population expressed MHC Class Ia molecules. We modified our adoptive EAE protocol by transferring WT MOG_{35-55}-specific CD4 T-cells into $K^bD^{b-/-}$ host mice and evaluated if autoregulatory CD8 T-cells could still suppress disease. Autoregulatory CD8 T-cells were found capable of suppressing WT adoptively transferred EAE when the recipient host was devoid of MHC Class I molecules (Figure 43).

Hence, I conclude that presentation of myelin antigen within the context of MHC Class I molecules is indeed activating autoregulatory CD8 T-cells. When antigen presentation was limited to pathogenic CD4 T-cells, there was a profound reduction in disease severity, suggesting that pathogenic CD4 T-cells could present myelin antigen directly to CD8 T-cells and thus potentiate their targeting.

Figure 43: Autoregulatory CD8 T-cell targeting of Pathogenic CD4 T-cells is Sufficient for EAE Amelioration



Autoregulatory CD8 (MOG_{35-55}-CD8+) or control CD8 ($OVA_{323-339}$-CD8+) T-cells were transferred i.v. into $K^bD^{b-/-}$ mice. The following day, WT MOG_{35-55}-specific CD4 T-cells were transferred into all recipient mice in order to induce adoptive EAE. Clinical disease was followed for 30 days using our two-way blinded evaluation scheme. Data are representative of two independent experiments, 5-7 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p \leq 0.05$.

4.2.4: Suppression/cytotoxicity of MOG-specific CD4 T-cells by Autoregulatory CD8 T-cells

Thereafter, I wanted to determine if autoregulatory CD8 T-cells were suppressive and/or cytotoxic to pathogenic CD4 T-cells *in vivo*. First, we determined if autoregulatory CD8 T-cells could kill MOG-loaded CD4 T-cells by performing an *in vitro* killing assay. ConA stimulated MOG-loaded splenocytes were used as target cells and cultured with an increasing number of MOG_{35-55}-specific CD8 T-cells in media alone or with cognate antigen for 48 hours. Killing was measured by evaluating the number of target cells normalized to control APC beads. For control purposes, MOG_{35-55}-specific CD8 T-cells were cultured with p815 cells decorated with murine anti-CD3 antibody. We observed a decrease in target cells as autoregulatory CD8 T-cell number increased. Using a previously described formula, we translated the loss in cell number to percent killing [61]. Hence, I concluded that autoregulatory CD8 T-cell killing increased as effector to target cell ratio increased, i.e. in a dose dependent manner (Figure 44).

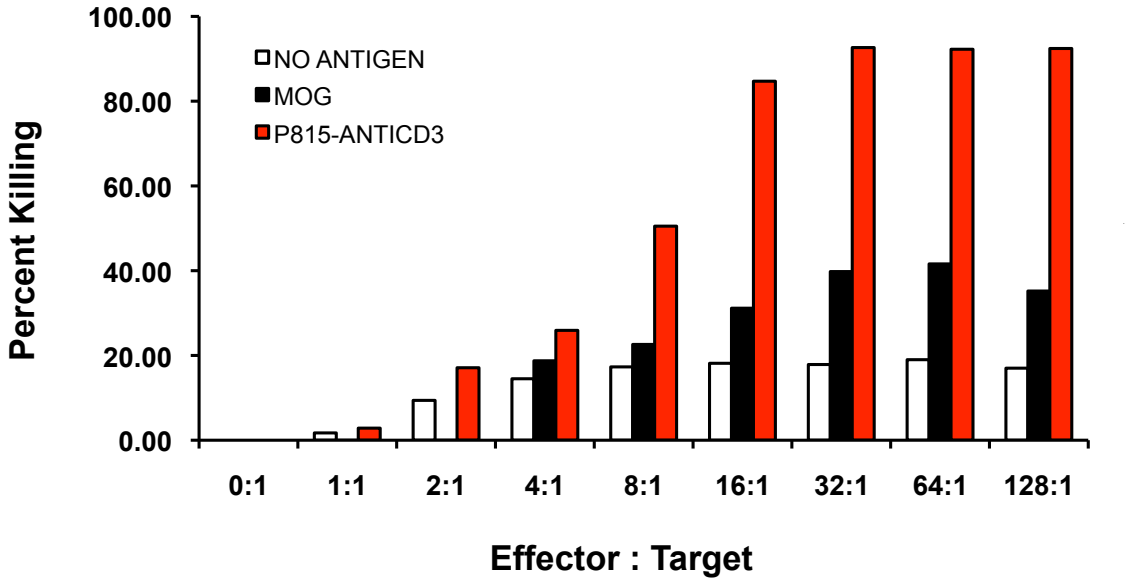
Next, I tried to determine if autoregulatory CD8 T-cells could suppress pathogenic CD4 T-cells *in vitro*. Several attempts resulted in inconclusive results. Thus, I proceeded to evaluate if suppression or cytotoxicity could be measured *in vivo*.

First, I evaluated *in vivo* suppression by transferring congenic CFSE-labeled MOG_{35-55}-specific CD4 T-cells into autoregulatory CD8 T-cell-treated mice and evaluating CFSE dilution. Donor myelin-specific CD4 T-cells were isolated from the draining lymph nodes of day 10 post MOG_{35-55}-immunized CD45.1+ mice. The cells where then adoptively transferred into naïve recipient CD45.2+ mice, which had been recipients of either, control $OVA_{323-339}$- or MOG_{35-55}-specific CD8 T-cells the previous day. Ten days later, cervical lymph node and spleen tissues were harvested and CFSE dilution of adoptively transferred CD45.1+ CD4 T-cells measured. A

significantly higher proportion of undiluted CFSE CD4+ T-cells were isolated from MOG-CD8-treated mice as compared to control (Figure 45). In fact, absolute counts demonstrated a higher number of CFSE hi CD4 T-cells in the cervical lymph nodes and spleens of MOG-CD8 treated mice (Figure 46). Although there was a discernable difference between the autoregulatory and control CD8 T-cell treated mice, there was a considerable amount of background noise (CFSE diluted cells) in both cohorts, probably due to the low precursor frequency (MOG_{35-55}-specific) of the adoptively transferred CD4 T-cells. Thus, I proceeded to evaluate if autoregulatory CD8 T-cells could suppress CD4 T-cells whose antigen specificity is directed towards a myelin antigen, transgenic MOG_{35-55}-specific (2D2) CD4 T-cells.

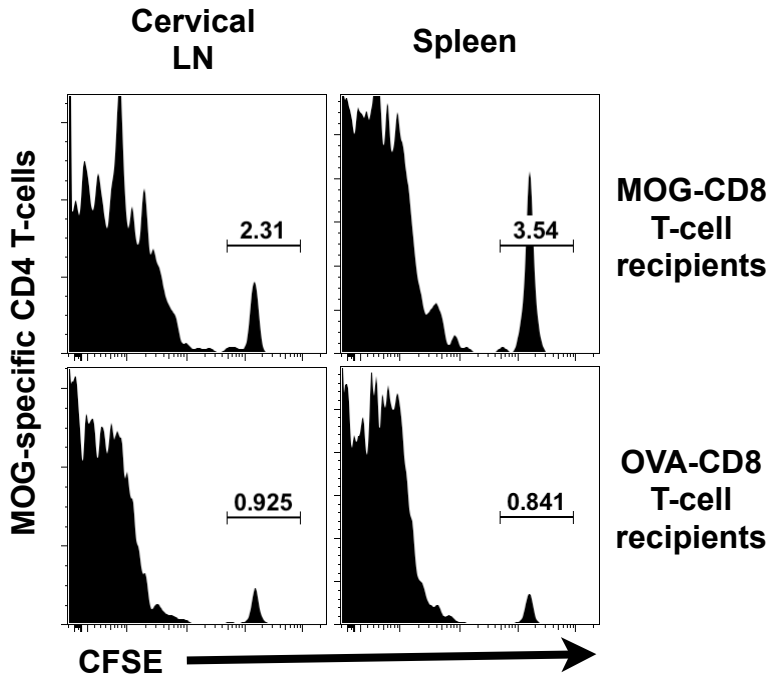
Determination of *in vivo* encephalitogenic CD4 T-cell suppression was assessed by adoptively transferred transgenic MOG-specific (2D2) CFSE-stained naïve CD4 T-cells (CD45.2+) into $OVA_{323-339}$- or MOG_{35-55}-specific CD8 T-cell treated mice (CD45.1+). All mice were then immunized with MOG_{35-55} peptide and ten days later, evaluation of CFSE dilution revealed an unimpeded 2D2-CD4 T-cell response to MOG_{35-55} in OVA-CD8 treated mice, while MOG-CD8 treated mice had a significant portion of undiluted CFSE 2D2 CD4 T-cells (Figure 47). In fact, evaluation of CFSE dilution at days 5 and 7 revealed a gradual increase in the suppression by autoregulatory CD8 T-cells (Figure 48).

Figure 44: *In vitro* Killing of MOG-loaded CD4 T-cells by Autoregulatory CD8 T-cells



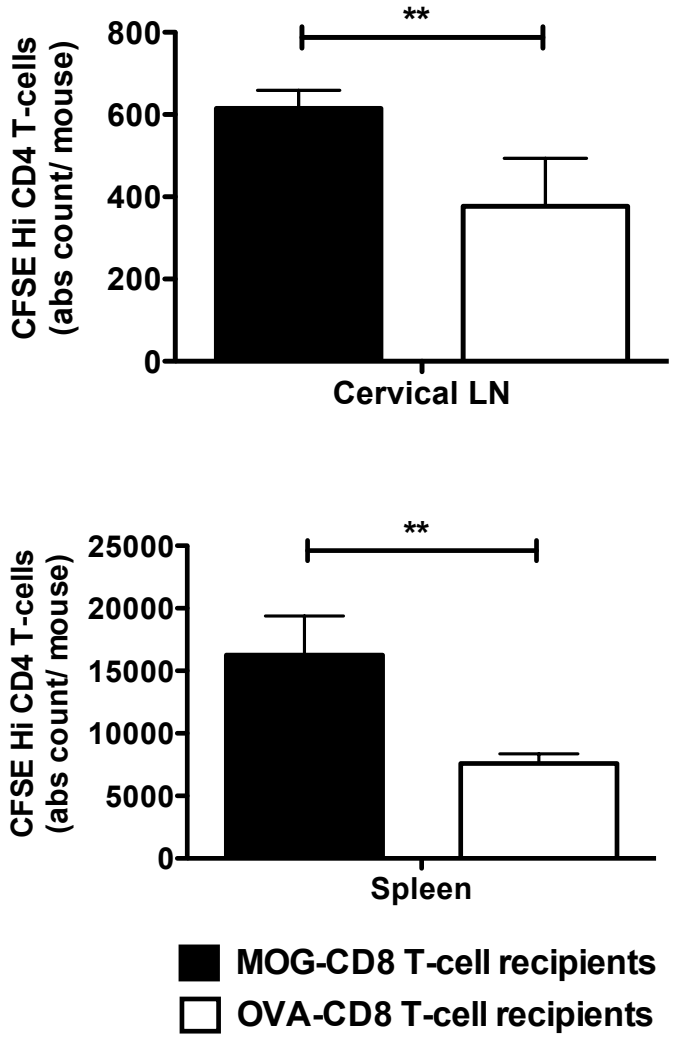
Assessment of *in vitro* killing by autoregulatory CD8 T-cells. Target cells are splenocytes that were ConA blasted and loaded with MOG_{35-55} peptide. Effector cells are MOG_{35-55}-specific CD8 T-cells isolated from day 20-post immunized mice. Effector cells were added in increasing numbers and cultured for 48 hours with no stimulation (NO ANTIGEN) or with MOG_{35-55} peptide (MOG). CD4+ T-cells were acquired and normalized with APCS beads (counting control). Percent killing was calculated using the formula previously described formula [61]. A redirect lysis condition was used as a positive control, whereby P815 cells were decorated with murine anti-CD3 and cultured with CD8 T-cells (P815-ANTICD3). *Courtesy of Dr. J. Mendoza.*

Figure 45: *In vivo* Suppression of MOG_{35-55}-specific CD4 T-cells by Autoregulatory CD8 T-cells



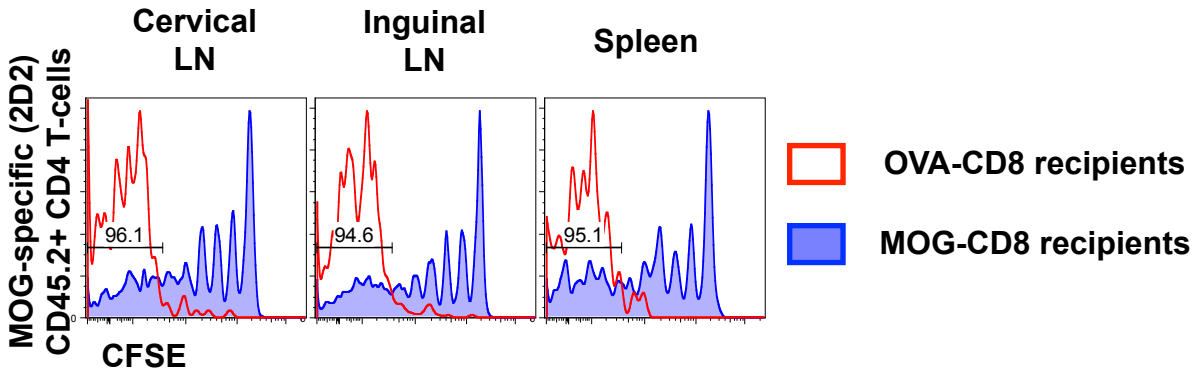
In vivo suppression of MOG_{35-55}-specific CD4 T-cells. Autoregulatory CD8 (MOG-CD8) or control (OVA-CD8) T-cells were transferred into naïve mice. CD45.1+ myelin-specific CD4 T-cells were isolated from day 10-post immunized B6 mice, CFSE stained and transferred i.p. into all mice. Ten days later, cervical and spleen tissues were harvested and processed into single cell suspensions and 500,000 events were acquired on a flow cytometer. Histogram shows events gated on CD3+CD4+CD8- cells and interval shows the percentage of CFSE hi cells within the gated population. Data are representative of two independent experiments, replicates within each experiment, five mice per condition.

Figure 46: Increased Number of Suppressed MOG-$_{35-55}$-specific CD4 T-cells in Autoregulatory CD8 T-cell-treated Mice



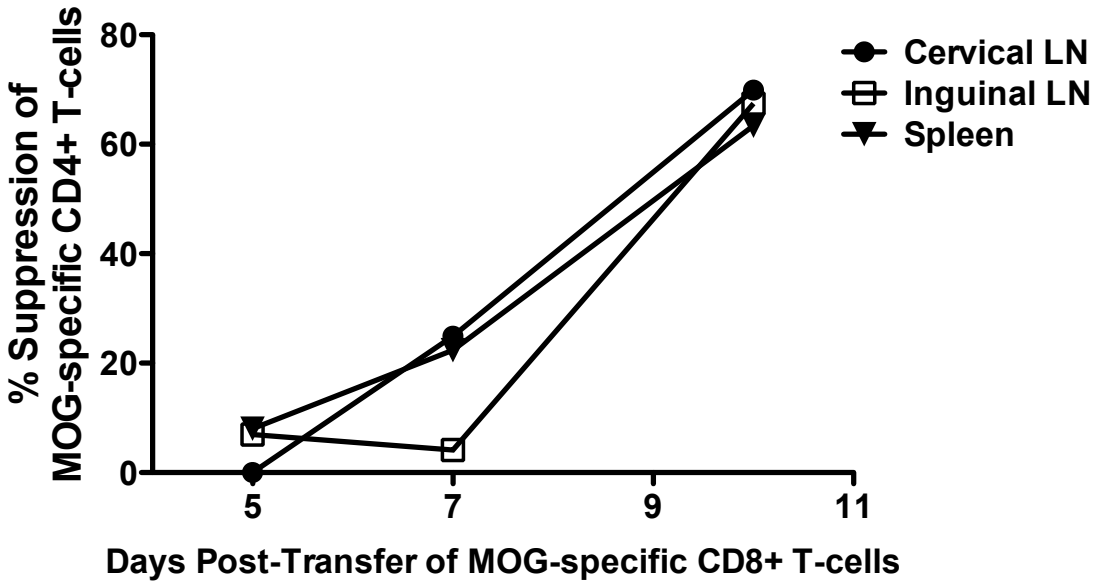
Absolute counts of MOG$_{35-55}$-specific CD4 T-cells in MOG$_{35-55}$-CD8 treated mice. Setup is similar to Figure 43. The number of CD4 CFSE hi is calculated based on the total count of events x the cell count number x the gated fraction within the CD3+CD4+CD8- fraction. Data are representative of two independent experiments, replicates within each experiment, five mice per condition. Error bars represent means ± standard error of the mean (SEM). **=$p<0.05$

Figure 47: *In vivo* Suppression of MOG (2D2)-CD4 T-cells by Autoregulatory CD8 T-cells



Autoregulatory CD8 (MOG-CD8) or control (OVA-CD8) T-cells were transferred into naïve CD45.1+ mice. All mice (CD45.1+) received transgenic (2D2) naïve CD4 T-cells that had been CFSE labeled and adoptively transferred i.p. Ten days later, peripheral immune compartment tissues, cervical lymph node (Cervical LN), inguinal lymph nodes (Inguinal LN) and spleens (Spleen) were harvested and CFSE dilution of myelin-specific CD4 T-cells measured by flow cytometry. Histograms are gated on TCRβ+CD4+CD8- cells and interval indicates the percentage of cells that have completely diluted their CFSE stain. Data are representative of two independent experiments, five mice per condition.

