NEUROANTIGEN-SPECIFIC CD8+ REGULATORY T-CELL FUNCTION IS DEFICIENT DURING ACUTE EXACERBATION OF MULTIPLE SCLEROSIS

APPROVED BY SUPERVISORY COMMITTEE

Nitin Karandikar MD PhD

Nancy Street PhD

Dwain Thiele MD

James Forman PhD DMD

Benjamin Greenberg MD MHSc

DEDICATION

I would like to dedicate this work in honor of my wife, daughter, and parents.

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by

ETHAN JAMES BAUGHMAN

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ETHAN JAMES BAUGHMAN, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2011

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

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS). MS is thought to be T-cell-mediated, with prior research predominantly focusing on CD4+ T-cells. There is a high prevalence of

CNS-specific CD8+ T-cell responses in MS patients and healthy subjects. However, the role of neuroantigen-specific CD8+ T-cells in MS is poorly understood, with the prevalent notion that these may represent pathogenic T-cells. We show here that healthy subjects and MS patients demonstrate similar magnitudes of CD8+ and CD4+ T-cell responses to various antigenic stimuli. Interestingly, CD8+ T-cells specific for CNS autoantigens, but not those specific for control foreign antigens, exhibit immune regulatory ability, suppressing proliferation of CD4+CD25- T-cells when stimulated by their cognate antigen. While CD8+ T-cell-mediated immune suppression is similar between healthy subjects and clinically quiescent treatment-naïve MS patients, it is significantly deficient during acute exacerbation of MS. Of note, the recovery of neuroantigen-specific CD8+ T-cell suppression correlates with disease recovery post-relapse. In healthy adult subjects, we observed that the CD62L- subset of CD8+ T cells harbored increased CNS- and Copaxone-specific suppressive ability, when compared to the CD62L+ subset and bulk CD8+ T cells, and that the CD28+ subset of CD8+ T cells harbored increased global suppressive ability, when compared to the CD28- subset. In contrast, we observed CD8+ T cells from neonates harbored increased global suppressive ability in the CD28- subset. The mechanism of neuroantigen-specific suppression by CD8+ T cells was dependent upon HLA class I, IFNγ, with possible partial involvement by NKG2D, PD-1, and IL10. These studies reveal a novel immune suppressor function for neuroantigen-specific CD8+ T-cells that is clinically relevant in the maintenance of peripheral tolerance and the intrinsic regulation of MS immune pathology

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LIST OF ABBREVIATIONS

ADEM (acute disseminated encephalomyelitis)
APC (antigen-presenting cells)
B cell (bone marrow-derived lymphocyte)
BBB (blood-brain barrier)
BDNF (bone-marrow derived neurotrophic factor)
CD4/8/25+ (cluster of differentiation 4, 8, 25, et cetera positive)
CNS (central nervous system)
CNTF (cytokine ciliary neurotrophic factor)
CSF (cerebrospinal fluid)
CTLA4 (cytotoxic T-lymphocyte antigen 4)
DMA (disease-modifying agents)
EAE (experimental autoimmune encephalomyelitis)
EBV (Epstein-Barr virus)
FDA (food and drug administration)
FOXP3 (forkhead box P3)
GA (copolymer-1/glatiramer acetate/Copaxone®)
H&E (hematoxylin and eosin)
HHV-6 (human herpes virus 6)
HLA (human leukocyte antigen)
IDO (indoleamine-pyrrole 2,3-dioxygenase)
IFNAR (interferon-α/β receptor)
IFNβ (type-one interferon beta)
IFNγ (interferon gamma)
IFNγR (IFNγ receptor)
Ig (immunoglobulins)
IL10/15 (interleukin 10, 15, et cetera)
IL27R (IL27 receptor)
IL2Rα (interleukin 2 receptor alpha)
iNOS (inducible nitric oxide synthase)
IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome)
IRF1 (interferon regulatory factor 1)
IVIG (intravenous Ig)
MAG (myelin-associated glycoprotein)
MBP (myelin basic protein)
MHC (major histocompatibility complex)
MMP (matrix metalloproteases)
MOG (myelin oligodendrocyte glycoprotein)
MRI (magnetic resonance imaging)
MS (multiple sclerosis)
MSRV (endogenous multiple sclerosis-associated retrovirus)
MX (mitoxantrone)
Myd88 (myeloid differentiation primary response gene 88)
PBMC (peripheral blood mononuclear cells)

PD-1 (programmed death 1)
PLP (proteolipid protein)
PML (progressive multifocal leukoencephalopthy)
PPD (Purified protein derivative of tuberculin)
PPMS (primary progressive MS)
RAG (recombinase-activating genes)
RORγt (retinoic acid receptor-related orphan receptor gamma isoform t)
RRMS (relapsing-remitting MS)
SNP (single nucleotide polymorphism)
SPMS (secondary or chronic progressive MS)
STAT1(signal transducers and activators of transcription protein 1)
steroids (corticosteroids)
T cell (thymus-derived lymphocyte)
T1DM (type one diabetes mellitus)
TBET (Th1-specific T box transcription factor)
TCR (T cell receptor)
Th1 (CD4+ helper type 1)
Th17 (CD4+ helper type 17)
Th2 (CD4+ helper type 2)
TNFRII (tumor necrosis factor receptor 2)
Treg (suppressor/regulatory T cell)
US (United States)
VCAM (vascular adhesion molecule)
VLA4 (very late antigen-4)

CHAPTER 1: INTRODUCTION

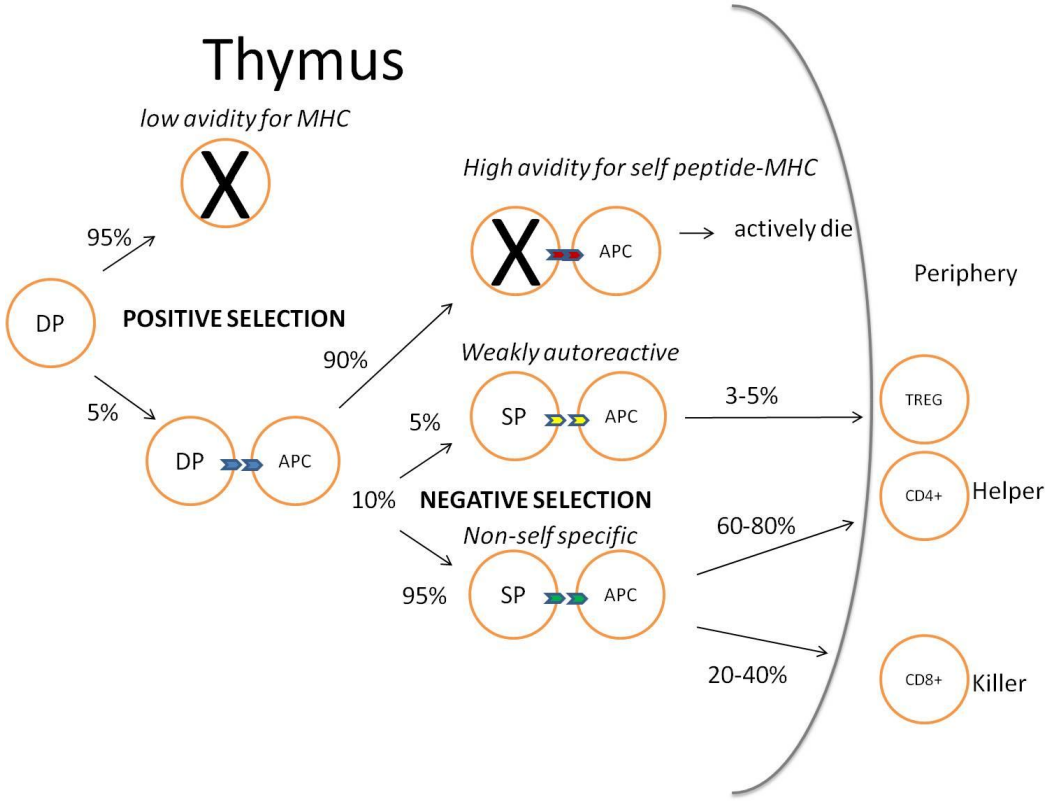
TOLERANCE

Conceptually, two major regulatory mechanisms exist to guard against immune-related pathology. Central tolerance and peripheral tolerance mechanisms maintain the distinction between self and non-self.