Figure 48: Temporal Increase in CD4 T-cell Suppression by Autoregulatory CD8 T-cells



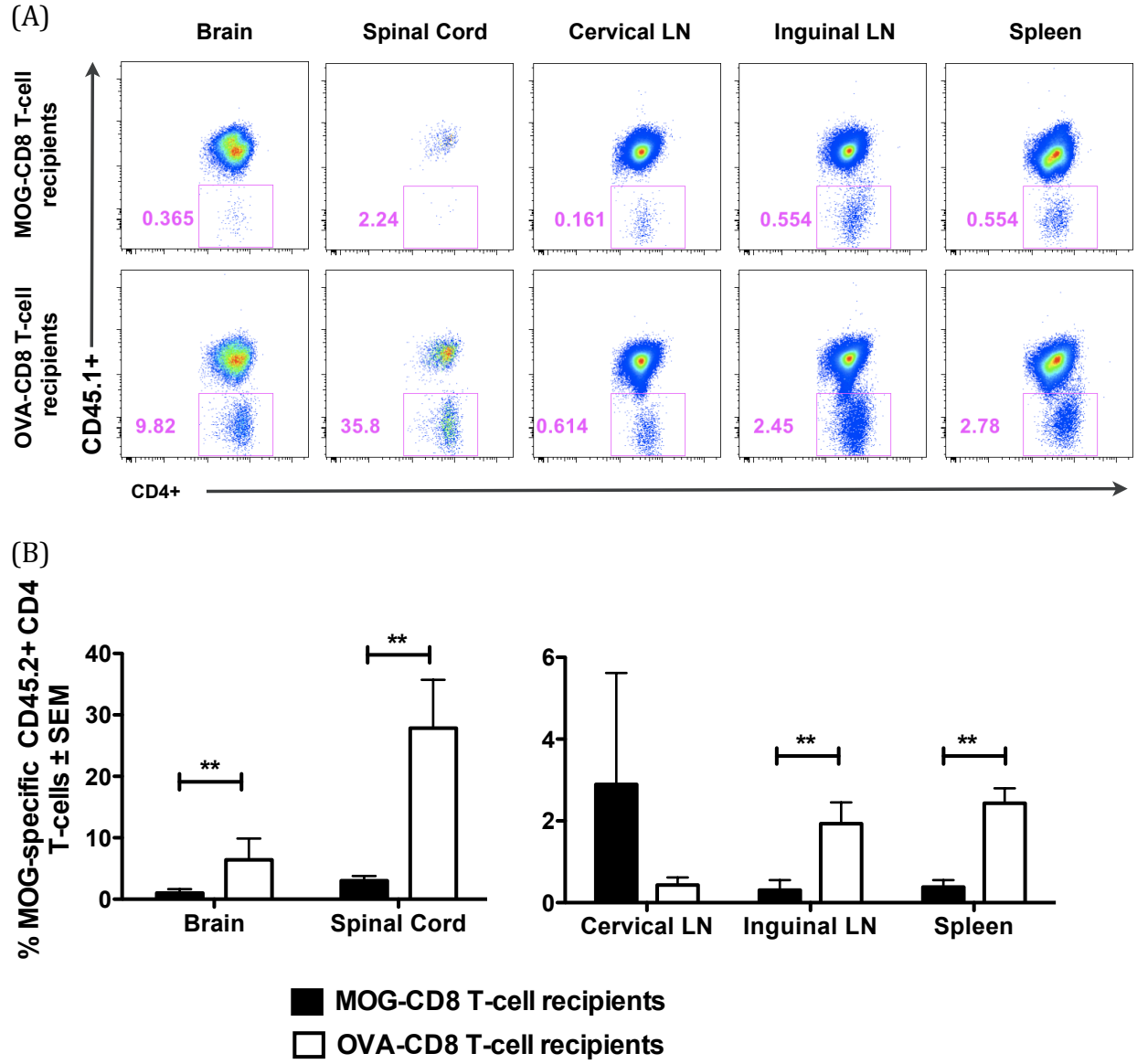
Autoregulatory (MOG_{35-55}-specific) or control (OVA323-339-specific) CD8 T-cells were transferred into naïve CD45.1+ mice. All mice (CD45.1+) received transgenic (2D2) naïve CD4 T-cells that had been CFSE labeled and adoptive transferred i.p. Five, seven and ten days later, peripheral immune compartment tissues: cervical lymph nodes (Cervical LN), inguinal lymph nodes (Inguinal LN) and spleens (Spleen) were harvested and CFSE dilution of myelin-specific CD4 T-cells measured by flow cytometry. Cumulative data of days 3, 5 and 7 post-transfer are shown and are representative of three independent experiments, five mice per condition.

4.2.5: Suppression/cytotoxicity of Pathogenic CD4 T-cells is Mediated in the CNS and Peripheral Immune Compartment

Next, I wanted to know where the modulation of CD4 T-cells was occurring, i.e. are autoregulatory CD8 T-cells suppressing the CD4 T-cells in the CNS or peripheral immune compartment? An *in vivo* trafficking experiment was setup where CD45.2+ CD4 T-cells were transferred into (CD45.1+) mice, which received either control $OVA_{323-339}$- or MOG_{35-55}-specific CD8 T-cells. Ten days later, tissues from the CNS (brain and spinal cord) and peripheral immune compartments (cervical, inguinal lymph node and spleen) were harvested and transferred CD4 T-cells enumerated. Both the CNS and peripheral immune compartments had a decrease in adoptively transferred (2D2) CD45.2+ CD4 T-cells (Figure 49A). The percentage of adoptively transferred CD4 T-cells was significantly decreased in the spinal cord of MOG-CD8 T-cell-treated mice (~17x), relative to OVA-CD8 T-cell control. The difference in percentage of adoptively transferred CD4 T-cells in the cervical lymph nodes did not reach statistical significance. In addition, the percentages of endogenous CD4 T-cells (CD45.1+) T-cells were also observed to be different between control and MOG-CD8 T-cell treated mice.

This observation correlated with our assessment of autoregulatory CD8 T-cell trafficking (Figure 32), where we did not observe a focusing of CD8 T-cell trafficking, suggesting that suppression by autoregulatory CD8 T-cells is indeed a global phenomenon.

Figure 49: Global Decrease of MOG-specific (2D2) CD4 T-cells in Autoregulatory CD8 T-cell-treated Mice



Autoregulatory (MOG_{35-55}-specific) or control ($OVA_{323-339}$-specific) CD8 T-cells were transferred into naïve CD45.1+ mice. All mice received transgenic (2D2) naïve CD4 T-cells by adoptive transfer. Ten days later the brain, spinal cord, cervical lymph node, inguinal lymph node, and spleens were harvested and presence of myelin-specific CD4 T-cells measured by flow cytometry. (A) Representative dot plots are gated on TCRβ+CD4+CD8- cells and gates indicate the percentage of MOG-specific T-cells (transferred cells) within the CD4 population. (B) Cumulative data are representative of two independent experiments, five mice per condition. Error bars represent mean ± standard error of the mean (SEM). **=$p<0.05$

4.3: Summary

We now provide strong support for the role of CD8 T-cells as suppressors by showing functional assays both in the murine model (in this study), as well as in MS samples [194], that indeed CD8 T-cells can function in a regulatory role. In this study, we show that autoregulatory CD8 T-cells can suppress the putative encephalitogenic CD4 T-cells *in vivo*. These observations set the foundation for our understanding of how the enigmatic autoregulatory CD8 T-cell can suppress an autoimmune disease. These observations are reflected in human studies, where the suppressor role has been shown to be absent in MS patients that are relapsing, suggesting that indeed autoregulatory CD8 T-cells are present in humans [194].

CHAPTER 5: AUGMENTING THE CLINICAL EFFICACY OF AUTOREGULATORY CD8 T-CELLS

5.1: Introduction

5.1.1: Current Treatment

The current repertoire of FDA approved drugs for MS include glatiramer acetate, interferon-β (various), mitoxantrone and natalizumab. Glatiramer acetate and interferon-β are approved for RRMS but only reduce the relapse rate by 30%. The most effective treatment is natalizumab but this drug was removed temporarily from the market due to the reactivation of the John Cunningham virus (JCV) in patients resulting in induction of Progressive Multifocal Leukoencephalopathy (PML). All of these drugs are labeled disease-modifying drugs because although they may suppress clinical symptoms temporarily, they are ineffective in curing the disease.

5.1.2: Objectives

We have observed and characterized a suppressive T-cell population that is capable of targeting the putative encephalitogenic CD4 T-cell in EAE, and probably in MS. The primary aim of the experimental work presented in this chapter is to evaluate methods, which can augment the generation and disease suppressing ability of autoregulatory CD8 T-cells. By potentiating the disease suppressing ability of autoregulatory CD8 T-cells, we hope to initiate the first steps towards an applicable cell-based immunotherapy.

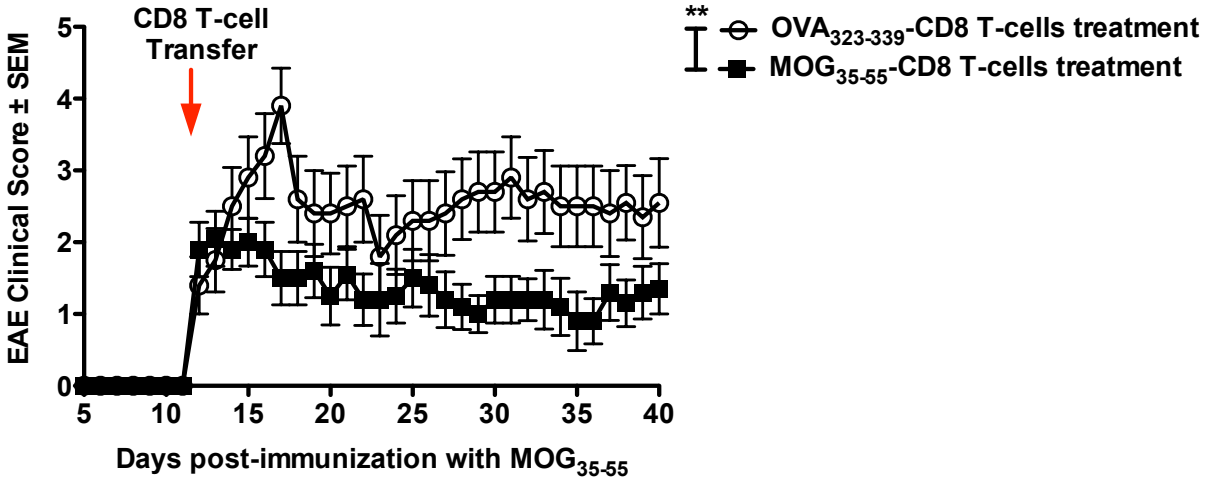
5.2: Results

5.2.1: Treatment of Established Clinical EAE Disease with Autoregulatory CD8 T-cells

We have previously shown and confirm here that MOG_{35-55}-specific CD8 T-cells are capable of suppressing established EAE, thus affirming their clinical efficacy [85]. This was performed by inducing EAE in two cohorts of mice and normalizing EAE in each cohort of mice at day 12. Autoregulatory or control CD8 T-cells were transferred into recipient mice by i.v. injection during peak EAE disease. Modulation of clinical disease was evaluated blindly for 40 days post immunization. We observed that mice treated with MOG_{35-55}-CD8 T-cells were found to have a significant reduction in EAE severity (Figure 50). In fact, the MOG_{35-55}-CD8 T-cell treated group began to show significant improvements within four days of administering autoregulatory CD8 T-cells.

Although we did see a significant reduction in disease severity, we did not observe a complete remission of paralysis. In order to augment the disease suppressing ability, we decided to increase the number of cells transferred into recipient mice. The transfer of 1.5X and 2X the number of autoregulatory CD8 T-cells did not show an increase in the reduction of disease severity. Since increasing the number of cells transferred did not alter clinical disease (data not shown), we hypothesized that we needed to optimize the generation of autoregulatory CD8 T-cells.

Figure 50: Autoregulatory CD8 T-cells can Suppress Established EAE



Clinical efficacy of autoregulatory CD8 T-cells was evaluated by transferring MOG_{35-55}-specific CD8 T-cells into mice that had established primary EAE (indicated by arrow - day 12 post immunization). EAE clinical disease was evaluate for 40 days and representative graph is shown. Data are representative of two independent experiments, five mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$

5.2.2: Generation of Autoregulatory CD8 T-cells

Immunization with a 15mer peptide, which has been shown capable of binding to MCH-II molecules, presents a slight problem when referring to CD8 T-cell activation. Since MHC Class I molecules can only bind up to 9mer peptides [3], the first question that needs to be answered is what portion of the MOG_{35-55} peptide is used to generate the disease ameliorating myelin-specific CD8 T-cell. First, we immunized mice with several described MOG_{35-55} epitopes and tested whether the generated CD8 T-cells were capable of ameliorating MOG_{35-55}-induced EAE. Within the MOG_{35-55} peptide, there are two described CD8 epitopes; MOG_{37-46} [204] and MOG_{44-54} [205], and one CD4 epitope MOG_{40-49} [206] (Table 5). Of the CD8 epitopes, we focused on the role of the MOG_{37-46} epitope, as this peptide has been shown by others to induce the highest production of IFN-γ by CD8 T-cells, relative to the other CD8 epitopes [197]. Although immunization with MOG_{37-46} peptide did not induce clinical EAE disease (Figure 51), we were able to detect a response to the peptide using the CFSE-based recall response assay (Figure 52). These CD8 T-cells were found to be ineffective in suppressing MOG_{35-55}-based EAE in B6 mice (Figure 53). For control purposes, a separate cohort of mice was immunized with the CD4 T-cell epitope, which did result in primary clinical disease (Figure 54A), no CD8 T-cell recall response (Figure 54B) and understandably no CD8-mediated disease protection (Figure 54C). It has also been described that CD8 T-cells responding to MOG_{35-55} peptide could be isolated using a MOG_{37-50}/H2-D^b MHC tetramer [204]. Immunization with MOG_{37-50} did result in primary EAE disease, and CFSE dilution assay did reveal a CD8 T-cell response for this peptide but protection from MOG_{35-55} induced-EAE was only partially effective, as only the acute phase of EAE disease was ameliorated (

Figure 55).

These data suggest that generation of autoregulatory CD8 T-cells requires the encephalitogenic CD4 epitope, as well as both CD8 epitopes of MOG_{35-55} peptide (Table 5).

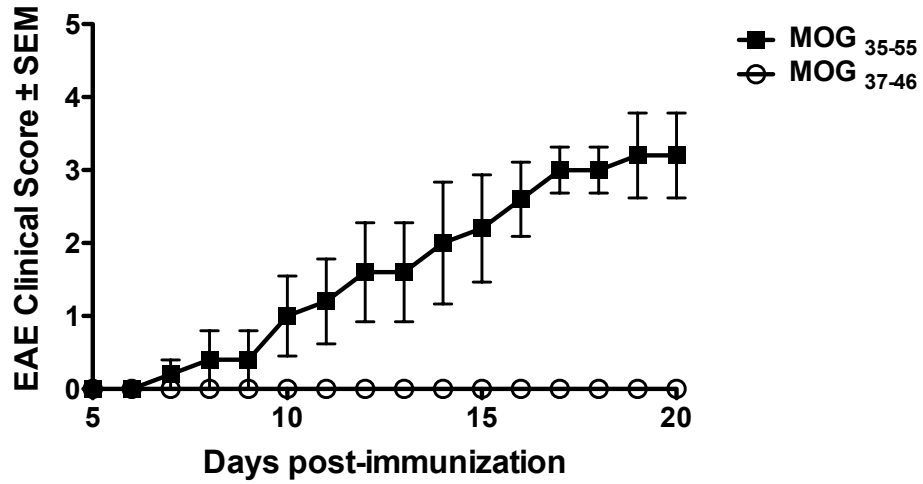
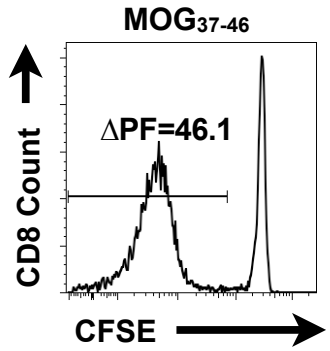


Figure 51: MOG_{37-46} Immunization does not induce Primary EAE

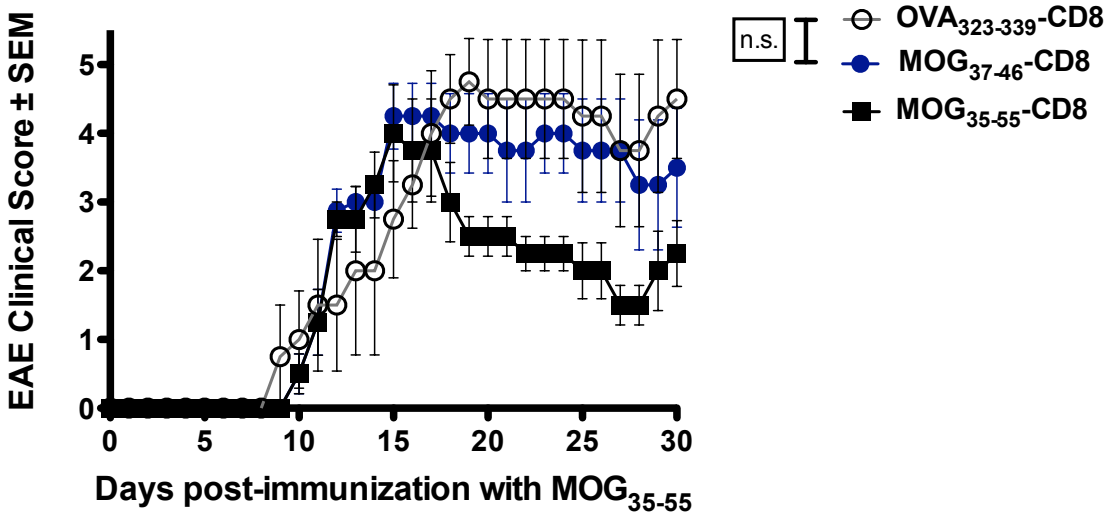
Induction of primary EAE disease with the CD8 T-cell epitope of MOG_{35-55} peptide. Naïve B6 mice were immunized with 100 µg of MOG_{35-55} or MOG_{37-46} peptide in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 25 days using our 2-way blinded manner. Data are representative of five independent experiments, 7-10 mice per condition. Error bars represent mean clinical score ± standard error of the mean (SEM).

Figure 52: MOG_{37-46}-specific Response from CD8 T-cells Isolated from MOG_{37-46} Immunized Mice



CD8 T-cell recall response to CD8 epitope of MOG_{35-55} was evaluated using the CFSE dilution assay. Following the immunization of B6 mice with MOG_{37-46}, ln cells were harvested and CFSE stained. Cells where then cultured *in vitro* with MOG_{37-46} and five days later CFSE dilution measured. ΔPF is the % of CFSE low CD8 T-cells in the MOG_{37-46} stimulated condition minus the no antigen (background) condition. Data are representative of three independent experiments, 5-7 mice per condition.

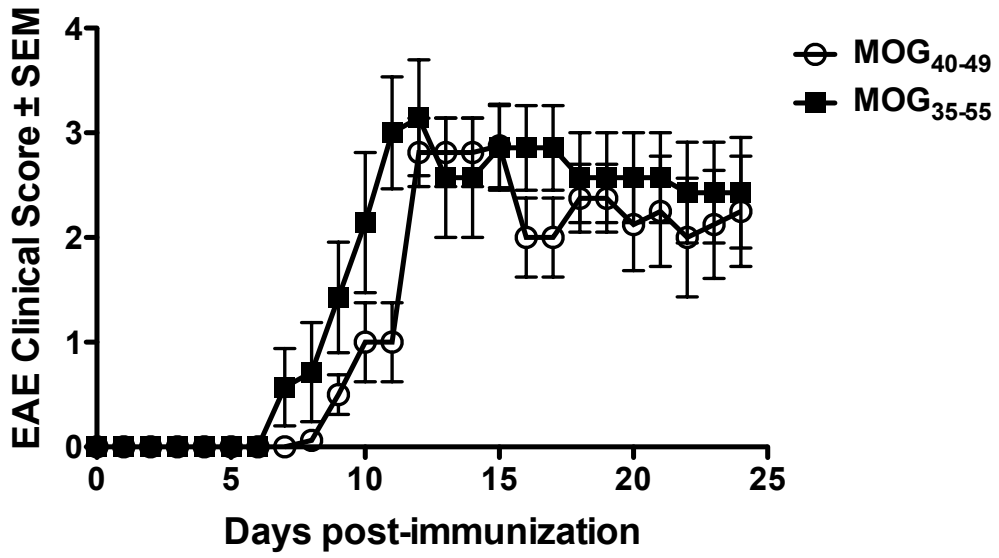
Figure 53: MOG_{37-46}-specific CD8 T-cells do not Suppress MOG_{35-55}-induced EAE



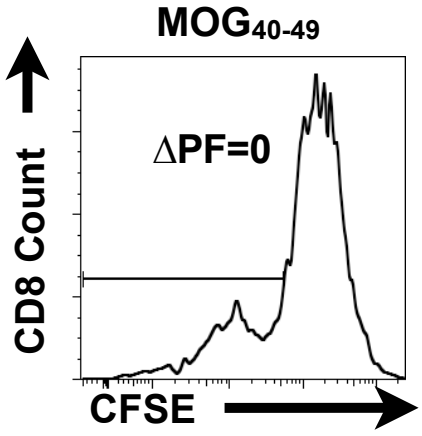
Evaluation of MOG_{37-46}-specific CD8 T-cell's ability to suppress primary EAE disease. MOG_{37-46}-specific CD8 T-cells were transferred i.v via tail vein at day 0. For controls purposes, MOG_{35-55}- and $OVA_{323-339}$-specific CD8 T-cells were transferred into two other cohort of mice. The recipient mice were immunized with 100 µg of MOG_{35-55} in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of three independent experiments, 7-10 mice per condition. Error bars represent means ± standard error of the mean (SEM). n.s. = not significant

Figure 54: MOG_{40-49}-specific CD8 T-cells are not Effective in Suppressing MOG_{35-55}-Induced EAE

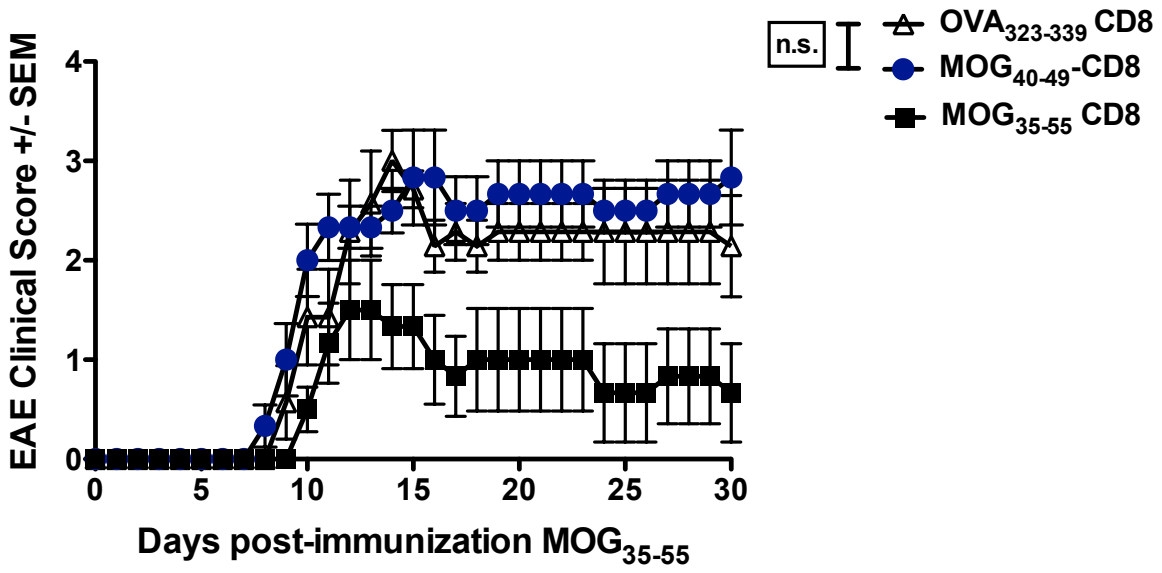
(A)



(B)



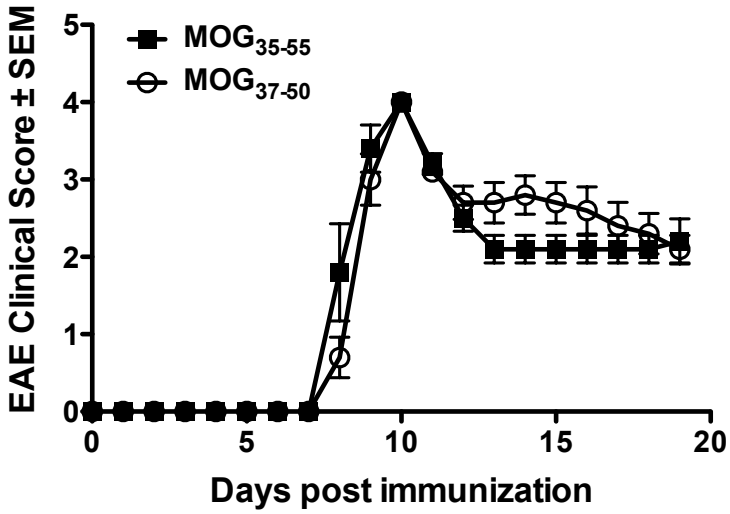
(C)



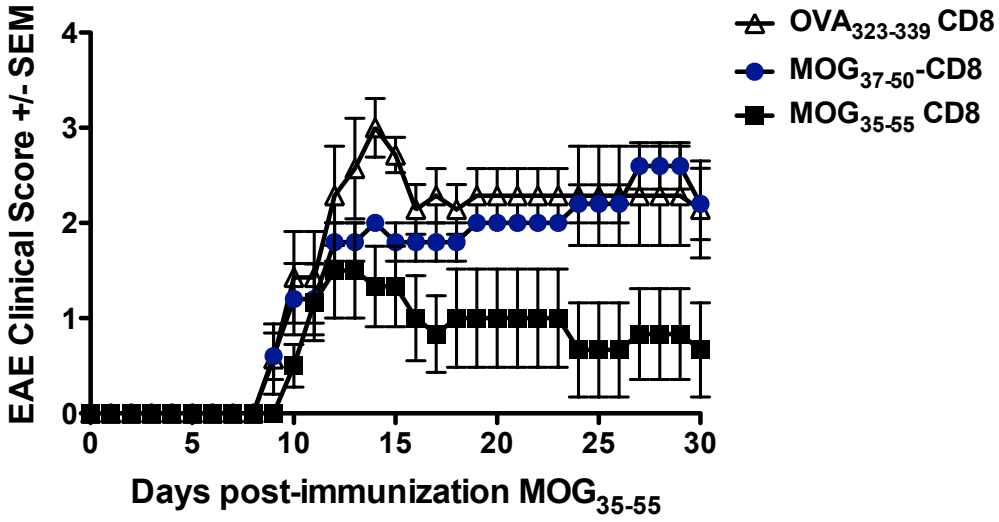
Evaluation of the disease suppressing ability of MOG_{40-49}-specific CD8 T-cells in primary EAE. (A) Naïve B6 mice were immunized with 100 µg of MOG_{35-55} or MOG_{40-49} peptide in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 25 days using our 2-way blinded manner. Data are representative of five independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). (B) Following the immunization of B6 mice with $MOG_{40-49,}$ ln. cells were harvested and CFSE stained. Cells where then cultured *in vitro* with MOG_{40-49} and five days later CFSE dilution measured. ΔPF is the % of CFSE low CD8 T-cells in the MOG_{37-46} stimulated condition minus the no antigen (background) condition. Data are representative of two independent experiments, 5-7 mice per condition. (C) MOG_{40-49}-specific CD8 T-cells were transferred i.v via tail vein at day 0. For controls purposes, MOG_{35-55}- and $OVA_{323-339}$-specific CD8 T-cells were transferred into five other cohorts of mice. The recipient mice were immunized with 100 µg of MOG_{35-55} in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of two independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of mean (SEM). n.s.=not significant.

Figure 55: MOG_{37-50}-specific CD8 T-cells are Partially Effective in MOG_{35-55}/EAE Suppression

(A)



(B)



Evaluation of the disease suppressing ability of MOG_{37-50}-specific CD8 T-cells in primary EAE. (A) Naïve B6 mice were immunized with 100 µg of MOG_{35-55} or MOG_{37-50} peptide in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 25 days using our 2-way blinded manner. Data are representative of five independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). (B) MOG_{37-50}-specific CD8 T-cells were transferred i.v via tail vein at day 0. For control purposes, MOG_{35-55}- and $OVA_{323-339}$-specific CD8 T-cells were transferred into two other cohort of mice. The recipient mice were immunized with 100 µg of MOG_{35-55} in CFA emulsion. All mice received PTX on days 0 and 2 post immunization. Clinical disease was evaluated for 30 days using our 2-way blinded manner. Data are representative of two independent experiments, 7-10 mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM).

Table 5: Autoregulatory CD8 T-cell Generation using Epitopes of MOG_{35-55}

Epitope name	Sequence	Primary EAE	Protection
MOG_{37-46}	MEVGWYRSPFSRVVHLYRNGK	-	-
MOG_{44-54}	MEVGWYRSPFSRVVHLYRNGK	-	-
MOG_{40-49}	MEVGWYRSPFSRVVHLYRNGK	++	-
MOG_{37-50}	MEVGWYRSPFSRVVHLYRNGK	++	+/-
MOG_{35-55}	MEVGWYRSPFSRVVHLYRNGK	++	++

MOG_{37-46} and MOG_{44-54} are the CD8 epitopes found within MOG_{35-55}. MOG_{40-49} is a CD4 epitope. MOG_{37-50} includes a CD8 epitope as well as a partial CD4 epitope. MOG_{35-55} includes both CD8 epitopes as well as a CD4 epitope. Primary EAE reflects the ability of the truncated MOG peptide to induce EAE, - = no primary disease and ++ indicates a normal induction of EAE. Protection indicates the ability of CD8 T-cells isolated from truncated MOG peptide immunized mice in protecting the mice from primary MOG_{35-55} EAE disease. - = no disease protection, +/- = partial disease protection and ++= full EAE protection.

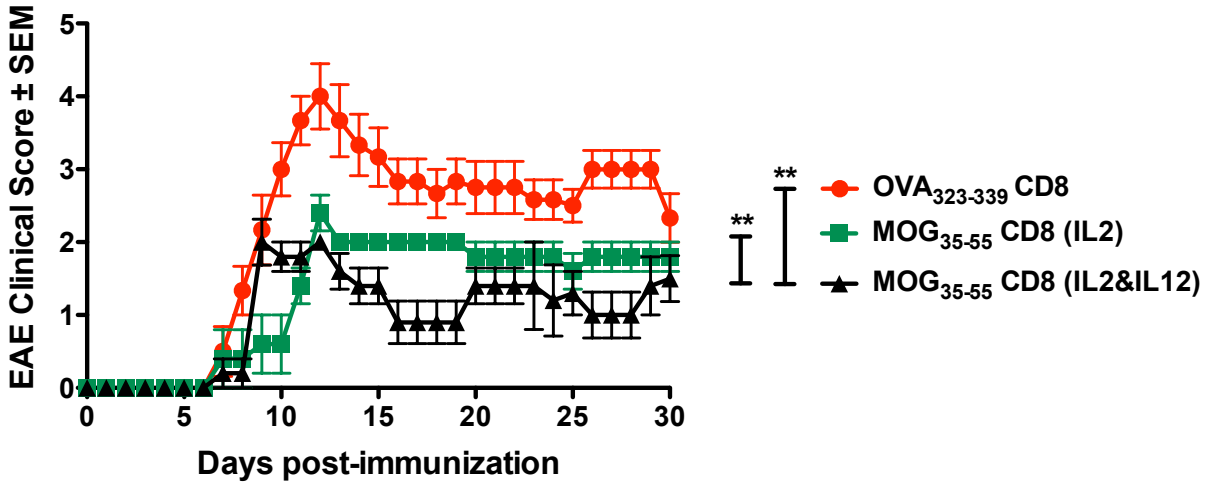
5.2.3: Autoregulatory CD8 T-cell Clinical Efficacy can be Augmented with *in vitro* IL12 Modulation

Although we have consistently observed disease modulation, our efforts to enhance disease suppression by increasing the number of autoregulatory CD8 T-cell transferred did not augment disease amelioration (data not shown). Recent observations [207]-[209], have revealed the role of IL12 in augmenting activation and cytotoxicity of CD8 T-cells. Thus, we evaluated whether IL12 could modulate the disease suppressing ability of autoregulatory CD8 T-cells. Similar to our adoptive protection experiments, we obtained WT MOG_{35-55}-specific CD8 T-cells, and cultured a portion with murine IL12 during the *in vitro* expansion phase. Next, these cells where transferred i.v. into WT naive mice, EAE induced and clinical efficacy compared to the WT-MOG_{35-55} (IL2 cultured) and WT-$OVA_{323-339}$-specific CD8 T-cell recipients. Those mice, which received IL2+IL12 modulated MOG_{35-55}-specific CD8 T-cells were found to have a significantly lower level of EAE disease as compared to our standard IL2-cultured MOG_{35-55}-specific CD8 T-cells (Figure 56). Analysis of post-*in vitro* cultures, revealed a slight expansion in the CD8 population within the T-cell population (gated on TCRβ+, first row), no significant differences in the differentiation (CD44 vs. CD62L) status of CD8 T-cells obtained from the OVA-IL2, MOG-IL2 and MOG-IIL2+L12 cohorts (2nd row), while activation status (CD25) was significantly increased (~3X) in the MOG-IL2+IL12 condition (Figure 57). Next, we evaluated the effects IL12 on autoreactive CD8 T-cells by using the CFSE dilution assay in conjunction with intracellular cytokine staining. Relative to the MOG-IL2 culture, there was an increase in IFN-γ producing MOG_{35-55}-specific CD8 T-cells, as well as more IFN-γ production on a per cell basis (Figure 58). This complements previous data, indicating that IFN-γ production was needed by autoregulatory CD8 T-cells in order to ameliorate EAE disease (Figure 33). For control purposes,

IL23, a cytokine that shares certain function with IL12, was evaluated for its potential modulation. *In vitro* modulation with IL23 led to a decrease in autoreactive CD8 T-cells producing IFN-γ while slightly increasing IL17+ CD8 T-cells. True to their purported roles, IL12 and IL23 potently augmented IFN-γ or IL17 production in autoreactive CD4 T-cells, respectively.

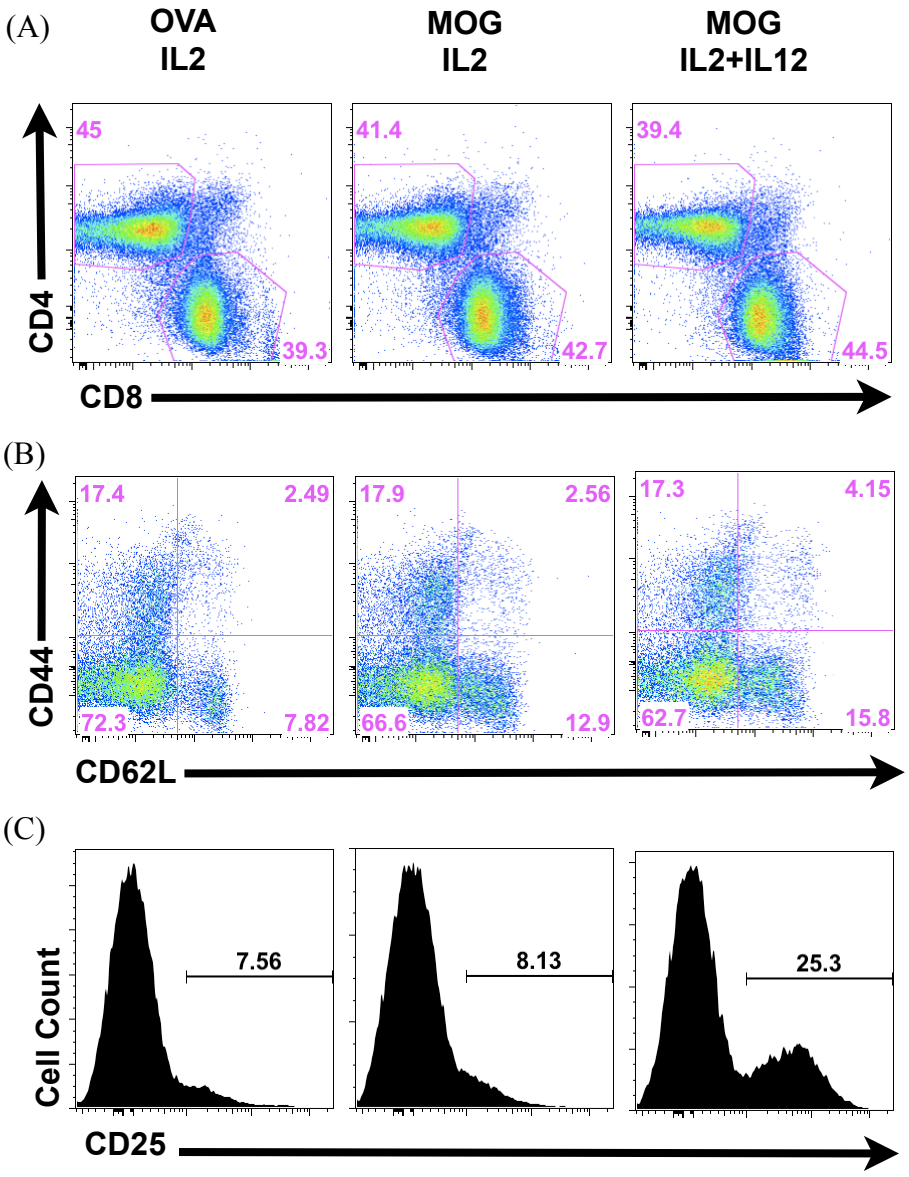
In summary, these data reveal a potent modulation of autoregulatory CD8 T-cells by IL12 during the *in vitro* expansion phase. This modulation resulted in a significant increase in disease amelioration, which correlated with an increase in activation and IFN-γ production by autoregulatory CD8 T-cells. These data also confirm that autoregulatory CD8 T-cells modulate clinical disease by producing IFN-γ

Figure 56: *In vitro* Modulation with IL12 Augments Autoregulatory CD8 T-cell Amelioration of EAE