CENTRAL TOLERANCE

Central tolerance mechanisms occur during thymic selection [5]. In the thymus, thymocytes, expressing a range of randomly-generated TCRs, undergo positive and negative selection (Figure 1). The thymus is most active during fetal life and atrophies with increasing age. Based on the specificity of the TCR, thymocytes are selected which bind weakly to HLA and do not strongly react with self. Thymocytes that do not bind HLA die of neglect, and thymocytes that strongly react with self are clonally deleted in an active manner. Clonal deletion in the thymus centrally promotes tolerance to self before educated thymocytes immigrate to the periphery and also promotes a repertoire of TCRs directed toward pathogens. The number of self-reactive T cells in the periphery is diminished compared to the pre-thymic selection pool.

Figure 1: Thymic selection diminishes the number of self-reactive T cells in the periphery



PERIPHERAL TOLERANCE

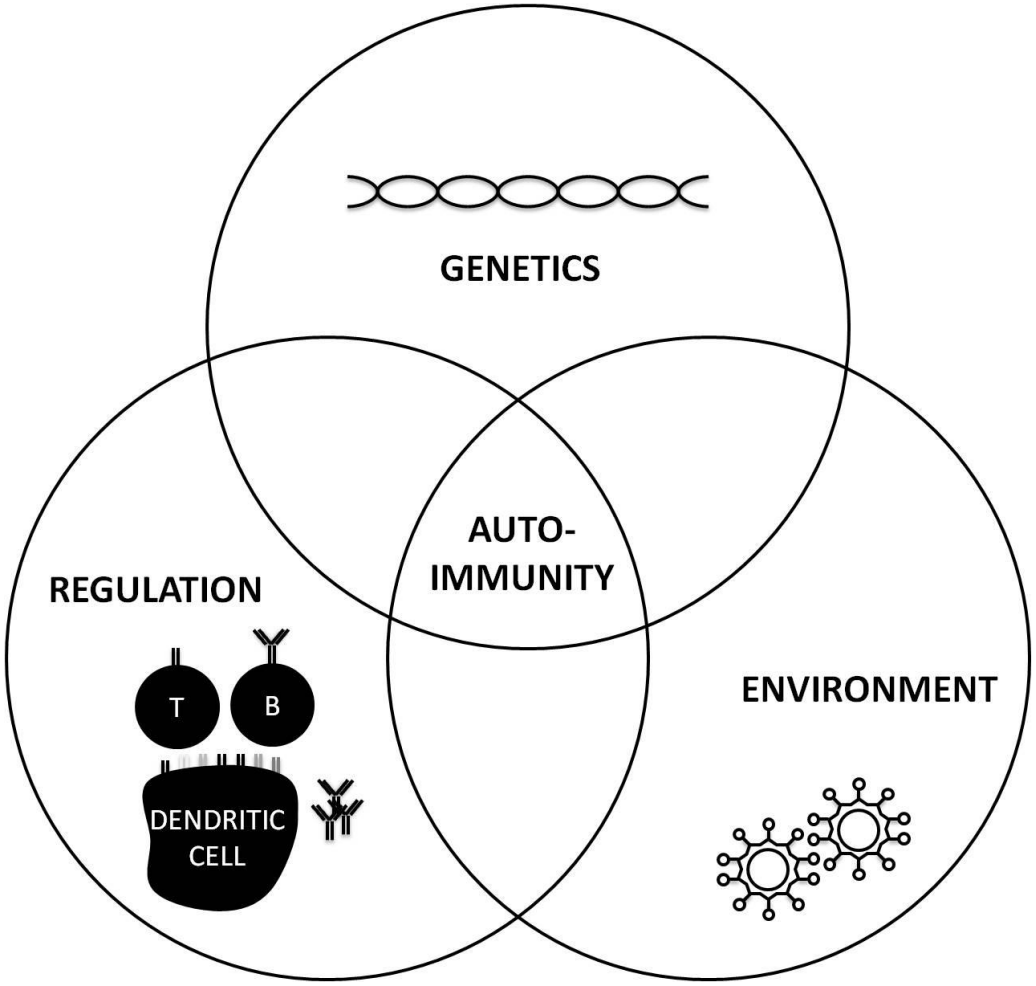
Mechanisms of peripheral tolerance deal with autoreactive T cells in the periphery [6]. Self-reactive T cells that survive thymic selection are detectable in the periphery and have lower TCR avidity for HLA compared to foreign antigen-specific counterparts. Limited numbers of self-reactive T cells in the periphery may serve a physiological function or cause autoimmunity if reactivated with inappropriate signals. Regulatory T cells may serve to counter balance the pathogenic potential of autoreactive T cells. Untoward immune responses are thought to result in absence of regulatory T cells. Through a puzzling mechanism, regulatory T cells may express low-avidity autoreactive TCRs. Autoimmunity may result from the dysfunctional conversion of autoreactive T cells away from a regulatory phenotype. Autoreactive, myelin-specific T cell responses are prevalent in healthy person and multiple sclerosis (MS) patients. Antigen exposure in the periphery and the lack of tolerogenic context of the encounter presumably are the critical immunological events in persons diagnosed with MS.

AUTOIMMUNITY

Approximately 1 in 31 Americans lives with an autoimmune disease [7]. The immune responses against self are often mediated by antigen-specific T cells, but controlled peripherally by a proper balance of Tregs (Figure 2). The experimental removal of the Treg subset can result in a variety of organ-specific autoimmune diseases [8]. Lethal autoimmune syndromes are characteristic phenotypes of several knockout mice: IL-Rα (CD25), IL-Rβ (CD122) [9],

forkhead transcription factor FOXP3 [10], and TGFβ1 [11]. The prevention of immune attack against autologous cells by adoptive transfer of Treg is the subject of numerous studies. Furthermore, adoptive transfer of CD25-depleted T cells into thymectomized mice results in general immune dysregulation and autoimmunity [12-14]. Defects in regulatory T cell function have been demonstrated in a wide variety of human disease [15]. In nearly all such cases, it is unclear whether an intrinsic defect lies in the ability of Tregs to suppress or in responder T cell hyporesponsiveness to Treg [16]. However, *ex vivo* generated and *in vivo* induced Treg can mediate therapy for T1DM [17-21]. A deficiency in the transcription factor FOXP3 is thought to result in defective regulatory T cell development and causes IPEX syndrome in humans [22]. IPEX neonates display T1DM, enteropathy, and endocrinopathy. The mouse form of disease, the scurvy mouse, exhibits a shortened lifespan, multiorgan inflammation, eosinophilia, cytokine storm, and hyperimmunoglobinemia. FOXP3 activity is regulated by TCR, CD3, CD28, CTLA4, TGFβ, PGE2, and others. FOXP3 is a global immune regulator, having modulatory effects on IL4, IL2, IL5, IL2RA, IgG2a, IgM, IgG1, and CD40LG [23].

Figure 2: Immune regulatory/tolerance mechanisms may fail in certain, genetically-susceptible individuals, when exposed to environmental risk factors



SUPPRESSOR T CELLS

The first report of a T cell population capable of suppressing other T cells was made some forty years ago while studying interactions between thymocytes in lethally irradiated mice lacking B cells [24]. The suppression assay measured total *in vivo* proliferative response to antigenic stimulation with sheep red blood cells (SRBC) by individual populations of T cells and combinations thereof. The proliferative response of primed (responder) cells was greater than the combined total proliferative response of primed (responder) cells plus unprimed (suppressor) T cells, suggesting that the unprimed (suppressor) T cells were capable of suppressing SRBC antigen-specific T cell responses.