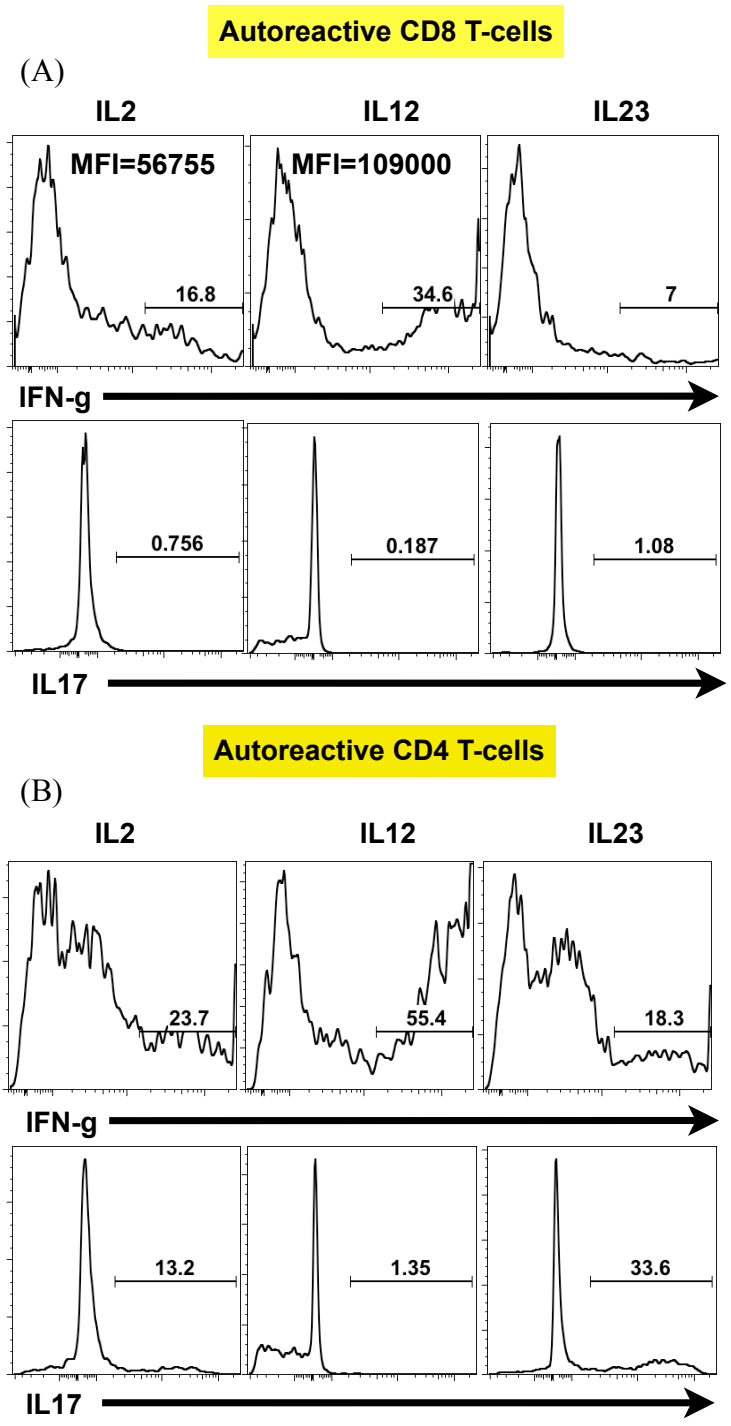
Clinical efficacy of autoregulatory CD8 T-cells can be augmented with IL12 modulation. Splenocytes from MOG_{35-55} immunized mice were cultured with IL2 or IL2/IL12 during the *in vitro* expansion phase. Subsequently, CD8 T-cells were transferred i.v. and primary EAE induced and disease evaluated for 30 days. Data are representative of two independent experiments, seven mice per condition. Error bars represent mean clinical disease ± standard error of the mean (SEM). **=$p<0.05$

Figure 57: *In vitro* IL12 Modulation Results in an Increase in Activated Autoregulatory CD8 T-cells



Comparison of CD25, CD44, and CD62L expression on gated TCRβ+ CD4- CD8+ T -cells post IL12/IL2 vs.. IL2 culture is shown. (A) Dot plots are gated on TCRβ+ cells. (B) Dot plots are gated on TCRβ+CD8+CD4- T-cells. (C) Histogram is gate on TCRβ+CD8+CD4- T-cells.

Figure 58: *In vitro* IL12 Modulation Augments IFN-γ Production by Autoregulatory CD8 T-cells



CFSE and intracellular cytokine staining were used to measure the levels of IFN-γ and IL17 production in MOG_{35-55}-specific CD4 and CD8 T-cells following IL2, IL12 or IL23 *in vitro* culture. MFI=mean fluorescence intensity. Representative histograms and dot plots of two independent experiments (2-4 mice per condition).

5.3: Summary

Selective activation of the immune system by immunizing mice with the CD8 T-cell epitope of MOG_{35-55} does not induce the generation of autoregulatory CD8 T-cells. These data indicate that the generation of autoregulatory CD8 T-cells is dependent on both CD8, as well as the CD4 T-cell epitopes of MOG_{35-55} peptide. The dependence of CD4 epitopes is probably due to the necessary cytokine milieu generated in the CNS by the activation of CD4 T-cells and not by direct activation of CD8 T-cells by the CD4 epitope. Interestingly, IL12 potentiated the disease-ameliorating role of CD8 T-cells by augmenting the production of IFN-γ and the activation status of autoregulatory CD8 T-cells; thus confirming our previous observations that indeed IFN-γ is required for disease amelioration.

CHAPTER 6: GENERAL DISCUSSION

Induction of an autoimmune response and failure to control its detrimental effects are believed to be the two crucial events leading to the debilitating neurological disease multiple sclerosis. In MS and EAE, there is ample evidence that neuroantigen-specific CD4 T-cells can initiate CNS inflammation and pathology. Currently, it is known that CD8 T-cells are abundantly present in the CNS lesions of MS patients but their role is poorly understood. It has been shown that MS patients harbor site-of-pathology-directed (i.e. myelin targeting) CD8 T-cells [84], thus it is plausible to assume that these cells are acting as pathological agents. But now there is increasing evidence, both in human studies and EAE models, of the existence of autoreactive regulatory CD8 T-cells. In fact, in this study we elucidate the immunophenotype and functional profile, as well as the mechanisms and cellular targets of autoregulatory CD8 T-cells. The clinical relevance of autoregulatory CD8 T-cells is addressed by evaluating their efficacy in established disease, methods of generation of autoregulatory CD8 T-cells and augmentation of their clinical efficacy by *in vitro* modulation.

6.1: Regulatory versus Pathogenic Role of Myelin-specific CD8 T-cells in EAE and MS

Our results demonstrate that, in general, EAE induces a population of autoreactive CD8 T-cells that are effective suppressors of autoimmune demyelinating disease. Generation of these cells were found to be a general phenomenon of EAE induction, as permutations of peptide or mouse strains revealed a consistent regulatory capacity of MOG_{35-55} and $PLP_{178-191}$-specific CD8 T-cells in B6 mice and $PLP_{178-191}$-specific CD8 T-cells in SJL mice. We did observe that

induction of EAE by $PLP_{139-151}$ did not generate a regulatory population of CD8 T-cells (nor did it produce a pathogenic population as we did not see an augmentation of primary EAE) and this observation will be the focus of future experiments. The regulatory role of CD8 T-cells were further confirmed by the augmented disease severity observed in EAE-induced $CD8^{-/-}$ B6 mice (Figure 7 and [181]). In fact, we were able to observe a higher response to myelin antigen, as well as an increase in inflammatory cytokine production from pathogenic CD4 T-cells in $CD8^{-/-}$ mice during active EAE. These autoregulatory CD8 T-cells were found to be sufficient in reversing the augmented disease severity. Thus, indicating that in the absence of CD8 T-cells, encephalitogenic CD4 T-cells are allowed to develop their full pathogenic potential.

These findings are somewhat controversial since others have demonstrated a pathogenic role for CD8 T-cells in transgenic and well as non-genetically manipulated mice. It is interesting to note that in transgenic mice expressing both neo-self antigens and CD8 T-cells directed toward these antigens, the level of clinical disease varies. For instance, mice expressing the TCR specific $OVA_{257-264}$ presented by H2-K^b succumbed to a lethal fulminant demyelinating CNS disease [210], while transgenic mice expressing the hemagglutinin-specific TCR, Cl4, that recognizes $HA_{512-520}$ presented by H2-K^d did not develop paralysis, although demyelination was present [211]. It is possible that in both cases, since the neo-self antigens were not expressed thymically, the neo-self reactive CD8 T-cells were not removed by negative selection and thus allowed to mature, migrate and target tissues expressing these new antigens. In our experiments, we use wild type B6 mice, which were immunized with MOG_{35-55} peptide in order to induce both diseases. In this setting, potentially pathogenic high avidity CD8 T-cells were deleted during negative thymic selection and mid and low avidity MOG-specific CD8 T-cells allowed to mature and possibly supply a pool of T-cells which could eventually become the autoregulatory CD8 T-

cells. This hypothesis could be tested by transferring CD8 T-cells MOG_{35-55} immunized $MOG^{-/-}$ B6 mice and determining if these cells could transfer disease. It is also possible that along with escape of central tolerance, other lab's protocols might select for high avidity CD8 T-cells which have been shown to induce disease in other models [195],[212]. There is also evidence in non-transgenic mice that autoreactive CD8 T-cells can play a pathogenic role, but disease severity (as compared to CD4 T-cell mediated disease) was revealed to be much milder and/or purity of adoptively transferred CD8 T-cell population was low [179],[204]. Conversely, there is increasing amount of data indicating a regulatory role for autoreactive CD8 T-cells in MS/EAE [85],[192],[194], as well as other autoimmune diseases [50],[195].

In human studies, proteins from the myelin sheath such as: MBP, PLP, MOG and myelin associated glycoprotein (MAG) have been evaluated in order to identify and enumerate the myelin-specific CD8 T-cell, with the presumption that these cells are pathogenic. What was revealed was that $MBP_{110-118}$ or transaldolase 168-176-specific CD8 T-cells were elevated in the peripheral blood of MS patients versus controls [213]-[215], although in separate studies, myelin-specific CD8 T-cell responses from healthy versus MS patients showed similar level of recall response to cognate antigen [216],[217]. In fact there have been reports that myelin-specific CD8 T-cells in MS patients exhibit an activated/effector phenotype [214],[216],[218], although levels of IFN-γ production were similar to healthy controls, indicating that these cells had been previously activated but not indicating their pathogenic role [219]. Up until recently, there has not been any evidence elucidating the effector function of autoreactive CD8 T-cells from MS patients. This changed, when MS patients that recently relapsed were shown to have a decrease in the suppressive ability of myelin-specific CD8 T-cell [194].

Much like the CD4 T-cell subset, there is convincing evidence for both regulatory, as well as pathogenic effector CD8 T-cells. Hence, it is becoming increasingly important to differentiate both sets of cells in order to better understand and develop more effective therapeutic protocols.

6.2: Characteristics of Autoregulatory CD8 T-cells in EAE

The immunophenotype data revealed a unique profile shared between myelin-specific regulatory CD8 T-cells: CD62L+CD44- CCR7+. This indicated a differentiation status of central memory and the ability to access secondary lymphoid tissues, thus suggesting that these cells exhibit their disease-suppressive ability within peripheral lymph nodes rather than within the CNS tissue. Similarly, MS patients have been described as having an increased level of CD8+CCR7+CD45RA- (central memory) in their peripheral blood compartment [220]. Although the authors speculate that this indicates a deregulation of CD8 T-cells, the disease status of the MS patients were unknown; thus it is possible that these CD8 T-cells may have been acting in a concerted effort to ameliorate disease.

We initially hypothesized that disease regulation was occurring in the periphery and thus performed a trafficking experiment to assess where autoregulatory CD8 T-cell were performing their regulatory role. We observed that myelin-specific CD8 T-cells were not infiltrating and accumulating in the CNS of naïve mice. This observation was reversed when EAE disease was induced post-transfer, possibly by either passive diffusion as a result of the blood brain barrier breakdown caused by EAE induction or the upregulation of chemokines on endothelial cells. Similar observations have been seen in MS patients where CD8 T-cells were reported as having an augmented capacity to roll and arrest on inflamed brain venules [221].

Numerous reports have shown that upon activation, CD8 T-cells can recognize and cytotoxically target CNS cells [222],[223]. In fact, my studies revealed that autoregulatory CD8 T-cells were suppressing EAE by a perforin dependent mechanism, thus confirming the *in vivo* killing of APCs and CD4 T-cells observed in York *et al.* [85]. Conversely, there have been documented cases where CD8 T-cells were able to target murine neurons by Fas/FasL

mechanism [224] or granzyme B mechanisms [225]. Although our CD8 T-cells were similar in their killing ability, the fact that they use a different mechanism and target a different cellular population implies that these autoregulatory CD8 T-cells are a distinct subset of CD8 T-cells.

Two important mechanisms of autoregulatory CD8 T-cells are noteworthy. The first is that these autoregulatory CD8 T-cells were found to produce IFN-γ and this production was necessary for *in vivo* disease amelioration. It is known that IFN-γ has a pluripotent role as it can alter the production of indoleamine-pyrrole 2,3-dioxygenase (IDO) and augment the regulatory capacity of DC [226], increase the expression of MHC Class I molecules on APCs and convert effector CD4 T-cells to CD4 Tregs [202], all potential avenues which might be used for inhibiting the presentation of myelin antigen and decreasing encephalitogenic CD4 T-cell activity (Figure 59). Similarly, perforin was found to be necessary for *in vivo* disease amelioration and we hypothesize that both molecules are used in tandem in order to inhibit APCs function but separately in order to target pathogenic CD4 T-cells. Again, unlike the regulatory Qa-1-restricted CD8 T-cells which secrete IL10 in order to exert their inhibitory activity, EAE generated autoregulatory CD8 T-cells were found to be IL10-independent (Figure 37).

6.3: MHC Class I Dependence of Autoregulatory CD8 T-cells

CD8 T-cells require antigen presentation within the context of MHC Class I molecules (main class I genes: HLA-A, B and C in humans and H-2D, H-2K and H-2L in mice) cite janeway. The MHC allele dictates which antigen it will present to the CD8 T-cell and this antigen must be able to stably bind to the MHC groove long enough for the TCR to recognize and elicit an immune response. In this study, it was observed that both MHC-Ia and Ib molecules were used to present myelin antigen to CD8 T-cells and induce activation (Figure 24). But these same cells were only dependent on MHC-Ia presentation in order to function in their disease-

suppressing role. This observation indicated to us that indeed these CD8 T-cells were unconventional, at least in that they did not use the Qa-1-dependent mechanism for disease suppression [185]. Similarly, in MS patients who express the MHC-I alleles HLA-A*0201 and HLA-C5, there is a decreased risk in developing MS, unlike HLA-A*0301 individuals who have an increase in risk. Thus, these observations suggest that MHC-Ia molecules impact the course of disease by their ability to present myelin-antigen to regulatory CD8 T-cells. It could be entertained that HLA-A*0201 and HLA-C5 form a more stable complex with their respective myelin peptides and thus are able to activate autoregulatory CD8 T-cells. In the case of HLA-A*0301, these molecules may either form unstable interaction between peptide and MHC molecules, which would be unable to activate autoregulatory CD8 T-cells and thus lead to increased disease susceptibility.

Interestingly, another difference between Qa-1 and MHC-I dependent regulatory CD8 T-cells was the observation that autoregulatory CD8 T-cells required cognate myelin antigen for disease suppression. Qa-1-mediated suppression involves the presentation of hsp60 (still a self antigen but not cognate myelin antigen) antigen within the context of Qa-1 on EAE-induced CD4 T-cells [227]. This presentation allows CD8 regulatory T-cells to cytotoxically target the pathogenic cell and reduce disease severity. As $PLP_{178-191}$-specific CD8 T-cells were unable to suppress disease in MOG_{35-55}-induced EAE (Figure 22), unless the $PLP_{178-191}$ antigen was present in the immunizing antigen (Figure 23), this excludes the possibility of hsp60 or other non-cognate antigen presentation by CD4 T-cells. It is tempting to appreciate the potential for antigen-specific regulatory CD8 T-cells, as their therapeutic application could avoid the shortfalls of current broad-spectrum immunosuppressive therapies.

In sum, our observations further confirm that autoreactive CD8 T-cells can function as a regulatory population, which ameliorates EAE by suppressor/cytotoxic mechanisms. Although distinct from their MHC Class Ib-restricted siblings, these autoregulatory CD8 T-cells reveal another potent immunomodulatory arm of the adaptive immune system, which may act in a concerted manner to ameliorate autoimmune demyelinating disease.

6.4: Antigen Presentation to Autoregulatory CD8 T-cells

Presentation of an exogenous antigen by the MHC-I pathway poses a problem. The MHC-I pathway loads only cytosolic peptides unto the MHC groove, while the MHC-II pathway loads peptides presented to it by endocytic vesicles, which have picked up extracellular debris. These two pathways are independent and no communication between them occurs, thus theoretically preventing an MHC-I molecule from presenting an exogenous peptide. Recently, a study showed that APCs can pick up antigen, migrate to the draining lymph node and through a process known as cross presentation, present exogenous peptide to a CD8 T-cell [228]. Even more interesting, upon antigen presentation, these APCs can communicate to the CD8 T-cells where the peptides are picked up and direct them to the originating tissue. CNS APCs also show a similar ability, as they are able phagocytize and cross-present myelin antigen to activated CD8 T-cells, thus initiating an effector response [229].

6.5: Cellular Targeting of Pathogenic CD4 T-cells

Earlier studies have described cytotoxic killing of target cells by CD8 T-cells both *in vivo* and *in vitro,* as a requirement for CD8 T-cell suppression [230]. In this report, we provide *in*

vitro and *in vivo* evidence that autoregulatory CD8 T-cells regulate disease by directly targeting encephalitogenic CD4 T-cells. First, autoregulatory CD8 T-cell transfer into AT-EAE induced mice resulted in a decrease in disease severity. As we had previously shown, these autoregulatory CD8 T-cells required MHC Class Ia *in vivo* for disease suppression, hence we proceeded to ask if autoregulatory CD8 T-cells could still suppress AT-EAE when only the adoptively transferred CD4 T-cells expressed MHC Class Ia. In both situations, disease suppression was significantly reduced. In fact, disease suppression correlated with *in vitro* killing of MOG-loaded CD4 T-cells, as well as *in vivo* suppression of encephalitogenic CD4 T-cells. Moreover, our *in vivo* trafficking experiments revealed a significant loss of encephalitogenic CD4 T-cells both within the CNS, as well as the peripheral immune compartment. It should be noted that targeting of encephalitogenic CD4 T-cells and other possible regulatory mechanisms, such as modulation and/or killing of APCs through expression of IFN-γ or perforin, need not be mutually exclusive. In fact, modulation of the APCs population is highly attractive mechanism of suppression as this would afford a much more significant and earlier modulation of disease, and is currently being actively pursued in our laboratory.

Pharmacological strategies targeting CD4 T-cells have produced little effect on MS disease and this may be due to several reasons such as: peripheral depletion of regulatory CD4 T-cells, inability of antibody to reach the target organ (CNS), tissue damage is already done, and a deficiency in the regulatory CD8 T-cell compartment. In this study, autoregulatory CD8 T-cell targeting of encephalitogenic CD4 T-cells was shown to be efficacious in down modulating CNS disease. In fact, this may prove to be very beneficial, as MS studies have already shown that CD8 T-cells are present at the site of pathology and are activated and oligoclonally expanded, although their function may be defective. Thus, a more effective therapy is needed which will

incorporate the use of adoptively transferring autoregulatory CD8 T-cells from healthy individuals or more likely, isolate dysfunctional myelin-specific CD8 T-cells from MS individuals, reactivate their suppressive function through *in vitro* modulation and re-insert them into relapsing MS patients.

6.6: Clinical Use of Autoregulatory CD8 T-cells

The clinical efficacy of autoregulatory CD8 T-cells was confirmed in this and a previous study [85], demonstrating that established disease could be modulated by the transfer of autoregulatory CD8 T-cells (Figure 50). The use of cellular based therapy offers many attractive advantages such as the ability to tailor the therapy to the patient, the cells can be relatively easily isolated and stored for relatively long periods of time using currently available cryogenic storage. Upon incursion of clinical disease, these cells can be activated, expanded and transferred into sites of inflammation. This is especially attractive in light of the fact that current approved therapies implement global immune suppression, which will impede the beneficial immune protection against tumor or viral infections. Additionally, current therapies are short term modulators of disease and in fact, over a period of time, CNS disease can become refractory to the therapeutic agent. Autoregulatory CD8 T-cell therapy is not only attractive because of their longevity but also because of its antigen specificity. This allows for the specific suppressesion of the deleterious immune responses to the inciting antigen, while allowing the rest of the immune system to maintain its protective role. Focusing on the clinical applicability of autoregulatory CD8 T-cells, we have endeavored to resolve the first two steps necessary in implicating this novel therapeutic agent. First we assessed the minimal myelin determinant needed for the generation of autoregulatory CD8 T-cells. We had initially speculated that we could immunize the mice with at least one CD8 epitope of MOG_{35-55} peptide and generate autoregulatory CD8 T-

cells. The advantages of this method include the generation of only a CD8 T-cell response, which would remove any possible contamination from myelin-reactive CD4 T-cells. More importantly, we would increase the cellularity obtained from immunized mice by removing the disease-causing epitope of MOG_{35-55}, thus maintaining the mice in a healthier state that would be more conducive to increasing the generation of autoregulatory CD8 T-cells. Unfortunately, as our data revealed, induction of regulatory CD8 T-cells does require the presentation of both the CD8 T-cell epitopes as well as the CD4 T-cell epitope in order to generate an effective regulatory CD8 T-cell population.

Next, as seen in our protection experiments, although we could suppress disease, we could never totally ameliorate the CNS autoimmunity. I initially performed adoptive transfer experiments where I transferred 1X, 1.5X and 2X number of autoregulatory CD8 T-cells and evaluated their suppressive ability. The increase in transferred CD8 T-cells did not have a significant effect on disease (data not shown). Curiously, an observation was made in another lab, where IL12 was found to augment the activation and cytotoxicity of CD8 T-cells. Thus, I proceeded to evaluate the role of IL12 in the generation and activation of autoregulatory CD8 T-cells. As our data revealed, *in vitro* IL-12 modulation augmented the disease suppressing ability of autoregulatory CD8 T-cells (Figure 58). This correlated with an increase in the activation status of CD8 T-cells, as well as IFN-γ production; a cytokine that we had previously shown to be required for disease amelioration (Figure 33).

Now we have a potential therapy that can be used to isolate defective myelin-specific CD8 T-cells from MS patients, *in vitro* modulate them to increase their suppressive function and re-insert them into patients. Although the field of cellular-based therapy is still young, further research into autoregulatory CD8 T-cells could be used to assure in a new type of therapy. I

predict that soon autoregulatory CD8 T-cells will be used for the primary purpose of alleviating patients from the grasp of the debilitating neurological disease called Multiple Sclerosis.

6.7: Proposed Model of Autoregulatory CD8 T-cells Mediated Suppression of EAE

Induction of EAE by immunization with a myelin peptide generates an encephalitogenic Th_1/Th_{17} CD4 T-cell that is capable of trafficking to the CNS and causing tissue pathology (Figure 60 A & B). Due to the inflammatory state (IL12) and myelin peptide (MOG) picked up by APCs and presented to naïve autoreactive CD8 T-cells, a cytotoxic autoreactive regulatory CD8 T-cell is generated by the immune system in an effort to down-modulate the CD4 T-cell mediated disease (Figure 60 C). This autoregulatory CD8 T-cell can traffic to the peripheral immune compartment and CNS. In these tissues, encephalitogenic CD4 T-cells can present cognate myelin antigen in the context of MHC-Ia to autoregulatory CD8 T-cells. This is turn activates the autoregulatory CD8 T-cell, which produces IFN-γ and perforin molecules in order to suppress the encephalitogenic CD4 T-cell (Figure 60 D). Loss of function of the encephalitogenic CD4 T-cells results in amelioration of disease. Due to the methods used in EAE induction, new waves of CD4 T-cells are constantly generated that the autoregulatory CD8 T-cell must target and this may be why we do not see a robust and permanent suppression of EAE. I hypothesized that in humans, the inciting antigen is transiently activating CD4 T-cells, and upon CD8 T-cell targeting of the encephalitogenic CD4 T-cells, this cellular based therapy would result in complete disease amelioration.

6.8: Future Directions

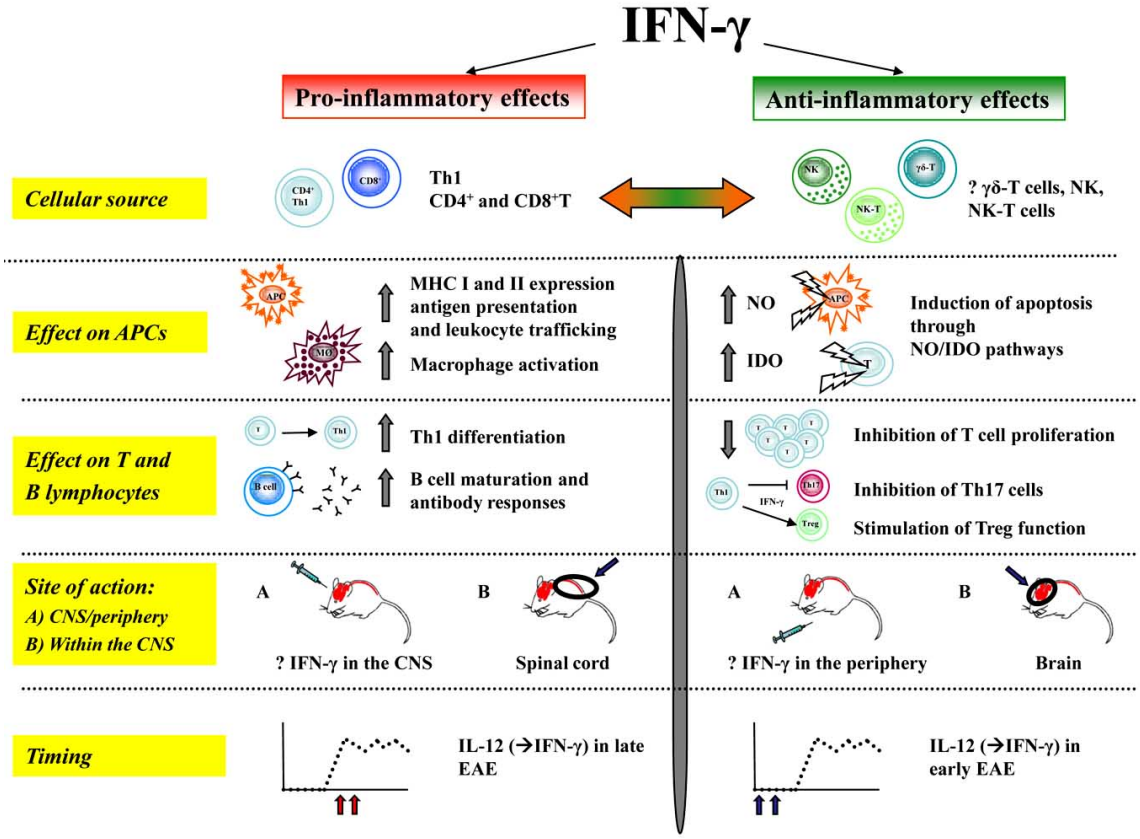
There are three distinct lines of evidence that need to be followed in order to further understand the role and mechanisms of disease regulation by autoregulatory CD8 T-cells. First we had observed that $PLP_{139-151}$-specific CD8 T-cells were unable to ameliorate this $PLP_{139-152}$-induced disease. One possible explanation for this observation could that the thymus does not express $PLP_{139-151}$ peptide and hence the $PLP_{139-151}$-specific CD8 T-cells in the SJL mice do not go through central tolerance. This is unlikely as removal of central tolerance selection would result in the presence of high avidity myelin-specific pathogenic CD8 T-cells that would potentiate disease. As an increase in disease severity was not seen in these experiments, I would speculate that thymic selection is not involved. A more likely scenario is that $PLP_{139-151}$-specific CD8 T-cells may have an alternate functional profile, which does not afford the ability to ameliorate disease. A functional profile comparison will need to be performed between definitive regulatory and non-regulatory CD8 T-cells.

Secondly, we have observed that autoregulatory CD8 T-cells were capable of targeting encephalitogenic CD4 T-cells directly. The following sets of questions would help confirm and elucidate how CD8 T-cells are directly targeting CD4 T-cells: 1) is there a preferential targeting of Th_1 or Th_{17} CD4 T-cells, 2) is perforin or IFN-γ preferentially used in the targeting of CD4 T-cells, 3) can CD8 T-cells be activated and directly kill encephalitogenic CD4 T-cells *in vitro*, 4) how are CD4 T-cells acquiring the machinery to present antigen to CD8 T-cells and 5) what portion(s) of the myelin peptide is being presented by CD4 T-cells

Finally, we were able to augment the disease ameliorating ability of autoregulatory CD8 T-cells by *in vitro* IL12 modulation. The results, although exciting, still do not show a full and impactful suppression of EAE. Thus, further optimization of IL12 or inclusion of other cytokines

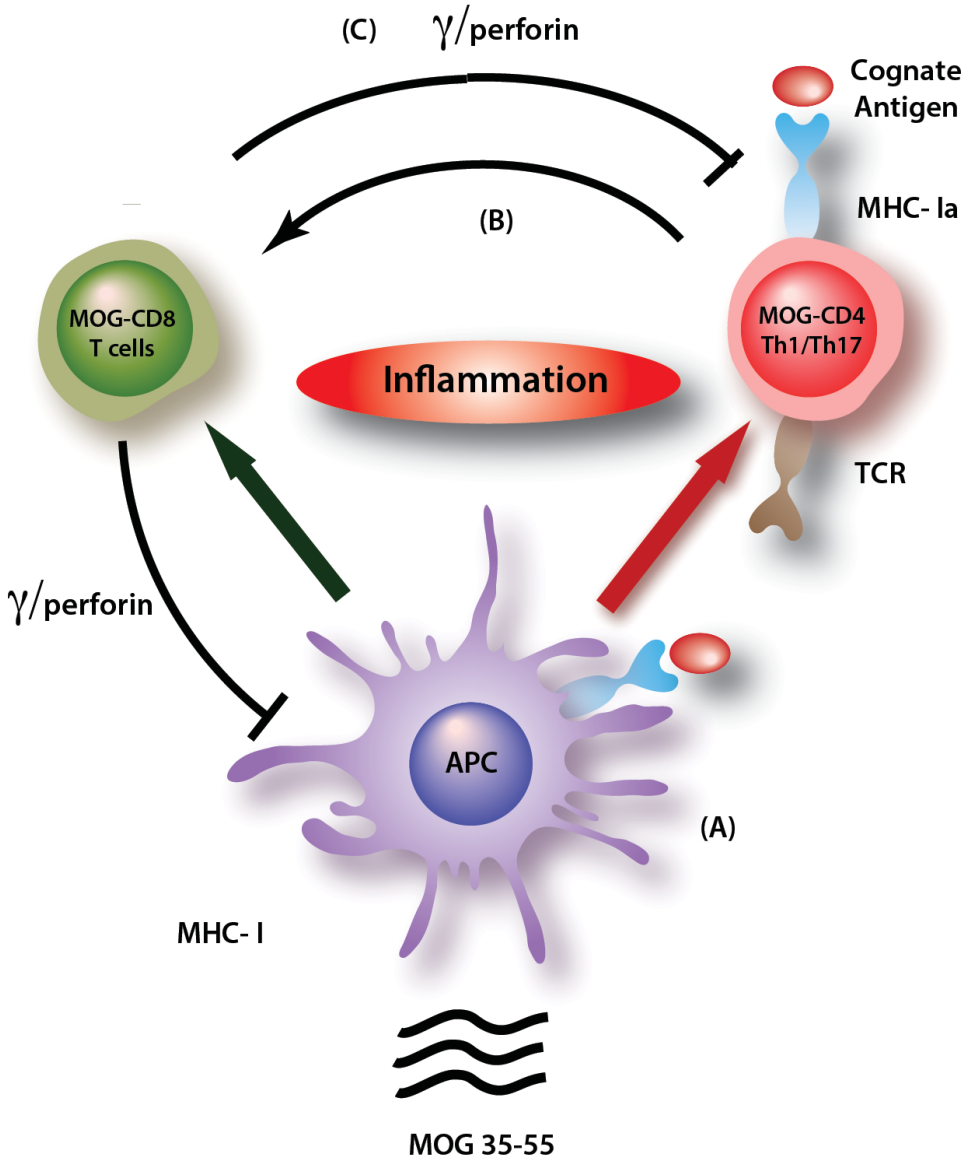
that can modulate CD8 T-cells, preferably by increasing the production of INF-γ or perforin, might result in full and complete suppression of EAE disease.

Figure 59: Overview of the pro-inflammatory and anti-inflammatory effects of IFN-Y in central nervous system autoimmune demyelination [151]



Effects depending on the cellular source. CD4+ and CD8+ T cells as well as γδT cells, CD56bright NK cells and NKT cells produce IFN-γ. All these cell types can be responsible for both pro-inflammatory and anti-inflammatory effects observed in animal models of disease (double arrow). One hypothesis is that IFN-γ producing -T cells, CD56bright NK cells and NKT cells could be protective in EAE/MS, whilst IFN-γ produced by encephalitogenic CD4 and CD8 T cells would exert pro-inflammatory effects. *Effects on antigen-presenting cells (APCs).* IFN-γ induces increased expression of MHC class I and class II molecules, increases antigen presentation, leukocyte trafficking and macrophage activation (left). IFN-γ is also able to increase nitric oxide (NO) and enzyme indolamine 2,3-dioxygenaseindolamine (IDO) that promote apoptosis of autoreactive T cells and APCs (right). *Effects on T and B lymphocytes.* Pro-inflammatory effects of IFN-γ include promotion of T cell differentiation and B cell maturation (left). IFN-γ exerts anti-inflammatory action since it reduces T cell proliferation, inhibits Th17 development and stimulates regulatory T cells (Treg) function (right). *Effects depending on the site of action.* A) Effects in the CNS or in the periphery. It has been hypothesised that locally produced IFN-γ promotes inflammation (left), whilst intraperitoneal administration of IFN-γ in mice results in disease suppression (right). B) Effects within the CNS. It has been hypothesised that IFN-γ promotes inflammation in the spinal cord (left), whilst it exerts regulatory action in the brain (right). *Effects depending on the time of administration during EAE.* Administration of IL-12 (and subsequent IFN-γ production) in late EAE promotes disease (left), whilst early administration of IL-12 results in disease limitation that is IFN-γ dependent (right) [151].

Figure 60: Model of Autoregulatory CD8 T-cell Suppression of CNS Demyelinating Disease



Model of generation of and disease suppression by autoregulatory CD8 T-cells in EAE. (A) Myelin peptides are picked up by APCs and presented to autoreactive CD4 T-cells. CD4 T-cells traffic to the CNS where they cause local inflammation and initiate a cascade of events which lead to inflammation and axonal demyelination. (B) APCs also present myelin antigen to naïve CD8 T-cells in the context of an inflammatory milieu resulting in the generation of autoreactive regulatory CD8 T-cell. (C) These autoregulatory CD8 T-cell can now inhibit both APCs function, as well as autoreactive CD4 T-cells in the periphery by using IFN-γ and perforin-dependent mechanisms. Its possible that these autoregulatory CD8 T-cells can also traffic to the CNS where they can target encephalitogenic CD4 T-cells, which present cognate myelin antigen in the context of MHC-Ia. Suppression/cytotoxicity of CD4 T-cells results in disease amelioration.

REFERENCES

1. **Janeway CA**, **Medzhitov R**. Innate immune recognition. *Annu Rev Immunol*. 2002; **20**:197–216.DOI: 10.1146/annurev.immunol.20.083001.084359.

2. **Akira S**, **Takeda K**. Toll-like receptor signalling. *Nat Rev Immunol*. 2004; **4**:499–511.DOI: 10.1038/nri1391.

3. **Janeway C**. *Immunobiology : the immune system in health and disease*. New York : Garland Science; 2005.

4. **Barry M**, **Bleackley RC**. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol*. 2002; **2**:401–409.DOI: 10.1038/nri819.

5. **Germain RN**. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. *Cell*. 1994; **76**:287–299.

6. **Jenkins MK**, **Khoruts A**, **Ingulli E**, **Mueller DL**, **McSorley SJ**, **Reinhardt RL**, **Itano A**, *et al.* In vivo activation of antigen-specific CD4 T cells. *Annu Rev Immunol*. 2001; **19**:23–45.DOI: 10.1146/annurev.immunol.19.1.23.

7. **Gordon S**, **Taylor PR**. Monocyte and macrophage heterogeneity. *Nat Rev Immunol*. 2005; **5**:953–964.DOI: 10.1038/nri1733.

8. **Murphy KM**, **Reiner SL**. The lineage decisions of helper T cells. *Nat Rev Immunol*. 2002; **2**:933–944.DOI: 10.1038/nri954.

9. **Agnello D**, **Lankford CSR**, **Bream J**, **Morinobu A**, **Gadina M**, **O'Shea JJ**, **Frucht DM**. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *Journal of clinical immunology*. 2003; **23**:147–161.

10. **Stockinger B**, **Veldhoen M**. Differentiation and function of Th17 T cells. *Current opinion in immunology*. 2007; **19**:281–286.DOI: 10.1016/j.coi.2007.04.005.

11. **Steinman L**. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med*. 2007; **13**:139–145.DOI: 10.1038/nm1551.

12. **Peterson P**, **Org T**, **Rebane A**. Transcriptional regulation by AIRE: molecular mechanisms of central tolerance. *Nat Rev Immunol*. 2008; **8**:948–957.DOI: 10.1038/nri2450.

13. **Lechler R**, **Chai JG**, **Marelli-Berg F**, **Lombardi G**. T-cell anergy and peripheral T-cell tolerance. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* 2001; **356**:625–637.DOI: 10.1098/rstb.2001.0844.

14. **Jiang H**, **Chess L**. Regulation of immune responses by T cells. *N. Engl. J. Med.* 2006; **354**:1166–1176.DOI: 10.1056/NEJMra055446.