A similar observation was also made using adoptive transfer of allogeneic (responder) cells into lethally irradiated hosts with and without autologous (suppressor) cells. Namely, the proliferative response of allogeneic (responder) cells was greater than the combined total proliferative response of allogeneic (responder) cells plus autologous (suppressor) host cells, suggesting that host cells could suppress graft-versus-host disease (GVHD)-causing alloantigen-specific T cell responses. These two important instances of antagonistic interactions between T cell populations highlighted the importance of suppressor T cells (Ts) for the first time. Competition for space in the spleen was thought to be unlikely as the authors had determined by other adoptive transfer experiments that the spleen's capacity was significantly higher than the cell numbers used in the suppression experiments. The authors hypothesized some cellular interaction was responsible for the suppression.

If the addition of unprimed T cell populations resulted in reduced total primed plus unprimed combined T cell proliferation, scientists thought that the removal of the source of unprimed T cells may increase T cell proliferation. The assertion turned out to be correct. Thymectomized animals were found to spontaneously develop oophoritsis, thyroiditis and auto-antibodies to thyroglobulin [25, 26]. Mice thymectomized between the second and fifth day of life have auto-immune reactions to multiple organs. CD4+ T cells infiltrate the affected organs, and auto-antibodies are generated [27]. CD90+CD5+ thymocytes were capable of adoptively transferring post-thymectomy autoimmune oophoritis [28]. Post-thymectomy autoimmunity was thought to develop as a result of depleted newly generated T lymphocytes. Normal lymphoid cells from euthymic mice transferred into athymic mice could prevent autoimmunity [29]. It remained unclear if other T populations were capable of suppressing T cells.

Other experiments showed that both CD4+ and CD8+ T cells were capable of suppression through unknown mechanisms. It was known that small amounts of antigen given orally inhibited immune responses upon re-exposure. Myelin basic protein (MBP), given orally, suppressed experimental autoimmune encephalomyelitis (EAE) through the transforming growth factor beta (TGFβ)-producing CD8+ T cells [30]. Hence, soluble cytokines were thought to mediate suppression. The surface phenotype of suppressor T cells was even more enigmatic. Thought to bear the CD8 marker, similar to cytotoxic T lymphocytes (CTL), the expected mechanism of suppression was thought to be cell-mediated

cytolytic ability. A few distinctions were thought to identify CD8+ suppressors from killers. CTLs were CD45RA+IL10(-) and lacked the ability to suppress Th1 and Th2 cells [31]. Suppressor CD8+ cells expressed IL10 and IFNγ and suppressed the proliferation of CD4+ cells.

I-J PARADOX

Perhaps the most contentious issue in the history of the suppressor T cell relates to the I-J paradox, a discrepancy between serological mapping of a presumed protein(s) called I-J and molecular genetic mapping [32]. Limited molecular technology limited the study of T suppressors in the 1970s and 1980s [33]. Early genetic linkage studies and Mendelian segregation experiments estimated the number and layout of the MHC loci on chromosome 17 [34, 35]. Suppressor T cells were thought to express an I-J molecule hypothetically located in the I-C region. In his shared 1980 Noble prize lecture in physiology or medicine “for their discoveries concerning genetically determined structures on the cell surface that regulate immunological reactions", Baruj Benacerraf described the presumed role of I-J in the suppressor T cell circuits. When the I region between the K and D loci was finally cloned and sequenced, no I-J gene could be identified in the local area [36]. Some thought that the I-J determinant was linked to MHC but the genetically mapped location was incorrect, possibly due to high frequency of multiple crossovers. The I-J paradox was never resolved and caused many immunologists to be skeptical of immune-regulatory phenomena and theory, including suppressor T cells. The old notions of the suppressor T network fell out of favor and became subject to necessary and

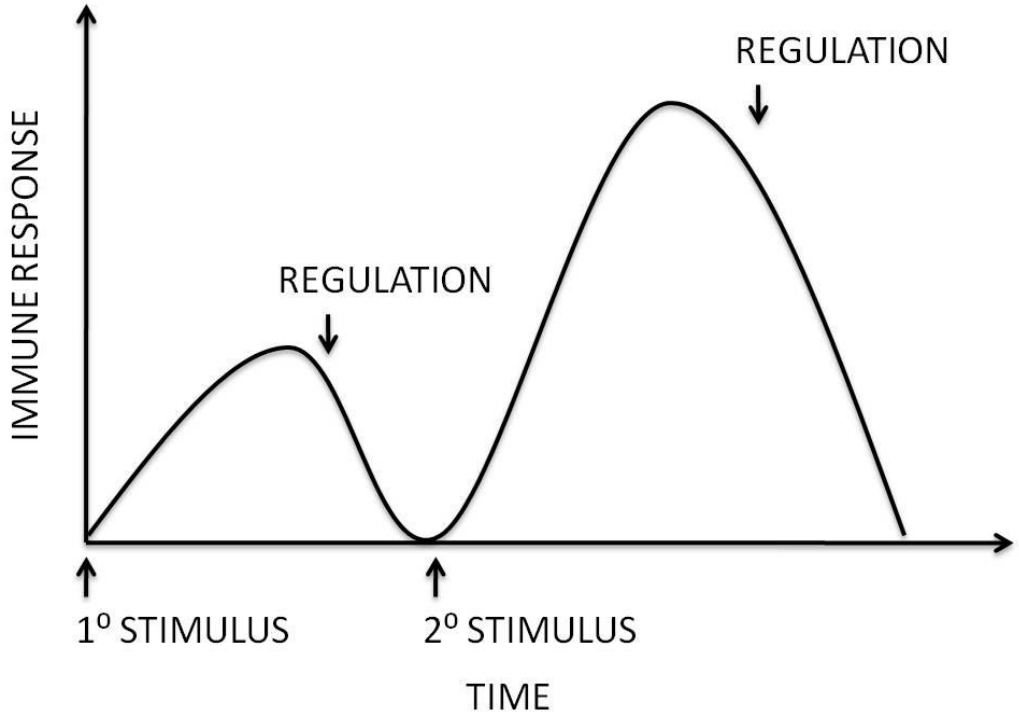
unnecessary criticism. For a time, discussion of suppression became synonymous with over-interpretation of scanty data. While much of the non-contentious work of these investigators has been forgotten or under-appreciated, past and recent evidence strongly supports the role for T cell mediated suppression in maintenance of peripheral tolerance.

REGULATORY T CELLS

Circulating throughout the blood and lymph are CD8+ and CD4+ T cells with suppressor potential (Figure 3). Suppressor cells (Ts) and regulatory T cells (T_{REG}(s)) are used synonymously here. The better-accepted and -understood variety of human T_{REG}s is thought to develop "naturally" from thymocyte selective processes. These natural Tregs (nT_{REG}s) are defined by their constitutive expression of the transcription factor FOXP3 and also express high levels the alpha chain of the IL2 receptor (CD25). Approximately 5-10% of CD4+ T cells that emigrate from the thymus into the blood stream exhibit the FOXP3+CD25+ regulatory phenotype. While much is known about CD4+ T_{REG}s, CD8+ T_{REG}s remain understudied. Tolerance to self antigen by Treg is crucial to prevent autoimmune tissue damage. In the context of hematopoetic or solid tissue transplant, graft rejection may be slowed or prevented through the use of donor antigen-specific T_{REG}s. T_{REG}s control amplified immune responses to innocuous foreign antigens and prevent allergic/atopic pathology. While most studies in the context of transplant implicate the CD28(-) subset of CD8+ T cells as having the most relevant regulatory potential [37-43], other studies point to other regulatory CD8+ T cells restricted to HLA-E [44], γδ-TCR CD8+ T cells [45], or CD8+

subsets expressing CD25 [46], CD122 [47, 48], CD103 [49] [50], GITR [51, 52], PD-1 [53] or FOXP3 [54] [55] [56]. The most well understood regulatory CD8+ T cells kill autoreactive, pathogenic CD4+ T cells that express HSP60 peptide-HLA-E complexes [44]. Naturally-occurring CD8+CD122+ T cells produce IL-10 and can only suppress autologous MHC-I-expressing T cell-IFNγ production in a Qa-1-independent manner. Alloantigen-specific CD8+CD28(-) T_{REG} suppress through upregulating ILT3 and ILT4 [54]. Human T_{REG} suppressive function can be abrogated with the use of TLR8 agonists [45]. Antigen-specific regulatory CD8+ T cells can be generated *ex vivo* [46, 57] and are capable of organ-specific immunosuppression. The phenomenon of T cells mediating suppression of immune responses has been observed for decades, while the mechanism(s) of such action has remained a topic of intense study and controversy.