15. **Vignali DAA**, **Collison LW**, **Workman CJ**. How regulatory T cells work. *Nat Rev Immunol*. 2008; **8**:523–532.DOI: 10.1038/nri2343.

16. **Tang Q**, **Bluestone JA**. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol*. 2008; **9**:239–244.DOI: 10.1038/ni1572.

17. **Santamaria P**. Development of Memory-Like Autoregulatory CD8+ T Cells Is CD4+ T Cell Dependent. *J Immunol*. 2011; **187**:2859–2866.DOI: 10.4049/jimmunol.1101117.

18. **McQualter JL**, **Bernard CCA**. Multiple sclerosis: a battle between destruction and repair. *J. Neurochem.* 2007; **100**:295–306.DOI: 10.1111/j.1471-4159.2006.04232.x.

19. **D'Cruz DP**, **Khamashta MA**, **Hughes GRV**. Systemic lupus erythematosus. *Lancet*. 2007; **369**:587–596.DOI: 10.1016/S0140-6736(07)60279-7.

20. **Rahman A**, **Isenberg DA**. Systemic Lupus Erythematosus. *N. Engl. J. Med.* 2012; **358**:929–939.DOI: doi: 10.1056/NEJMra071297.

21. **Cooke DW**, **Plotnick L**. Type 1 diabetes mellitus in pediatrics. *Pediatr Rev*. 2008; **29**:374–84– quiz 385.DOI: 10.1542/pir.29-11-374.

22. **King C**, **Sarvetnick N**. Organ-specific autoimmunity. *Current opinion in immunology*. 1997; **9**:863–871.

23. **Clanet M**. *Jean-Martin Charcot. 1825 to 1893.*; 2008:59–61.

24. **Anderson DW**, **Ellenberg JH**, **Leventhal CM**, **Reingold SC**, **Rodriguez M**, **Silberberg DH**. Revised estimate of the prevalence of multiple sclerosis in the United States. *Ann. Neurol.* 1992; **31**:333–336.DOI: 10.1002/ana.410310317.

25. **Compston A**, **Coles A**. Multiple sclerosis. *The Lancet*. 2008; **372**:1502–1517.DOI: 10.1016/S0140-6736(08)61620-7.

26. **Alonso A**, **Hernán MA**. Temporal trends in the incidence of multiple sclerosis: a systematic review. *Neurology*. 2008; **71**:129–135.DOI: 10.1212/01.wnl.0000316802.35974.34.

27. **Miller DH**, **Leary SM**. Primary-progressive multiple sclerosis. *Lancet Neurol*. 2007; **6**:903–912.DOI: 10.1016/S1474-4422(07)70243-0.

28. **Rosati G**. The prevalence of multiple sclerosis in the world: an update. *Neurol. Sci.* 2001; **22**:117–139.

29. **Compston A**, **Coles A**. Multiple sclerosis. *The Lancet*. 2002; **359**:1221–1231.DOI:

10.1016/S0140-6736(02)08220-X.

30. **Olerup O**, **Hillert J**. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. *Tissue Antigens*. 1991; **38**:1–15.

31. **Marrosu MG**, **Muntoni F**, **Murru MR**, **Costa G**, **Pischedda MP**, **Pirastu M**, **Sotgiu S**, *et al.* HLA-DQB1 genotype in Sardinian multiple sclerosis: evidence for a key role of DQB1 *0201 and *0302 alleles. *Neurology*. 1992; **42**:883–886.

32. **Gregory SG**, **Schmidt S**, **Seth P**, **Oksenberg JR**, **Hart J**, **Prokop A**, **Caillier SJ**, *et al.* Interleukin 7 receptor alpha chain (IL7R) shows allelic and functional association with multiple sclerosis. *Nat. Genet.* 2007; **39**:1083–1091.DOI: 10.1038/ng2103.

33. Risk Alleles for Multiple Sclerosis Identified by a Genomewide Study. *N. Engl. J. Med.* 2007; **357**:851–862.DOI: 10.1056/NEJMoa073493.

34. **Lundmark F**, **Duvefelt K**, **Iacobaeus E**, **Kockum I**, **Wallström E**, **Khademi M**, **Oturai A**, *et al.* Variation in interleukin 7 receptor alpha chain (IL7R) influences risk of multiple sclerosis. *Nat. Genet.* 2007; **39**:1108–1113.DOI: 10.1038/ng2106.

35. **International Multiple Sclerosis Genetics Consortium (IMSGC)**. Refining genetic associations in multiple sclerosis. *Lancet Neurol*. 2008; **7**:567–569.DOI: 10.1016/S1474-4422(08)70122-4.

36. **Yeo TW**, **De Jager PL**, **Gregory SG**, **Barcellos LF**, **Walton A**, **Goris A**, **Fenoglio C**, *et al.* A second major histocompatibility complex susceptibility locus for multiple sclerosis. *Ann. Neurol.* 2007; **61**:228–236.DOI: 10.1002/ana.21063.

37. **Dean G**, **Yeo TW**, **Goris A**, **Taylor CJ**, **Goodman RS**, **Elian M**, **Galea-Debono A**, *et al.* HLA-DRB1 and multiple sclerosis in Malta. *Neurology*. 2008; **70**:101–105.DOI: 10.1212/01.wnl.0000284598.98525.d7.

38. **Ramagopalan SV**, **Morris AP**, **Dyment DA**, **Herrera BM**, **DeLuca GC**, **Lincoln MR**, **Orton SM**, *et al.* The inheritance of resistance alleles in multiple sclerosis. *PLoS Genet.* 2007; **3**:1607–1613.DOI: 10.1371/journal.pgen.0030150.

39. **Dean G**, **Kurtzke JF**. On the risk of multiple sclerosis according to age at immigration to South Africa. *Br Med J*. 1971; **3**:725–729.

40. **ALTER M**, **HALPERN L**, **KURLAND LT**, **BORNSTEIN B**, **LEIBOWITZ U**, **SILBERSTEIN J**. Multiple sclerosis in Israel. Prevalence among immigrants and native inhabitants. *Arch. Neurol.* 1962; **7**:253–263.

41. **Detels R**, **Visscher BR**, **Malmgren RM**, **Coulson AH**, **Lucia MV**, **Dudley JP**. Evidence for lower susceptibility to multiple sclerosis in Japanese-Americans. *Am. J. Epidemiol.* 1977; **105**:303–310.

42. **Elian M**, **Nightingale S**, **Dean G**. Multiple sclerosis among United Kingdom-born

children of immigrants from the Indian subcontinent, Africa and the West Indies. *J. Neurol. Neurosurg. Psychiatr.* 1990; **53**:906–911.

43. **Martyn CN**, **Cruddas M**, **Compston DA**. Symptomatic Epstein-Barr virus infection and multiple sclerosis. *J. Neurol. Neurosurg. Psychiatr.* 1993; **56**:167–168.

44. **Serafini B**, **Rosicarelli B**, **Franciotta D**, **Magliozzi R**, **Reynolds R**, **Cinque P**, **Andreoni L**, *et al.* Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain. *J Exp Med*. 2007; **204**:2899–2912.DOI: 10.1084/jem.20071030.

45. **Ramagopalan SV**, **Dyment DA**, **Valdar W**, **Herrera BM**, **Criscuoli M**, **Yee IML**, **Sadovnick AD**, *et al.* Autoimmune disease in families with multiple sclerosis: a population-based study. *Lancet Neurol*. 2007; **6**:604–610.DOI: 10.1016/S1474-4422(07)70132-1.

46. **Fisniku LK**, **Brex PA**, **Altmann DR**, **Miszkiel KA**, **Benton CE**, **Lanyon R**, **Thompson AJ**, *et al.* Disability and T2 MRI lesions: a 20-year follow-up of patients with relapse onset of multiple sclerosis. *Brain*. 2008; **131**:808–817.DOI: 10.1093/brain/awm329.

47. **Rovaris M**, **Confavreux C**, **Furlan R**, **Kappos L**, **Comi G**, **Filippi M**. Secondary progressive multiple sclerosis: current knowledge and future challenges. *Lancet Neurol*. 2006; **5**:343–354.DOI: 10.1016/S1474-4422(06)70410-0.

48. **Lublin FD**, **Reingold SC**. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. *Neurology*. 1996; **46**:907–911.

49. **Andersen O**, **Lygner PE**, **Bergström T**, **Andersson M**, **Vahlne A**. Viral infections trigger multiple sclerosis relapses: a prospective seroepidemiological study. *J. Neurol.* 1993; **240**:417–422.

50. **Kang H-K**, **Michaels MA**, **Berner BR**, **Datta SK**. Very low-dose tolerance with nucleosomal peptides controls lupus and induces potent regulatory T cell subsets. *J Immunol*. 2005; **174**:3247–3255.

51. **Correale J**, **Farez M**. Association between parasite infection and immune responses in multiple sclerosis. *Ann. Neurol.* 2007; **61**:97–108.DOI: 10.1002/ana.21067.

52. **Vukusic S**, **Hutchinson M**, **Hours M**, **Moreau T**, **Cortinovis-Tourniaire P**, **Adeleine P**, **Confavreux C**, *et al.* Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain*. 2004; **127**:1353–1360.DOI: 10.1093/brain/awh152.

53. **Heesen C**, **Mohr DC**, **Huitinga I**, **Bergh FT**, **Gaab J**, **Otte C**, **Gold SM**. Stress regulation in multiple sclerosis: current issues and concepts. *Mult. Scler.* 2007; **13**:143–148.DOI: 10.1177/1352458506070772.

54. **Brønnum-Hansen H**, **Koch-Henriksen N**, **Stenager E**. Trends in survival and cause of death in Danish patients with multiple sclerosis. *Brain*. 2004; **127**:844–850.DOI: 10.1093/brain/awh104.

55. **Kurtzke JF**. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology*. 1983; **33**:1444–1452.

56. **Gold R**. Combination therapies in multiple sclerosis. *J. Neurol.* 2008; **255 Suppl 1**:51–60.DOI: 10.1007/s00415-008-1008-2.

57. **Jarius S**, **Hohlfeld R**. [Interferon therapy of multiple sclerosis. Synopsis of various dosage forms]. *Nervenarzt*. 2004; **75**:1226–1230.DOI: 10.1007/s00115-004-1834-0.

58. **Sorensen PS**, **Ross C**, **Clemmesen KM**, **Bendtzen K**, **Frederiksen JL**, **Jensen K**, **Kristensen O**, *et al.* Clinical importance of neutralising antibodies against interferon beta in patients with relapsing-remitting multiple sclerosis. *Lancet*. 2003; **362**:1184–1191.DOI: 10.1016/S0140-6736(03)14541-2.

59. **Duda PW**, **Schmied MC**, **Cook SL**, **Krieger JI**, **Hafler DA**. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest*. 2000; **105**:967–976.DOI: 10.1172/JCI8970.

60. **Vieira PL**, **Heystek HC**, **Wormmeester J**, **Wierenga EA**, **Kapsenberg ML**. Glatiramer acetate (copolymer-1, copaxone) promotes Th2 cell development and increased IL-10 production through modulation of dendritic cells. *J Immunol*. 2003; **170**:4483–4488.

61. **Tennakoon DK**, **Mehta RS**, **Ortega SB**, **Bhoj V**, **Racke MK**, **Karandikar NJ**. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J Immunol*. 2006; **176**:7119–7129.

62. **Karandikar NJ**, **Crawford MP**, **Yan X**, **Ratts RB**, **Brenchley JM**, **Ambrozak DR**, **Lovett-Racke AE**, *et al.* Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest*. 2002; **109**:641–649.DOI: 10.1172/JCI14380.

63. **Putheti P**, **Soderstrom M**, **Link H**, **Huang Y-M**. Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis. *J Neuroimmunol*. 2003; **144**:125–131.

64. **Jacobs LD**, **Cookfair DL**, **Rudick RA**, **Herndon RM**, **Richert JR**, **Salazar AM**, **Fischer JS**, *et al.* Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann. Neurol.* 1996; **39**:285–294.DOI: 10.1002/ana.410390304.

65. **Johnson KP**, **Brooks BR**, **Cohen JA**, **Ford CC**, **Goldstein J**, **Lisak RP**, **Myers LW**, *et al.* Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial.

The Copolymer 1 Multiple Sclerosis Study Group. *Neurology*. 1995; **45**:1268–1276.

66. **Paty DW**, **Li DK**. Interferon beta-1b is effective in relapsing-remitting multiple sclerosis. II. MRI analysis results of a multicenter, randomized, double-blind, placebo-controlled trial. UBC MS/MRI Study Group and the IFNB Multiple Sclerosis Study Group. *Neurology*. 1993; **43**:662–667.

67. **Steinman L**. Blocking adhesion molecules as therapy for multiple sclerosis: natalizumab. *Nat Rev Drug Discov*. 2005; **4**:510–518.DOI: 10.1038/nrd1752.

68. **Kappos L**, **Bates D**, **Hartung H-P**, **Havrdová E**, **Miller D**, **Polman CH**, **Ravnborg M**, *et al.* Natalizumab treatment for multiple sclerosis: recommendations for patient selection and monitoring. *Lancet Neurol*. 2007; **6**:431–441.DOI: 10.1016/S1474-4422(07)70078-9.

69. **Rudick RA**, **Stuart WH**, **Calabresi PA**, **Confavreux C**, **Galetta SL**, **Radue E-W**, **Lublin FD**, *et al.* Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N. Engl. J. Med.* 2006; **354**:911–923.DOI: 10.1056/NEJMoa044396.

70. **Hartung H-P**. New cases of progressive multifocal leukoencephalopathy after treatment with natalizumab. *Lancet Neurol*. 2009; **8**:28–31.DOI: 10.1016/S1474-4422(08)70281-3.

71. **Edan G**, **Morrissey S**, **Le Page E**. Rationale for the use of mitoxantrone in multiple sclerosis. *J. Neurol. Sci.* 2004; **223**:35–39.DOI: 10.1016/j.jns.2004.04.017.

72. **Kappos L**, **Radue E-W**, **O'Connor P**, **Polman C**, **Hohlfeld R**, **Calabresi P**, **Selmaj K**, *et al.* A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. *N. Engl. J. Med.* 2010; **362**:387–401.DOI: 10.1056/NEJMoa0909494.

73. **Paugh SW**, **Payne SG**, **Barbour SE**, **Milstien S**, **Spiegel S**. The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS LETTERS*. 2003; **554**:189–193.

74. **McAlpine D**, **Compston A**. *McAlpine's Multiple Sclerosis*. Churchill Livingstone; 2005.

75. **Lalive PH**, **Neuhaus O**, **Benkhoucha M**, **Burger D**, **Hohlfeld R**, **Zamvil SS**, **Weber MS**. Glatiramer acetate in the treatment of multiple sclerosis: emerging concepts regarding its mechanism of action. *CNS Drugs*. 2011; **25**:401–414.DOI: 10.2165/11588120-000000000-00000.

76. **Joller N**, **Peters A**, **Anderson AC**, **Kuchroo VK**. Immune checkpoints in central nervous system autoimmunity. *Immunol Rev*. 2012; **248**:122–139.DOI: 10.1111/j.1600-065X.2012.01136.x.

77. **Fletcher JM**, **Lalor SJ**, **Sweeney CM**, **Tubridy N**, **Mills KHG**. T cells in multiple sclerosis and experimental autoimmune encephalomyelitis. *Clin Exp Immunol*. 2010;

162:1–11.DOI: 10.1111/j.1365-2249.2010.04143.x.

78. **Mix E**, **Meyer-Rienecker H**, **Hartung H-P**, **Zettl UK**. Animal models of multiple sclerosis-Potentials and limitations. *Progress in Neurobiology*. 2010; **92**:386–404.DOI: 10.1016/j.pneurobio.2010.06.005.

79. **Furtado GC**, **Marcondes MCG**, **Latkowski J-A**, **Tsai J**, **Wensky A**, **Lafaille JJ**. Swift entry of myelin-specific T lymphocytes into the central nervous system in spontaneous autoimmune encephalomyelitis. *The Journal of Immunology*. 2008; **181**:4648–4655.

80. **Sospedra M**, **Martin R**. Immunology of multiple sclerosis. *Annu Rev Immunol*. 2005; **23**:683–747.DOI: 10.1146/annurev.immunol.23.021704.115707.

81. **El-Behi M**, **Dubucquoi S**, **Lefranc D**, **Zéphir H**, **De Seze J**, **Vermersch P**, **Prin L**. New insights into cell responses involved in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol. Lett.* 2005; **96**:11–26.DOI: 10.1016/j.imlet.2004.07.017.

82. **McFarland HF**, **Martin R**. Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol*. 2007; **8**:913–919.DOI: 10.1038/ni1507.

83. **t Hart BA**, **Hintzen RQ**, **Laman JD**. Multiple sclerosis - a response-to-damage model. *Trends in Molecular Medicine*. 2009; **15**:235–244.DOI: 10.1016/j.molmed.2009.04.001.

84. **Crawford MP**, **Yan SX**, **Ortega SB**, **Mehta RS**, **Hewitt RE**, **Price DA**, **Stastny P**, *et al.* High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood*. 2004; **103**:4222–4231.DOI: 10.1182/blood-2003-11-4025.

85. **York NR**, **Mendoza JP**, **Ortega SB**, **Benagh A**, **Tyler AF**, **Firan M**, **Karandikar NJ**. Immune regulatory CNS-reactive CD8+T cells in experimental autoimmune encephalomyelitis. *J Autoimmun*. 2010.DOI: 10.1016/j.jaut.2010.01.003.

86. **Sallusto F**, **Impellizzieri D**, **Basso C**, **Laroni A**, **Uccelli A**, **Lanzavecchia A**, **Engelhardt B**. T-cell trafficking in the central nervous system. *Immunol Rev*. 2012; **248**:216–227.DOI: 10.1111/j.1600-065X.2012.01140.x.

87. **Viglietta V**, **Baecher-Allan C**, **Weiner HL**, **Hafler DA**. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med*. 2004; **199**:971–979.DOI: 10.1084/jem.20031579.

88. **Shi Y**, **Feng Y**, **Kang J**, **Liu C**, **Li Z**, **Li D**, **Cao W**, *et al.* Critical regulation of CD4+ T cell survival and autoimmunity by beta-arrestin 1. *Nat Immunol*. 2007; **8**:817–824.DOI: 10.1038/ni1489.

89. **Langrish CL**, **Chen Y**, **Blumenschein WM**, **Mattson J**, **Basham B**, **Sedgwick JD**, **McClanahan T**, *et al.* IL-23 drives a pathogenic T cell population that induces

autoimmune inflammation. *J Exp Med*. 2005; **201**:233–240.DOI: 10.1084/jem.20041257.

90. **Kebir H**, **Kreymborg K**, **Ifergan I**, **Dodelet-Devillers A**, **Cayrol R**, **Bernard M**, **Giuliani F**, *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med*. 2007; **13**:1173–1175.DOI: 10.1038/nm1651.