Figure 3: T_{REG}s are essential for controlling immune responses to self and contracting the immune response after clearance of foreign antigen



ANTIGEN-SPECIFIC CD8+ TREGS

Antigen-specific CD4+ and CD8+ T_{REG}s are essential for controlling immune responses to self and contracting the immune response after clearance of foreign antigen [58]. Mice deficient in the CD8 molecule suffered clinically more severe EAE compared to wild-type mice. As a potential mechanism of peripheral tolerance, CD8+ T cells' TCRs specifically recognize a unique set of self-peptides presented by the MHC class Ib molecule Qa-1, HLA-E in humans, differentially expressed on T cells as a function of the affinity/avidity of T cell activation [59]. Challenging notions about the definition of an effector cytokine, antigen-specific CD8+ T_{REG}s require IFNγ for induction or suppressive function during viral infection, cancer, or MS therapy [60]. As a means of maintaining the immunologic privilege of the eye, anterior chamber-injected antigen induces CD8+ T_{REG}s, which depend on CD94/NKG2A-Qa-1 interactions to reduce ocular DTH responses [61]. Ocular iris pigment epithelia constitutively express CD80/86 and membrane-bound active TGFβ to induce CD8+B7+ Tregs [62]. Injection of antigen into the anterior chamber of an eye induces splenic CD8+ T cells whose suppressor function requires IFNγ receptor stimulation [63]. Antigen injection into the ocular anterior chamber induces CD8+CD103+ T_{REG}s [64]. The context of antigen presentation has important consequences toward the generations of T_{REG}s. Vasoactive intestinal peptide (VIP)-treated monocytes differentiate into toleragenic DC, which are capable of inducing IL-10-producing CD8+CD28-CTLA4+ T cells capable of suppressing antigen-specific Th1 cells [65].

In the context of autoimmune disease, T_{REG}s have been the subject of intense study and controversy. Present at a higher frequency in ankylosing spondylitis, autoreactive CD8+CD25+ FOXP3+CTLA-4+ T cells produce IL-4, IL-5, IL-13 and TGFβ and suppress CD4+ T cell proliferation and IFNγ production in a CTLA-4- and HLA-I-dependent manner [66]. During MS exacerbations, CD8+ T cell clones express significantly more killer-inhibitory receptor CD94/NKG2A, which IL-15 and IFNγ further increase, and exhibit reduced cytolytic ability towards MBP- and MOG-specific CD4+ T cells [67]. Human CD8+ T cells, derived from CD45RA+ CD27- cells, suppress glutamic acid decarboxylase 65-specific CD4+ T cells in a contact- and IL-10-dependent manner [68]. The potential for therapeutic advantage from these and other studies will be discussed in further detail.

GLOBALLY SUPPRESSIVE, NON-SPECIFIC CD8+ TREGS

Polyclonal CD4+ and CD8+ Tregs have suppressive ability in several models of autoimmunity, transplant, and GvHD [69]. Antigen non-specific Type 2 CD8+ T suppressors, which inhibit T cell proliferation and are cytolytic by soluble factors, are deficient in multiple sclerosis, systemic lupus erythematosus, or systemic sclerosis as well as HIV or chronic HCV infected patients [70]. In $MHCII^{-/-}$ mice, CD8+CD25+ T cells are GITR+CTLA+FOXP3+IL-10+, inhibit anti-CD3 stimulated CD25- T cells, and proliferated and produced IFNγ as well [71]. The intestinal parasite, Heligmosomoides polygyrus, induced CD8+ T cells in the intestinal lamina propria having suppressive ability toward splenocyte anti-CD3 stimulation [72]. Even in the steady-state, intraepithelial CD8+CD28-

CD103+ T cells appear to possess suppressive function, and their absence may predispose to inflammatory bowel disease [73]. The phenomenon of non-specific T_{REG} suppression suggests that entirely different mechanisms of action differentiate many distinct subsets of T_{REG}s.

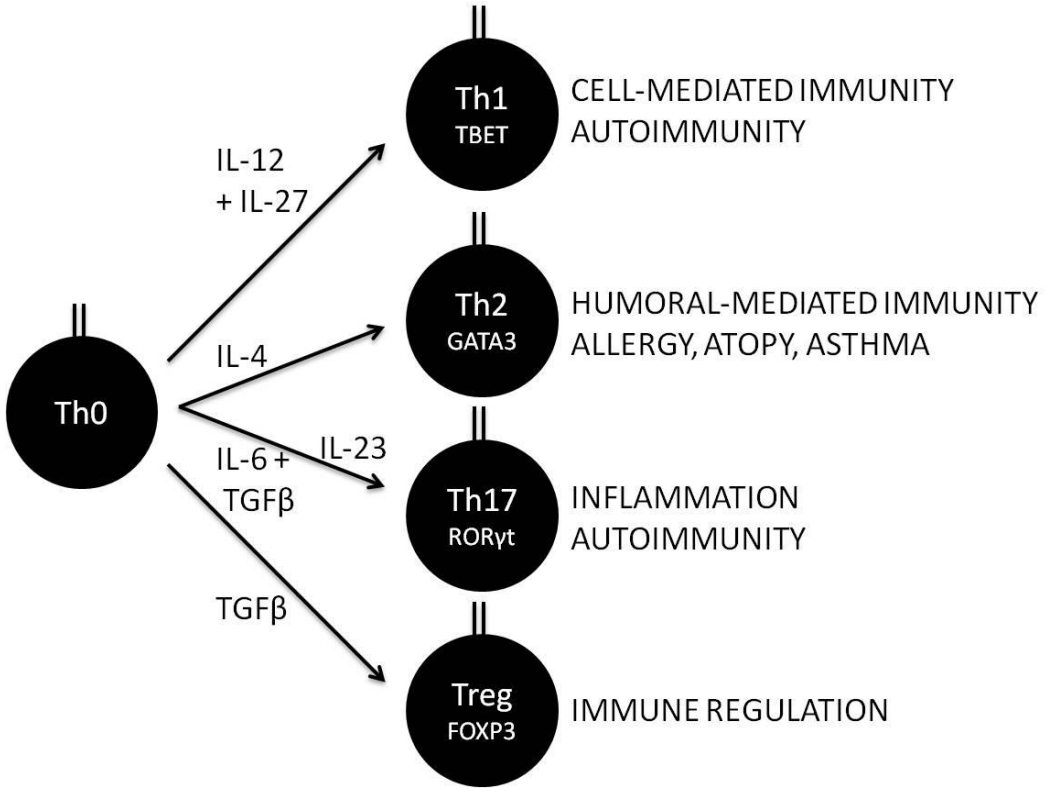
T CELL ACTIVATION AND DIFFERENTIATION

T cell activation and its context are generally thought to involve three external signals. The first signal is TCR stimulation. The second is co-stimulation, and the third signals are cytokines. T cell activation and differentiation is thought to be regulated by several families of signaling molecules and transcription factors. Upon TCR activation, CD3 and Lck signal to ZAP70 [74]. ZAP70 is phosphorylated, which signals to phospholipase Cγ1 to generate inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 causes calcium release intracellularly, which activates the nuclear factor of activated T cell (NFAT) pathway. DAG leads to the phosphorylation of protein kinase Cθ, which activates the TCR-induced IκB kinase (IKK) and NF-κB pathway. Stimulation of TCR or CD28 alone is insufficient to activate naïve T cells [75]. Co-stimulatory signals from the CD28 family are also needed to activate PDK1, which through Akt completely activates NF-κB. The third exterior signal, cytokines, is transmitted through cytokine receptors and the JAK/STAT pathway. While CD4+ T cell differentiation has been studied extensively (Figure 4), CD8+ T cell differentiation is poorly understood and may be incorrectly presumed to mirror CD4+ T cell differentiation.