91. **Tzartos JS**, **Friese MA**, **Craner MJ**, **Palace J**, **Newcombe J**, **Esiri MM**, **Fugger L**. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am. J. Pathol.* 2008; **172**:146–155.DOI: 10.2353/ajpath.2008.070690.

92. **Ousman SS**, **Tomooka BH**, **van Noort JM**, **Wawrousek EF**, **O'Connor KC**, **Hafler DA**, **Sobel RA**, *et al.* Protective and therapeutic role for alphaB-crystallin in autoimmune demyelination. *Nature*. 2007; **448**:474–479.DOI: 10.1038/nature05935.

93. **Mathey EK**, **Derfuss T**, **Storch MK**, **Williams KR**, **Hales K**, **Woolley DR**, **Al-Hayani A**, *et al.* Neurofascin as a novel target for autoantibody-mediated axonal injury. *J Exp Med*. 2007; **204**:2363–2372.DOI: 10.1084/jem.20071053.

94. **Määttä JA**, **Käldman MS**, **Sakoda S**, **Salmi AA**, **Hinkkanen AE**. Encephalitogenicity of myelin-associated oligodendrocytic basic protein and 2',3“-cyclic nucleotide 3”-phosphodiesterase for BALB/c and SJL mice. *Immunology*. 1998; **95**:383–388.

95. **Trapp BD**, **Peterson J**, **Ransohoff RM**, **Rudick R**, **Mörk S**, **Bö L**. Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* 1998; **338**:278–285.DOI: 10.1056/NEJM199801293380502.

96. **Coman I**, **Aigrot MS**, **Seilhean D**, **Reynolds R**, **Girault JA**, **Zalc B**, **Lubetzki C**. Nodal, paranodal and juxtaparanodal axonal proteins during demyelination and remyelination in multiple sclerosis. *Brain*. 2006; **129**:3186–3195.DOI: 10.1093/brain/awl144.

97. **Eshed Y**, **Feinberg K**, **Poliak S**, **Sabanay H**, **Sarig-Nadir O**, **Spiegel I**, **Bermingham JR**, *et al.* Gliomedin mediates Schwann cell-axon interaction and the molecular assembly of the nodes of Ranvier. *Neuron*. 2005; **47**:215–229.DOI: 10.1016/j.neuron.2005.06.026.

98. **Scolding N**, **Franklin R**, **Stevens S**, **Heldin CH**, **Compston A**, **Newcombe J**. Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis. *Brain*. 1998; **121 (Pt 12)**:2221–2228.

99. **Wolswijk G**. Oligodendrocyte regeneration in the adult rodent CNS and the failure of this process in multiple sclerosis. *Prog. Brain Res.* 1998; **117**:233–247.

100. **Chandran S**, **Hunt D**, **Joannides A**, **Zhao C**, **Compston A**, **Franklin RJM**. Myelin repair: the role of stem and precursor cells in multiple sclerosis. *Philos. Trans. R.*

Soc. Lond., B, Biol. Sci. 2008; **363**:171–183.DOI: 10.1098/rstb.2006.2019.

101. **Chari DM**. Remyelination in multiple sclerosis. *Int. Rev. Neurobiol.* 2007; **79**:589–620.DOI: 10.1016/S0074-7742(07)79026-8.

102. **Sabin AB**, **Wright AM**. ACUTE ASCENDING MYELITIS FOLLOWING A MONKEY BITE, WITH THE ISOLATION OF A VIRUS CAPABLE OF REPRODUCING THE DISEASE. *J Exp Med*. 1934; **59**:115–136.

103. **Rivers TM**, **Sprunt DH**, **Berry GP**. OBSERVATIONS ON ATTEMPTS TO PRODUCE ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MONKEYS. *J Exp Med*. 1933; **58**:39–53.

104. **Rivers TM**, **Schwentker FF**. ENCEPHALOMYELITIS ACCOMPANIED BY MYELIN DESTRUCTION EXPERIMENTALLY PRODUCED IN MONKEYS. *J Exp Med*. 1935; **61**:689–702.

105. **Constantinescu CS**, **Farooqi N**, **O'Brien K**, **Gran B**. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *Br. J. Pharmacol.* 2011; **164**:1079–1106.DOI: 10.1111/j.1476-5381.2011.01302.x.

106. **Teixeira SA**, **Varriano AA**, **Bolonheis SM**, **Muscará MN**. Experimental autoimmune encephalomyelitis: A heterogeneous group of animal models to study human multiple sclerosis. *Drug Discovery Today: Disease Models*. 2005; **2**:127–134.DOI: 10.1016/j.ddmod.2005.05.017.

107. **Stromnes IM**, **Goverman JM**. Active induction of experimental allergic encephalomyelitis. *Nat Protoc*. 2006; **1**:1810–1819.DOI: 10.1038/nprot.2006.285.

108. **Stromnes IM**, **Goverman JM**. Passive induction of experimental allergic encephalomyelitis. *Nat Protoc*. 2006; **1**:1952–1960.DOI: 10.1038/nprot.2006.284.

109. **Pettinelli CB**, **McFarlin DE**. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Lyt 1+ 2- T lymphocytes. *J Immunol*. 1981; **127**:1420–1423.

110. **Miller SD**, **Karpus WJ**, **Davidson TS**. Current Protocols in Immunology. 2010:1–20.DOI: 10.1002/0471142735.im1501s88.

111. **McRae BL**, **Vanderlugt CL**, **Dal Canto MC**, **Miller SD**. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. *J Exp Med*. 1995; **182**:75–85.

112. **Steinman L**, **Zamvil SS**. Virtues and pitfalls of EAE for the development of therapies for multiple sclerosis. *Trends Immunol*. 2005; **26**:565–571.DOI: 10.1016/j.it.2005.08.014.

113. **O'Connor RA**, **Prendergast CT**, **Sabatos CA**, **Lau CWZ**, **Leech MD**, **Wraith DC**, **Anderton SM**. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *The Journal of Immunology*. 2008; **181**:3750–3754.

114. **Aranami T**, **Yamamura T**. Th17 Cells and autoimmune encephalomyelitis (EAE/MS). *Allergol Int*. 2008; **57**:115–120.DOI: 10.2332/allergolint.R-07-159.

115. **Stromnes IM**, **Cerretti LM**, **Liggitt D**, **Harris RA**, **Goverman JM**. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med*. 2008; **14**:337–342.DOI: 10.1038/nm1715.

116. **Matusevicius D**, **Kivisäkk P**, **He B**, **Kostulas N**, **Ozenci V**, **Fredrikson S**, **Link H**. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult. Scler.* 1999; **5**:101–104.

117. **Durelli L**, **Conti L**, **Clerico M**, **Boselli D**, **Contessa G**, **Ripellino P**, **Ferrero B**, *et al.* T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. *Ann. Neurol.* 2009; **65**:499–509.DOI: 10.1002/ana.21652.

118. **Lock C**, **Hermans G**, **Pedotti R**, **Brendolan A**, **Schadt E**, **Garren H**, **Langer-Gould A**, *et al.* Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat Med*. 2002; **8**:500–508.DOI: 10.1038/nm0502-500.

119. **Montes M**, **Zhang X**, **Berthelot L**, **Laplaud D-A**, **Brouard S**, **Jin J**, **Rogan S**, *et al.* Oligoclonal myelin-reactive T-cell infiltrates derived from multiple sclerosis lesions are enriched in Th17 cells. *Clin Immunol*. 2009; **130**:133–144.DOI: 10.1016/j.clim.2008.08.030.

120. **Bar-Or A**, **Oliveira EM**, **Anderson DE**, **Hafler DA**. Molecular pathogenesis of multiple sclerosis. *J Neuroimmunol*. 1999; **100**:252–259.

121. **Sawcer S**, **Ban M**, **Maranian M**, **Yeo TW**, **Compston A**, **Kirby A**, **Daly MJ**, *et al.* A high-density screen for linkage in multiple sclerosis. *Am. J. Hum. Genet.* 2005; **77**:454–467.DOI: 10.1086/444547.

122. **Friese MA**, **Fugger L**. Pathogenic CD8(+) T cells in multiple sclerosis. *Ann. Neurol.* 2009; **66**:132–141.DOI: 10.1002/ana.21744.

123. **Sobottka B**, **Harrer MD**, **Ziegler U**, **Fischer K**, **Wiendl H**, **Hünig T**, **Becher B**, *et al.* Collateral bystander damage by myelin-directed CD8+ T cells causes axonal loss. *American Journal Of Pathology*. 2009; **175**:1160–1166.DOI: 10.2353/ajpath.2009.090340.

124. **Franciotta D**, **Salvetti M**, **Lolli F**, **Serafini B**, **Aloisi F**. B cells and multiple sclerosis. *Lancet Neurol*. 2008; **7**:852–858.DOI: 10.1016/S1474-4422(08)70192-3.

125. **Goverman J**. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol*. 2009; **9**:393–407.DOI: 10.1038/nri2550.

126. **Hohlfeld R**, **Meinl E**, **Dornmair K**. B- and T-cell responses in multiple sclerosis: novel approaches offer new insights. *J. Neurol. Sci.* 2008; **274**:5–8.DOI: 10.1016/j.jns.2008.07.006.

127. **Martin MDP**, **Monson NL**. Potential role of humoral immunity in the pathogenesis of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). *Front. Biosci.* 2007; **12**:2735–2749.

128. **Ziemssen T**, **Ziemssen F**. The role of the humoral immune system in multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). *Autoimmun Rev*. 2005; **4**:460–467.DOI: 10.1016/j.autrev.2005.03.005.

129. **Hafler DA**, **Slavik JM**, **Anderson DE**, **O'Connor KC**, **De Jager P**, **Baecher-Allan C**. Multiple sclerosis. *Immunol Rev*. 2005; **204**:208–231.DOI: 10.1111/j.0105-2896.2005.00240.x.

130. **O'Connor RA**, **Anderton SM**. Foxp3+ regulatory T cells in the control of experimental CNS autoimmune disease. *J Neuroimmunol*. 2008; **193**:1–11.DOI: 10.1016/j.jneuroim.2007.11.016.

131. **Bynoe MS**, **Bonorino P**, **Viret C**. Control of experimental autoimmune encephalomyelitis by CD4+ suppressor T cells: peripheral versus in situ immunoregulation. *J Neuroimmunol*. 2007; **191**:61–69.DOI: 10.1016/j.jneuroim.2007.09.010.

132. **Ben Nun A**, **Mendel I**, **Bakimer R**, **Fridkis-Hareli M**, **Teitelbaum D**, **Arnon R**, **Sela M**, *et al.* The autoimmune reactivity to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis is potentially pathogenic: effect of copolymer 1 on MOG-induced disease. *J. Neurol.* 1996; **243**:S14–22.

133. **Willing A**, **Friese MA**. CD8-mediated inflammatory central nervous system disorders. *Curr Opin Neurol*. 2012; **25**:316–321.DOI: 10.1097/WCO.0b013e328352ea8b.

134. **Abreu SL**. Suppression of experimental allergic encephalomyelitis by interferon. *Immunol. Commun.* 1982; **11**:1–7.

135. **Jacobs L**, **O'Malley J**, **Freeman A**, **Murawski J**, **Ekes R**. Intrathecal interferon in multiple sclerosis. *Arch. Neurol.* 1982; **39**:609–615.

136. **Teitelbaum D**, **Webb C**, **Meshorer A**, **Arnon R**, **Sela M**. Suppression by several synthetic polypeptides of experimental allergic encephalomyelitis induced in guinea pigs and rabbits with bovine and human basic encephalitogen. *Eur J Immunol*. 1973; **3**:273–279.DOI: 10.1002/eji.1830030505.

137. **Teitelbaum D**, **Meshorer A**, **Hirshfeld T**, **Arnon R**, **Sela M**. Suppression of

experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur J Immunol*. 1971; **1**:242–248.DOI: 10.1002/eji.1830010406.

138. **Teitelbaum D**, **Webb C**, **Bree M**, **Meshorer A**, **Arnon R**, **Sela M**. Suppression of experimental allergic encephalomyelitis in Rhesus monkeys by a synthetic basic copolymer. *Clin. Immunol. Immunopathol.* 1974; **3**:256–262.

139. **Yednock TA**, **Cannon C**, **Fritz LC**, **Sanchez-Madrid F**, **Steinman L**, **Karin N**. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature*. 1992; **356**:63–66.DOI: 10.1038/356063a0.

140. **Theien BE**, **Vanderlugt CL**, **Eagar TN**, **Nickerson-Nutter C**, **Nazareno R**, **Kuchroo VK**, **Miller SD**. Discordant effects of anti-VLA-4 treatment before and after onset of relapsing experimental autoimmune encephalomyelitis. *J Clin Invest*. 2001; **107**:995–1006.DOI: 10.1172/JCI11717.

141. **Theien BE**, **Vanderlugt CL**, **Nickerson-Nutter C**, **Cornebise M**, **Scott DM**, **Perper SJ**, **Whalley ET**, *et al.* Differential effects of treatment with a small-molecule VLA-4 antagonist before and after onset of relapsing EAE. *Blood*. 2003; **102**:4464–4471.DOI: 10.1182/blood-2003-03-0974.

142. **Miller DH**, **Khan OA**, **Sheremata WA**. A Controlled Trial of Natalizumab for Relapsing Multiple Sclerosis — NEJM. *... England Journal of ...*. 2003.

143. **Hauser SL**, **Dawson DM**, **Lehrich JR**, **Beal MF**, **Kevy SV**, **Propper RD**, **Mills JA**, *et al.* Intensive immunosuppression in progressive multiple sclerosis. A randomized, three-arm study of high-dose intravenous cyclophosphamide, plasma exchange, and ACTH. *N. Engl. J. Med.* 1983; **308**:173–180.DOI: 10.1056/NEJM198301273080401.

144. **Massacesi L**, **Parigi A**, **Barilaro A**, **Repice AM**, **Pellicanò G**, **Konze A**, **Siracusa G**, *et al.* Efficacy of azathioprine on multiple sclerosis new brain lesions evaluated using magnetic resonance imaging. *Arch. Neurol.* 2005; **62**:1843–1847.DOI: 10.1001/archneur.62.12.1843.

145. **Casetta I**, **Iuliano G**, **Filippini G**. Azathioprine for multiple sclerosis. *Cochrane Database Syst Rev*. 2007:CD003982.DOI: 10.1002/14651858.CD003982.pub2.

146. **Elkhalifa A**, **Weiner H**. Cyclophosphamide Treatment of MS: Current Therapeutic Approaches and Treatment Regimens. *Int MS J*. 2010; **17**:12–18.

147. **Hartung H-P**, **Gonsette R**, **König N**, **Kwiecinski H**, **Guseo A**, **Morrissey SP**, **Krapf H**, *et al.* Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. *The Lancet*. 2002; **360**:2018–2025.DOI: 10.1016/S0140-6736(02)12023-X.

148. **Weiner HL**. Immunosuppressive treatment in multiple sclerosis. *J. Neurol. Sci.* 2004; **223**:1–11.DOI: 10.1016/j.jns.2004.04.013.

149. **Martinelli Boneschi F**, **Rovaris M**, **Capra R**, **Comi G**. Mitoxantrone for multiple sclerosis. *Cochrane Database Syst Rev*. 2005:CD002127.DOI: 10.1002/14651858.CD002127.pub2.

150. **Weiner HL**, **Mackin GA**, **Matsui M**, **Orav EJ**, **Khoury SJ**, **Dawson DM**, **Hafler DA**. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science*. 1993; **259**:1321–1324.

151. **Sanvito L**, **Constantinescu CS**, **Gran B**. The multifaceted role of interferon-gamma in central nervous system autoimmune demyelination. *Open Autoimmun …*. 2010.

152. **Ferber IA**, **Brocke S**, **Taylor-Edwards C**, **Ridgway W**, **Dinisco C**, **Steinman L**, **Dalton D**, *et al.* Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol*. 1996; **156**:5–7.

153. **Bettelli E**, **Sullivan B**, **Szabo SJ**, **Sobel RA**, **Glimcher LH**, **Kuchroo VK**. Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med*. 2004; **200**:79–87.DOI: 10.1084/jem.20031819.

154. **Panitch HS**, **Hirsch RL**, **Haley AS**, **Johnson KP**. Exacerbations of multiple sclerosis in patients treated with gamma interferon. *The Lancet*. 1987; **1**:893–895.

155. **Segal BM**, **Constantinescu CS**, **Raychaudhuri A**. ScienceDirect.com - The Lancet Neurology - Repeated subcutaneous injections of IL12/23 p40 neutralising antibody, ustekinumab, in patients with relapsing-remitting multiple sclerosis: a phase II, double-blind, placebo-controlled, randomised, dose-ranging study. *The Lancet*. 2008.

156. **Nishizuka Y**, **Sakakura T**. Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science*. 1969; **166**:753–755.

157. **Gershon RK**, **Cohen P**, **Hencin R**, **Liebhaber SA**. Suppressor T Cells. *The Journal of …*. 1972.

158. **Cantor H**, **Hugenberger J**, **McVay-Boudreau L**, **Eardley DD**, **Kemp J**, **Shen FW**, **Gershon RK**. Immunoregulatory circuits among T-cell sets. Identification of a subpopulation of T-helper cells that induces feedback inhibition. *J Exp Med*. 1978; **148**:871–877.

159. **Sakaguchi S**, **Sakaguchi N**, **Asano M**, **Itoh M**, **Toda M**. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*. 1995; **155**:1151–1164.

160. **Weyand CM**. Immunopathologic aspects of rheumatoid arthritis: who is the conductor and who plays the immunologic instrument? *J Rheumatol Suppl*. 2007; **79**:9–14.

161. **Weyand CM**, **Goronzy JJ**. Ectopic germinal center formation in rheumatoid

synovitis. *Ann N Y Acad Sci*. 2003; **987**:140–149.

162. **Davila E**, **Kang YM**, **Park YW**, **Sawai H**, **He X**, **Pryshchep S**, **Goronzy JJ**, *et al.* Cell-based immunotherapy with suppressor CD8+ T cells in rheumatoid arthritis. *J Immunol*. 2005; **174**:7292–7301.

163. **Klimiuk PA**, **Goronzy JJ**, **Weyand CM**. IL-16 as an anti-inflammatory cytokine in rheumatoid synovitis. *J Immunol*. 1999; **162**:4293–4299.

164. **Seo SK**, **Choi JH**, **Kim YH**, **Kang WJ**, **Park HY**, **Suh JH**, **Choi BK**, *et al.* 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nat Med*. 2004; **10**:1088–1094.DOI: 10.1038/nm1107.

165. **Filaci G**, **Bacilieri S**, **Fravega M**, **Monetti M**, **Contini P**, **Ghio M**, **Setti M**, *et al.* Impairment of CD8+ T suppressor cell function in patients with active systemic lupus erythematosus. *J Immunol*. 2001; **166**:6452–6457.

166. **Singh RR**, **Ebling FM**, **Sercarz EE**, **Hahn BH**. Immune tolerance to autoantibody-derived peptides delays development of autoimmunity in murine lupus. *J Clin Invest*. 1995; **96**:2990–2996.DOI: 10.1172/JCI118371.