Naïve CD4+ T helper cells were once thought to differentiate into two subsets, Th1 and Th2, each with a distinct function and cytokine production profile [76]. IL12 signals through STAT4 to promote Th1 cell differentiation [77]. T-bet expression activates the Th1 genetic program that commits the T cell to producing IL-2 and IFNγ. Th1 cells are thought to have an important role in cell-mediated immunity. Th1 development attenuates the Th2 program and vice versa [78]. IL4 signals through STAT6 to promote Th2 cell differentiation. GATA3 expression activates the Th2 genetic program that commits the T cell to produce IL4, IL5, and IL13 [79]. Th2 cells are thought to have an important role in humoral-mediated immunity.

More recently, important roles for FOXP3 and retinoid-related orphan receptor (ROR) γ in effector T cell differentiation have been recognized [80]. RORγ expression activates the Th17 genetic program that commits the T cell to producing IL17 [81]. Th17 cells are thought to have an important pro-inflammatory role in autoimmunity and gut homeostasis [82] [83]. FOXP3 expression is thought to activate the Treg genetic program that commits the T cell to produce TGFβ [84]. Tregs have an important role in down-regulating Th1, Th2, and Th17 cells [85] [86]. IL6 is thought promote either the Th17 or Treg lineage when combined with other cytokines [87]. Combined with IL6, TGFβ or IL23 promote the generation of Treg or Th17 cells, respectively [88] [89]. While many of the master regulatory transcription factors of T cells have been worked out in CD4+ T cells, differentiation and lineage commitment of CD8+ T cell remains less clear.

Figure 4: CD4+ T cell differentiation



TRANSCRIPTIONA.L REGULATION OF TREG

The roles of master regulator transcription factors that globally regulate T cell gene expression are still under investigation. FOXP3 was recently recognized as a master regulator of T cell gene expression. FOXP3+CD25+ T cells down-regulate or suppress untoward immune responses by other non-FOXP3+ T cell populations, playing a role in autoimmunity, transplant, allergy, tumors, and infections. CD4+CD25+FOXP+ cells are the most well accepted phenotype of CD4+ Tregs. CD8+CD25+FOXP3+ T cells can be generated by continuous antigen stimulation, which induces upregulation of costimulatory and cytotoxic molecules, enabling inhibition of CD4+ and CD8+ T cell proliferation and cytokine production, independent of CTLA4, CD80/86, prostaglandin E(2), IL-10 and TGF-beta, yet in a contact-dependent manner [46]. PPD-primed CD8+CD25+FOXP3+ Tregs suppressed live bacillus Calmette Guérin-specific responses and produced CCL4 [90]. CD8 + CD28- T cells, which express FOXP3, are expanded in blood of lung cancer patients [55]. The FOXP3 aspect of peripheral tolerance is important for contracting the immune response and lessening the activity of potentially harmful autoreactive T cells. While FOXP3 is thought to be the most specific marker known for human regulatory T cells (Tregs), *in vitro* suppressive activity remains the benchmark for any lineage of regulatory T cells. Natural Tregs are derived from the thymus. Adaptive Tregs are induced in the periphery; FOXP3 expression is induced by virtually any stimulus that activates T cells. Both CD8+FOXP3(-) and CD4+FOXP3(-) human T cells transiently express FOXP3 and CD25 upon activation. While T cells

attain the FOXP3+CD25+ status, the cells inhibit *in vitro* proliferation of autologous T cells, suppressing IFNγ production by responders cells by expressing TNFα and IL10 [91]. Activation-induced FOXP3 expression is down-regulated along with suppressive ability in all but a fraction of circulating T cells; the FOXP3+ remainder are possibly the source of *de novo* peripherally induced Tregs. Activated FOXP3 expressing T cells may regulate excessive immune activation *in vivo.* FOXP3+ T cells may represent a plastic and dynamic population, potentially informed by the pool of activated T cells. It remains unclear what effector function remains armed in FOXP3 + T cells and how much clinical potential Tregs possess.

SUPPRESSIVE PROPERTY OF ACTIVATED FOXP3+ T CELLS

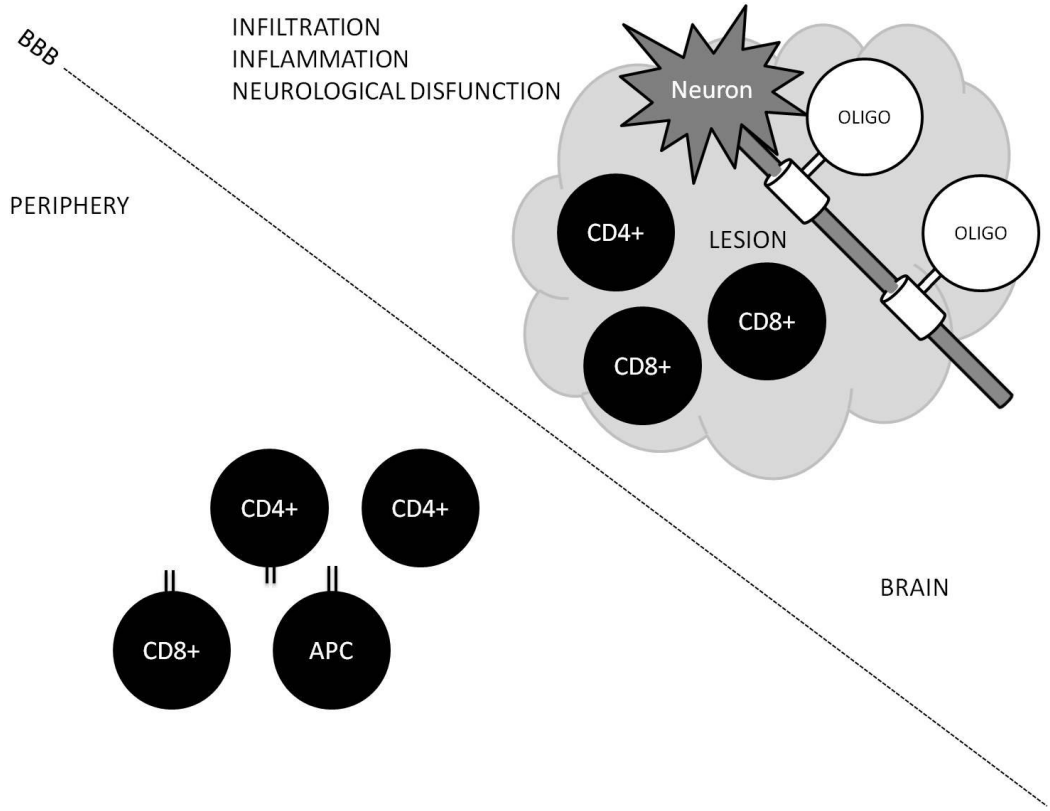
Whether activated human T-cells that express FOXP3 have regulatory properties is a subject of intense study and controversy. While a few groups have previously shown suppressive activity in activated T-cells [91], many other studies have concluded that activated/induced $FOXP3^+$ T-cells do not acquire suppressive properties at all or acquire it only if there is sustained FOXP3 expression. There are several possible reasons for such disparate results. It may be the use of an insensitive suppression assay, using bulk cultures of activated cells measured by total tritiated thymidine uptake of all cells in the culture. The insensitivity of the assay may be explained by impure suppressors cells contaminated by non-anergic responders cells. Suppressive property of the regulatory population may be masked by the proliferation of the other non-anergic cells in the mix. Suppressor cells may also not be completely anergic. To cope

with these and other challenges, we devised a novel flow cytometry-based suppression assay (described in the methods and results), whereby the putative suppressor cells were excluded from the analysis of proliferation, avoiding the pitfalls of the thymidine-based assay through taking advantage of proliferation dyes and long-term cellular tracking dyes.