167. **Hahn BH**, **Singh RR**, **Wong WK**, **Tsao BP**, **Bulpitt K**, **Ebling FM**. Treatment with a consensus peptide based on amino acid sequences in autoantibodies prevents T cell activation by autoantigens and delays disease onset in murine lupus. *Arthritis Rheum.* 2001; **44**:432–441.DOI: 10.1002/1529-0131(200102)44:2<432::AID-ANR62>3.0.CO;2-S.

168. **Hahn BH**, **Singh RP**, **La Cava A**, **Ebling FM**. Tolerogenic treatment of lupus mice with consensus peptide induces Foxp3-expressing, apoptosis-resistant, TGFbeta-secreting CD8+ T cell suppressors. *J Immunol*. 2005; **175**:7728–7737.

169. **Singh RP**, **La Cava A**, **Wong M**, **Ebling F**, **Hahn BH**. CD8+ T cell-mediated suppression of autoimmunity in a murine lupus model of peptide-induced immune tolerance depends on Foxp3 expression. *J Immunol*. 2007; **178**:7649–7657.

170. **Ménager-Marcq I**, **Pomié C**, **Romagnoli P**, **van Meerwijk JPM**. CD8+CD28- regulatory T lymphocytes prevent experimental inflammatory bowel disease in mice. *Gastroenterology*. 2006; **131**:1775–1785.DOI: 10.1053/j.gastro.2006.09.008.

171. **Ho J**, **Kurtz CC**, **Naganuma M**, **Ernst PB**, **Cominelli F**, **Rivera-Nieves J**. A CD8+/CD103high T cell subset regulates TNF-mediated chronic murine ileitis. *J Immunol*. 2008; **180**:2573–2580.

172. **James EA**, **Kwok WW**. CD8+ suppressor-mediated regulation of human CD4+ T cell responses to glutamic acid decarboxylase 65. *Eur J Immunol*. 2007; **37**:78–86.DOI: 10.1002/eji.200636383.

173. **Herold KC**, **Gitelman SE**, **Masharani U**, **Hagopian W**, **Bisikirska B**, **Donaldson**

D, **Rother K**, *et al.* A single course of anti-CD3 monoclonal antibody hOKT3gamma1(Ala-Ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes. *Diabetes*. 2005; **54**:1763–1769.

174. **Bisikirska B**, **Colgan J**, **Luban J**, **Bluestone JA**, **Herold KC**. TCR stimulation with modified anti-CD3 mAb expands CD8+ T cell population and induces CD8+CD25+ Tregs. *J Clin Invest*. 2005; **115**:2904–2913.DOI: 10.1172/JCI23961.

175. **Jilek S**, **Schluep M**, **Rossetti AO**, **Guignard L**, **Le Goff G**, **Pantaleo G**, **Pasquier Du RA**. CSF enrichment of highly differentiated CD8+ T cells in early multiple sclerosis. *Clin Immunol*. 2007; **123**:105–113.DOI: 10.1016/j.clim.2006.11.004.

176. **Hauser SL**, **Bhan AK**, **Gilles F**, **Kemp M**, **Kerr C**, **Weiner HL**. Immunohistochemical analysis of the cellular infiltrate in multiple sclerosis lesions. *Ann. Neurol.* 1986; **19**:578–587.DOI: 10.1002/ana.410190610.

177. **Babbe H**, **Roers A**, **Waisman A**, **Lassmann H**, **Goebels N**, **Hohlfeld R**, **Friese M**, *et al.* Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *J Exp Med*. 2000; **192**:393–404.

178. **Huseby ES**, **Liggitt D**, **Brabb T**, **Schnabel B**, **Ohlén C**, **Goverman J**. A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med*. 2001; **194**:669–676.

179. **Sun D**, **Whitaker JN**, **Huang Z**, **Liu D**, **Coleclough C**, **Wekerle H**, **Raine CS**. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol*. 2001; **166**:7579–7587.

180. **Jiang H**, **Zhang SI**, **Pernis B**. Role of CD8+ T cells in murine experimental allergic encephalomyelitis. *Science*. 1992; **256**:1213–1215.

181. **Koh DR**, **Fung-Leung WP**, **Ho A**, **Gray D**, **Acha-Orbea H**, **Mak TW**. Less mortality but more relapses in experimental allergic encephalomyelitis in CD8-/- mice. *Science*. 1992; **256**:1210–1213.

182. **Linker RA**, **Rott E**, **Hofstetter HH**, **Hanke T**, **Toyka KV**, **Gold R**. EAE in beta-2 microglobulin-deficient mice: axonal damage is not dependent on MHC-I restricted immune responses. *Neurobiol Dis*. 2005; **19**:218–228.DOI: 10.1016/j.nbd.2004.12.017.

183. **Jiang H**, **Braunstein NS**, **Yu B**, **Winchester R**, **Chess L**. CD8+ T cells control the TH phenotype of MBP-reactive CD4+ T cells in EAE mice. *Proc Natl Acad Sci USA*. 2001; **98**:6301–6306.DOI: 10.1073/pnas.101123098.

184. **Jiang H**, **Curran S**, **Ruiz-Vazquez E**, **Liang B**, **Winchester R**, **Chess L**. Regulatory CD8+ T cells fine-tune the myelin basic protein-reactive T cell receptor V beta repertoire during experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci*

USA. 2003; **100**:8378–8383.DOI: 10.1073/pnas.1432871100.

185. **Hu D**, **Ikizawa K**, **Lu L**, **Sanchirico ME**, **Shinohara ML**, **Cantor H**. Analysis of regulatory CD8 T cells in Qa-1-deficient mice. *Nat Immunol*. 2004; **5**:516–523.DOI: 10.1038/ni1063.

186. **Beeston T**, **Smith TRF**, **Maricic I**, **Tang X**, **Kumar V**. Involvement of IFN-γ and perforin, but not Fas/FasL interactions in regulatory T cell-mediated suppression of experimental autoimmune encephalomyelitis. *J Neuroimmunol*. 2010:1–7.DOI: 10.1016/j.jneuroim.2010.07.007.

187. **Najafian N**, **Chitnis T**, **Salama AD**, **Zhu B**, **Benou C**, **Yuan X**, **Clarkson MR**, *et al.* Regulatory functions of CD8+CD28- T cells in an autoimmune disease model. *J Clin Invest*. 2003; **112**:1037–1048.DOI: 10.1172/JCI17935.

188. **Lee Y-H**, **Ishida Y**, **Rifa'i M**, **Shi Z**, **Isobe K-I**, **Suzuki H**. Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. *J Immunol*. 2008; **180**:825–832.

189. **Antel JP**, **Bania MB**, **Reder A**, **Cashman N**. Activated suppressor cell dysfunction in progressive multiple sclerosis. *The Journal of* .... 1986.

190. **Antel J**, **Brown M**, **Nicholas MK**, **Blain M**, **Noronha A**, **Reder A**. Activated suppressor cell function in multiple sclerosis--clinical correlations. *J Neuroimmunol*. 1988; **17**:323–330.

191. **Chou YK**, **Henderikx P**, **Jones RE**, **Kotzin B**, **Hashim GA**, **Offner H**, **Vandenbark AA**. Human CD8+ T cell clone regulates autologous CD4+ myelin basic protein specific T cells. *Autoimmunity*. 1992; **14**:111–119.

192. **Correale J**, **Villa A**. Isolation and characterization of CD8+ regulatory T cells in multiple sclerosis. *J Neuroimmunol*. 2008; **195**:121–134.DOI: 10.1016/j.jneuroim.2007.12.004.

193. **Crucian B**, **Dunne P**, **Friedman H**, **Ragsdale R**, **Pross S**, **Widen R**. Alterations in levels of CD28-/CD8+ suppressor cell precursor and CD45RO+/CD4+ memory T lymphocytes in the peripheral blood of multiple sclerosis patients. *Clin. Diagn. Lab. Immunol.* 1995; **2**:249–252.

194. **Baughman EJ**, **Mendoza JP**, **Ortega SB**, **Ayers CL**, **Greenberg BM**, **Frohman EM**, **Karandikar NJ**. Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis. *J Autoimmun*. 2011; **36**:115–124.DOI: 10.1016/j.jaut.2010.12.003.

195. **Tsai S**, **Shameli A**, **Yamanouchi J**, **Clemente-Casares X**, **Wang J**, **Serra P**, **Yang Y**, *et al.* Reversal of Autoimmunity by Boosting Memory-like Autoregulatory T Cells. *Immunity*. 2010.DOI: 10.1016/j.immuni.2010.03.015.

196. **Biegler BW**, **Yan SX**, **Ortega SB**, **Tennakoon DK**, **Racke MK**, **Karandikar NJ**. Clonal composition of neuroantigen-specific CD8+ and CD4+ T-cells in multiple sclerosis. *J Neuroimmunol*. 2011:1–10.DOI: 10.1016/j.jneuroim.2011.02.001.

197. **Bettini M**, **Rosenthal K**, **Evavold BD**. Pathogenic MOG-reactive CD8+ T cells require MOG-reactive CD4+ T cells for sustained CNS inflammation during chronic EAE. *J Neuroimmunol*. 2009:1–9.DOI: 10.1016/j.jneuroim.2009.05.017.

198. **Dinesh RK**, **Skaggs BJ**, **Cava AL**, **Hahn BH**, **Singh RP**. CD8+ Tregs in lupus, autoimmunity, and beyond. *Autoimmun Rev*. 2010; **9**:560–568.DOI: 10.1016/j.autrev.2010.03.006.

199. **Niederkorn JY**. Emerging concepts in CD8(+) T regulatory cells. *Current opinion in immunology*. 2008; **20**:327–331.DOI: 10.1016/j.coi.2008.02.003.

200. **Faunce DE**, **Terajewicz A**, **Stein-Streilein J**. Cutting edge: in vitro-generated tolerogenic APC induce CD8+ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis. *J Immunol*. 2004; **172**:1991–1995.

201. **Saikali P**, **Antel JP**, **Pittet CL**, **Newcombe J**, **Arbour N**. Contribution of astrocyte-derived IL-15 to CD8 T cell effector functions in multiple sclerosis. *J Immunol*. 2010; **185**:5693–5703.DOI: 10.4049/jimmunol.1002188.

202. **Wang Z**, **Hong J**, **Sun W**, **Xu G**, **Li N**, **Chen X**, **Liu A**, *et al.* Role of IFN-gamma in induction of Foxp3 and conversion of CD4+ CD25- T cells to CD4+ Tregs. *J Clin Invest*. 2006; **116**:2434–2441.DOI: 10.1172/JCI25826.

203. **Chen M**, **Yan B**, **Kozoriz D**, **Weiner H**. Novel CD8(+) regulatory T cells suppress experimental autoimmune encephalomyelitis by TGF-beta- and IFN-gamma-dependent mechanisms. *Eur J Immunol*. 2009; **39**:–3435.DOI: 10.1002/eji.200939441.

204. **Ford ML**, **Evavold BD**. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur J Immunol*. 2005; **35**:76–85.DOI: 10.1002/eji.200425660.

205. **Sun D**, **Zhang Y**, **Wei B**, **Peiper SC**, **Shao H**, **Kaplan HJ**. Encephalitogenic activity of truncated myelin oligodendrocyte glycoprotein (MOG) peptides and their recognition by CD8+ MOG-specific T cells on oligomeric MHC class I molecules. *Int. Immunol.* 2003; **15**:261–268.

206. **Mendel Kerlero de Rosbo N**, **Ben Nun A**. Delineation of the minimal encephalitogenic epitope within the immunodominant region of myelin oligodendrocyte glycoprotein: diverse V beta gene usage by T cells recognizing the core epitope encephalitogenic for T cell receptor V beta b and T cell receptor V beta a H-2b mice. *Eur J Immunol*. 1996; **26**:2470–2479.DOI: 10.1002/eji.1830261030.

207. **Tajima M**, **Wakita D**, **Satoh T**, **Kitamura H**, **Nishimura T**. IL-17/IFN-γ double producing CD8+ T (Tc17/IFN-γ) cells: a novel cytotoxic T-cell subset converted from

Tc17 cells by IL-12. *Int. Immunol.* 2011; **23**:751–759.DOI: 10.1093/intimm/dxr086.

208. **Chowdhury FZ**, **Ramos HJ**, **Davis LS**, **Forman J**, **Farrar JD**. IL-12 selectively programs effector pathways that are stably expressed in human CD8+ effector memory T cells in vivo. *Blood*. 2011; **118**:3890–3900.DOI: 10.1182/blood-2011-05-357111.

209. **Gran B**, **Chu N**, **Zhang G-X**, **Yu S**, **Li Y**, **Chen X-H**, **Kamoun M**, *et al.* Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *J Neuroimmunol*. 2004; **156**:123–131.DOI: 10.1016/j.jneuroim.2004.07.019.

210. **Na S-Y**, **Cao Y**, **Toben C**, **Nitschke L**, **Stadelmann C**, **Gold R**, **Schimpl A**, *et al.* Naive CD8 T-cells initiate spontaneous autoimmunity to a sequestered model antigen of the central nervous system. *Brain*. 2008; **131**:2353–2365.DOI: 10.1093/brain/awn148.

211. **Saxena A**, **Bauer J**, **Scheikl T**, **Zappulla J**, **Audebert M**, **Desbois S**, **Waisman A**, *et al.* Cutting edge: Multiple sclerosis-like lesions induced by effector CD8 T cells recognizing a sequestered antigen on oligodendrocytes. *The Journal of Immunology*. 2008; **181**:1617–1621.

212. **Han B**, **Serra P**, **Yamanouchi J**, **Amrani A**, **Elliott JF**, **Dickie P**, **Dilorenzo TP**, *et al.* Developmental control of CD8 T cell-avidity maturation in autoimmune diabetes. *J Clin Invest*. 2005; **115**:1879–1887.DOI: 10.1172/JCI24219.

213. **Tsuchida T**, **Parker KC**, **Turner RV**, **McFarland HF**, **Coligan JE**, **Biddison WE**. Autoreactive CD8+ T-cell responses to human myelin protein-derived peptides. *Proc Natl Acad Sci USA*. 1994; **91**:10859–10863.

214. **Zang YCQ**, **Li S**, **Rivera VM**, **Hong J**, **Robinson RR**, **Breitbach WT**, **Killian J**, *et al.* Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. *J Immunol*. 2004; **172**:5120–5127.

215. **Niland B**, **Banki K**, **Biddison WE**, **Perl A**. CD8+ T cell-mediated HLA-A*0201-restricted cytotoxicity to transaldolase peptide 168-176 in patients with multiple sclerosis. *J Immunol*. 2005; **175**:8365–8378.

216. **Crawford MP**. High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood*. 2004; **103**:4222–4231.DOI: 10.1182/blood-2003-11-4025.

217. **Huizinga R**, **Hintzen RQ**, **Assink K**, **van Meurs M**, **Amor S**. T-cell responses to neurofilament light protein are part of the normal immune repertoire. *Int. Immunol.* 2009; **21**:433–441.DOI: 10.1093/intimm/dxp011.

218. **Rus H**, **Pardo CA**, **Hu L**, **Darrah E**, **Cudrici C**, **Niculescu T**, **Niculescu F**, *et al.* The voltage-gated potassium channel Kv1.3 is highly expressed on inflammatory infiltrates in multiple sclerosis brain. *Proc Natl Acad Sci USA*. 2005; **102**:11094–11099.DOI: 10.1073/pnas.0501770102.

219. **Berthelot L**, **Laplaud D-A**, **Pettré S**, **Ballet C**, **Michel L**, **Hillion S**, **Braudeau C**, *et al.* Blood CD8+ T cell responses against myelin determinants in multiple sclerosis and healthy individuals. *Eur J Immunol*. 2008; **38**:1889–1899.DOI: 10.1002/eji.200838023.

220. **Liu G-Z**, **Fang L-B**, **Hjelmström P**, **Gao X-G**. Increased CD8+ central memory T cells in patients with multiple sclerosis. *Mult. Scler.* 2007; **13**:149–155.DOI: 10.1177/1352458506069246.

221. **Battistini L**, **Piccio L**, **Rossi B**, **Bach S**, **Galgani S**, **Gasperini C**, **Ottoboni L**, *et al.* CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1. *Blood*. 2003; **101**:4775–4782.DOI: 10.1182/blood-2002-10-3309.

222. **Oldstone MB**, **Blount P**, **Southern PJ**, **Lampert PW**. Cytoimmunotherapy for persistent virus infection reveals a unique clearance pattern from the central nervous system. *Nature*. 1986; **321**:239–243.DOI: 10.1038/321239a0.

223. **Melzer N**, **Meuth S**, **Wiendl H**. CD8+ T cells and neuronal damage: direct and collateral mechanisms of cytotoxicity and impaired electrical excitability. *FASEB J.* 2009.DOI: 10.1096/fj.09-136200.

224. **Medana IM**, **Gallimore A**, **Oxenius A**, **Martinic MM**, **Wekerle H**, **Neumann H**. MHC class I-restricted killing of neurons by virus-specific CD8+ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. *Eur J Immunol*. 2000; **30**:3623–3633.DOI: 10.1002/1521-4141(200012)30:12<3623::AID-IMMU3623>3.0.CO;2-F.

225. **Wang T**, **Allie R**, **Conant K**, **Haughey N**, **Turchan-Chelowo J**, **Hahn K**, **Rosen A**, *et al.* Granzyme B mediates neurotoxicity through a G-protein-coupled receptor. *FASEB J.* 2006; **20**:1209–1211.DOI: 10.1096/fj.05-5022fje.

226. **Jürgens B**, **Hainz U**, **Fuchs D**, **Felzmann T**, **Heitger A**. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. *Blood*. 2009; **114**:3235–3243.DOI: 10.1182/blood-2008-12-195073.

227. **Wu Y**, **Zheng Z**, **Jiang Y**, **Chess L**, **Jiang H**. The specificity of T cell regulation that enables self-nonself discrimination in the periphery. *Proc Natl Acad Sci USA*. 2009; **106**:534–539.DOI: 10.1073/pnas.0811843106.

228. **Calzascia T**, **Masson F**, **Di Berardino-Besson W**, **Contassot E**, **Wilmotte R**, **Aurrand-Lions M**, **Rüegg C**, *et al.* Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. *Immunity*. 2005; **22**:175–184.DOI: 10.1016/j.immuni.2004.12.008.

229. **Calzascia T**, **Di Berardino-Besson W**, **Wilmotte R**, **Masson F**, **de Tribolet N**, **Dietrich P-Y**, **Walker PR**. Cutting edge: cross-presentation as a mechanism for efficient recruitment of tumor-specific CTL to the brain. *J Immunol*. 2003; **171**:2187–2191.

230. **Lu L**, **Kim H-J**, **Werneck MBF**, **Cantor H**. Regulation of CD8+ regulatory T cells: Interruption of the NKG2A-Qa-1 interaction allows robust suppressive activity and resolution of autoimmune disease. *Proc Natl Acad Sci USA*. 2008; **105**:19420–19425.DOI: 10.1073/pnas.0810383105.