IMMUNE-MEDIATED DYMELINATION

The preponderant belief is that multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) are mediated through central nervous system (CNS)-specific CD4+ helper type 1 (Th1) and type 17 (Th17) responses and regulated by CD4+ regulatory T-cells [92]. Considerable evidence, however, points to an important role for CD8+ T-cells in the pathogenesis and/or regulation [93] of MS and EAE [94, 95] [96] [97] [98] [99] [100] [101] [44] [17, 102, 103] [47, 104, 105] [106]. While granzyme B-expressing CD8+ T cells predominate in demyelinated areas of acute MS lesions, CD8+CD122+ T cells also regulate the recovery phase of EAE, as shown through adoptive transfer and anti-CD122 mAb experiments [47]. While it is thought that these cells represent a key pathogenic element of MS lesions, neither the antigenic specificity of CD8+ T cells nor their role has been elucidated.

Figure 5: T cells become activated in the periphery and cross the blood-brain barrier

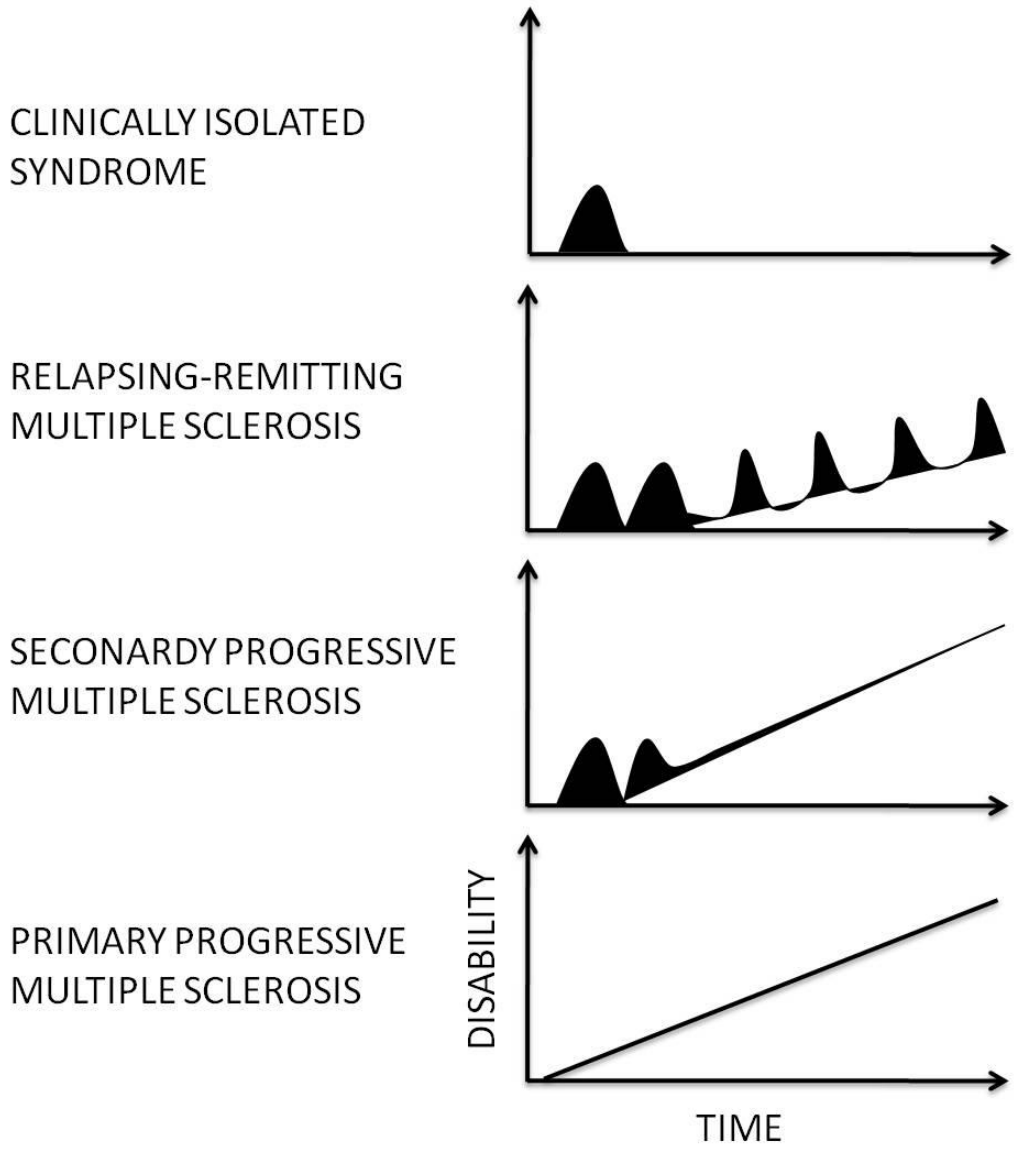


While CD8+ T cells are outnumbered by CD4+ T cells in the periphery, CD8+ T cells are predominant in MS lesions of the CNS

MULTIPLE SCLEROSIS

MS is characterized by inflammation, demyelination, and degeneration within the CNS [107], and is the most common debilitating neurological disease of young people [108]. Although, the exact etiology remains unknown, autoimmunity and immune dysfunction is thought to play a role. The result is dysfunctional myelin, the lipid-rich insulation of neural tissue. The myelin of the brain and spinal cord are affected, leading to a variety of neurological defects (Figure 6). Young adults, predominantly Caucasian women, between their third and fifth decades of life are most commonly affected. Approximately one in every thousand persons in the United States (US) is diagnosed with MS. MS is associated with significant impairment caused by neurologic defects in motor, sensory, cognitive, and autonomic tracts. The clinical presentation corresponds to temporally and spatially separated lesions of the CNS.

Figure 6: Demyelination syndrome subtypes are classified by clinical progression of neurological disability



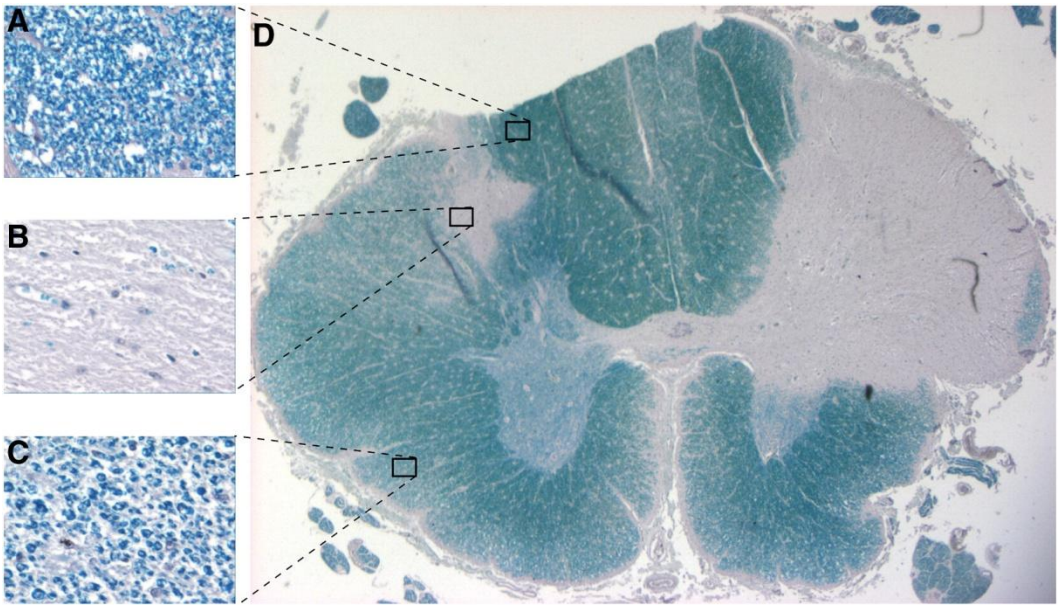
Symptoms may include, but are not limited to fatigue, optic neuritis, weakness, pain, cognitive impairment, depression, spasms, ataxia, paraesthesias, and incontinence.

BASIS OF PATHOLOGY

Lesions or plaques of the CNS are a critical functional requirement for the pathologic diagnosis of MS [109]. White matter, a specialized neurological tissue especially rich in myelin, is primarily affected. Post-mortem and imaging studies reveal infiltration by mononuclear cells into mostly the white matter, but also grey matter of the CNS [110]. Luxol fast blue, histological sections from tissue taken from MS patients (Figure 7), reveal decreased myelination, and hematoxylin and eosin (H&E) stains demonstrate increased cellularity of lesions as compared to healthy subjects [111]. Plaques exhibit sharply demarcated margins of inflammation and demyelination [112]. Active demyelination plaques contain lymphocytes and histiocytes concentrated around blood vessels. Grossly, demyelination is multifocal, located throughout the CNS, but concentrated perivascularly and periventricularly. The size and shape of the plaque(s) can be variable and are asymmetrically distributed. Lymphocytes and macrophages must first cross the open, damaged blood-brain barrier (BBB) and attack myelin and myelin-associated antigens. Glial cells, such as the oligodendrocytes and Schwann cells, are destroyed, thereby impeding conduction of neuronal impulses. Even if spared of transection, axons may still function, albeit poorly [113]. Episodic attacks of demyelination are separated by periods of inactivity or remission. Once the level of inflammatory cells has decreased, astrocytes attempt to repair the active lesion and a gliotic process produces scar tissue. Oligodendrocyte progenitor cells are active in re-myelination. Proliferation by astrocytes, also competent APCs, and further lymphocyte infiltration may lead to

further immune cell-reactivation in the lesion [114]. Usually widespread, multiple lesions often develop distributed over space and time. The optic tracts, cerebrum, brain stem, cerebellum, and spinal cord are frequently affected. End stage effects are atrophy and degeneration of the brain and spinal cord.

Figure 7: Axial luxol-fast blue staining of cervical spinal cord



(D) exhibits variable degrees of demyelination in an MS subject: (A) normal myelination, (B) severe demyelination, (C) mild demyelination [115]

Four distinct subtypes of MS histopathology have been observed [116]. Type one lesions contain mostly T cells with early preservation of oligodendrocytes. Type two lesions contain T cells, IgG, and complement components with intact oligodendrocytes. Unlike type one and two type lesions, type three lesions are not perivascular and exhibit marked loss of myelin and oligodendrocytes. Type four lesions are perivascular with marked loss of oligodendrocytes. Any firm relationship among the subtypes may be tentative. Clinical subtypes lack strong correlation to pathological subtypes. Individual patient lesions are heterogeneously composed of various subtypes. The dynamics and evolution among subtypes of the MS lesion are still unclear.

CLINICAL PRESENTATION

MS affects mostly young, Caucasian, adult women [117]. Based on the location and frequency of lesions, neurological dysfunction varies widely among MS patients. The most common chief medical complaints are spontaneous visual impairment in one eye, fatigue, heat sensitivity, spasticity, and urinary sphincter dysfunction. However, nearly all MS patients exhibit one of two major forms of MS (Figure 6). The relapsing-remitting form (RRMS) is most common, making up approximately eighty-five to ninety percent. RRMS is characterized by periods of remission that are punctuated by acute exacerbations, which remit with limited accumulation of disability. The acute exacerbation symptoms can be caused by new lesions in new areas of the CNS or reoccurring inflammation in pre-existing lesions. Over two to four decades, the majority of RRMS patients convert to a malignant disease course, known as secondary or chronic progressive

MS (SPMS) [118]. SPMS patients suffer from a non-remitting accumulation of neurological defects. While RRMS is characterized by frequent new lesions, SPMS, in contrast, exhibits chronic lesions with disseminating degeneration along neural tracts away from the lesion with increasingly widespread atrophy and gliosis. Cumulative axonal degeneration is thought to cause clinical decline in SPMS. Approximately one in three is wheel-chair bound, one in two requires medical assistive devices, and two in three are unable to work due to disability. Men and African Americans are at higher risk of progressive disease. The processes that lead away from inflammatory processes to develop degeneration of SPMS are poorly understood.

Other patients present at the onset with accumulating disability with unremitting new lesions. Known as primary progressive MS (PPMS), this group of patients is so different in clinical nature from RRMS, it has led to speculation that it may be better considered a distinct disorder [119]. Relative to RRMS, progressive MS is understudied and poorly understood.

Heat or infection may often slow or block transmission of neuronal impulse through a pre-existing, dormant lesion. Clinically, this manifests as a worsening of previously observed symptoms, which can be difficult to differentiate from a new lesion. The diagnostic laboratory provides the physician with some tools to address these concerns.

LABORATORY DIAGNOSTICS

A major class of immunoglobulins (Ig) and a major component of myelin, myelin basic protein (MBP), are found at increased levels in the cerebrospinal

fluid (CSF) during acute exacerbations of MS [120]. CSF oligoclonal bands of Ig are useful for detecting the immune processes that underlie MS. Magnetic resonance imaging (MRI) of the CNS is useful for detecting white matter lesions of MS [121, 122].

Using Koch's postulates, many etiologic agents have been ruled out. MS is neither contagious, nor monophasic. No conclusive evidence points to a single virus, bacteria, spirochete, simple genetic process, dietary deficiency, coagulopathy, poison, or vascular anomaly to cause MS. To date, no single autoimmune or other marker exists to simply differentiate the clinical presentation of MS from its many mimics. Thus, MS requires an exhaustive effort on the part of physicians to exclude other diseases of myelin, including vascular, degenerative, infectious, inflammatory, oncologic, structural, or other self-limiting etiologies. If no better explanation exists for at least two demyelinating lesions in different areas or times within the CNS, a diagnosis of MS can be made. The differential diagnosis of MS challenges even the most skilled teams of neurologists. In order to determine lesion dissemination in space, MRI provides definitive data needed for the diagnosis of MS [123].

FAMILY HISTORY

Genetic factors are significant in MS [124]. First-degree relatives of MS patients are twenty to fifty times more likely than the general population to also develop MS. Certain alleles of human leukocyte antigen (HLA), also known as major histocompatibility complex (MHC), class II represent the strongest genetic risk factor for MS but are not a surrogate for the previously mentioned criteria for

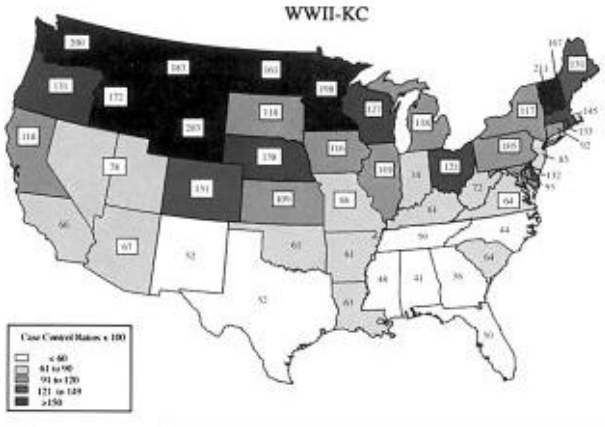
the diagnosis of MS. Implicated in the pathogenesis of MS, HLA class II is required for presentation of antigen to CD4+ T cells. HLA-DR and -DQ loci account for up to sixty percent of genetic risk for MS. Caucasians, Japanese, and many others carrying the DRB1*1501, DRB5*0101, DQA1*0102, and DQB1*0602 alleles are at higher risk of developing MS (odds ratios in the range of 2-6). Alleles of HLA class I also predict clinical outcome. Implicated in the modulation of MS, HLA class I is required for presentation of antigen to CD8+ T cells [125]. A*0301, B*07, and B*12 alleles are associated with a poor outcome. Alleles A*02, B*44, and C*05 are protective, with odds ratios in the range of 0.45-0.81 ($p < 10^{-3}$). CD8+ T cell lines from MS patients with progressive disease were first reported as having defective ability to suppress during a time when "suppressor" cells were a rather poorly accepted phenomenon [126]. Several genomes-wide single nucleotide polymorphism (SNP) analyses have also implicated other molecules of immune regulation, including the interleukin 2 receptor (IL2Rα), also known as (CD25), and IL7Rα, also known as CD127 [127]. The mechanisms underlying the associated risk inherent to certain alleles are unknown.

ENVIRONMENTAL FACTORS

MS is distributed geographically in mostly developed and westernized countries [128]. Monozygotic twins are concordant in only approximately twenty-five percent of cases, suggesting that environmental effects are significant as well. Natives of higher latitudes, such as Canada, northern Europe, and northern US (Figure 8), carry an increased risk of developing MS. This elevated

risk persists even in individuals who migrate toward lower latitudes after childhood. Based on these geographical correlations, respiratory illnesses and vitamin D deficiency have been implicated, but not definitive. A continued search for a viral cause of MS endures, including human herpes virus 6 (HHV-6), Epstein-Barr virus (EBV), and endogenous multiple sclerosis-associated retrovirus (MSRV).

Figure 8: The prevalence of MS is increased at higher latitudes



Adjusted case–control ratios (×100) were calculated per state for white male veterans of World War II–Korean Conflict (WWII-KC) for MS at entry into active duty [129]

Other demyelinating syndromes that exhibit similarity to MS are sometimes observed after flu-like illnesses or the administration of experimental vaccines. These demyelinating processes are not a direct result of the pathogen, but rather, are the result of the immune activation. The host immune response is erroneously directed toward self myelin antigens as a result of priming by pathogen antigen, which are similar to self antigens, a microbiologic phenomenon known as molecular mimicry. Paradoxically, vaccination efforts meaning to protect against a viral illness led to a discovery about MS [130, 131]. People and animals vaccinated for rabies and smallpox sometimes develop an ADEM-like syndrome. Both vaccines were initially prepared experimentally using neural tissue. In 1935, studies of *rabies vaccine-induced demyelinating disease* led many to think for the first time that MS was immune-related. Sterile rabbit-brain emulsions, given intramuscularly without any transmissible agent to macaques, lead to myelin destruction [132]. This demyelinating disease, experimental encephalomyelitis, and experimental autoimmune type one diabetes mellitus (T1DM) are cornerstones of the immune processes thought to be underlying MS [133]. However, a monophasic demyelinating illness, acute disseminated encephalomyelitis (ADEM) is now thought to more directly resemble the current animal models of MS [134] [135]. Classic EAE is monophasic, affects the spinal cord, and displays mild disability that is chronic. Atypical EAE, as well, is monophasic affects the brain. The SJL mouse strain immunized with the epitope of proteolipid protein (PLP) amino acids 139-151, however, exhibits a relapsing-remitting form, as a result of epitope spreading [136] [137]. Nonetheless, much

of what is known about the immunologic processes that underlie MS derives from work in murine EAE, wherein T cells are major mediators of the acute phase of early disease [138, 139]. However, it is clear that MS is far more complex than EAE, in that insidious, degenerative processes also contribute to MS disability. EAE is monophasic, while MS is relapsing-remitting. In particular, research toward the immunology of MS, the use of MRI, and the development of new therapies have provided value able insights into MS pathology.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Induced by immunization with myelin-associated antigens, EAE exhibits a variety of neurological defects similar to MS, but predominately an ascending paralysis [139]. Adoptively transferred CD4+ T cells reactive to various CNS antigens are also sufficient for induction of symptoms [138]. EAE is thought to be a Th1-mediated autoimmune disease. However, several studies have suggested pathogenic as well as regulatory involvement of CD8+ T cell responses. The effect of CD8+ T cells in EAE is largely strain-, vector-, and antigen-specific [140] [141]. The most successful methods for demonstrating the encephalitic potential of CD8+ T cells utilize recombinant virus constructs to express myelin protein [142] [96]. Histological sections of the CNS plaques in EAE show perivascular, mononuclear infiltrates and demyelination, suggestive of immune-mediated pathology similar to RRMS and monophasic ADEM.

KNOCKOUT AND MONOCLONAL STUDIES

EAE performed in a variety of genetic knockout or antibody-treated mice reveals a detailed window into the important players of the disease [143].

Compared to wild-type mice, a number of knockout mice, mice treated systemically with monoclonal antibodies or biologic factors display earlier induction and heightened peak disease, underscoring the importance of the following in the prevention, down-regulation, and recovery of EAE: cytokine ciliary neurotrophic factor (CNTF) [144], inducible nitric oxide synthase (iNOS) [145], perforin [146], interferon gamma (IFNγ) [147], IFNγ receptor (IFNγR), tumor necrosis factor receptor 2 (TNFRII) [148], interferon-α/β receptor (IFNAR) [149], interleukin 10 (IL10) [150], IL15 [151, 152], IL27 receptor (IL27R) [153], CD19 [154], CD8 [155, 156], β2 microglobulin [157], HLA-E [158], CD1 [159], cytotoxic T-lymphocyte antigen 4 (CTLA4) [160, 161], programmed death 1 (PD1) [162], CD25 [9], STAT1 [163], and STAT6 [164]. These studies suggest that these markers, transcription factors, and cell types play a regulatory or protective role, and their absence in the knockout is marked by a severe EAE phenotype compared to wild-type.

In contrast, a number of knockout mice, mice treated with monoclonal antibodies and biologic factors display delayed induction, reduced peak disease, or a complete resistance to EAE induction, underscoring the impotance of the following in the initiation, maintenance, and exacerbation of EAE: Fas, Fas ligand (FasL) [165], IL1 [166], IL2 [167], IL4R [150], IL6 [168, 169], IL7 [170], p40p19 subunit double knockout of IL12 and IL23 [171], IL17 [172, 173], p40 subunit of IL23 [174], recombinase-activating genes (RAG) [175], CD4 [155], HLA class II [176], CD28 [177-179], Tbet [180], RORγt [181], IRF1 [182], STAT4 [164], and Myd88 [183]. This suggests that these players are

inflammatory and pathogenic in EAE, and that their absence in the knockout exhibits a mild EAE or complete lack thereof compared to wild-type. This information paints in broad strokes the major aspects of the innate and adaptive immune system necessary in order to swell delayed-type hypersensitivity, or cell-mediated immunity, and the means necessary to contract, slow, and a reverse that immune response. EAE studies have elucidated the fundamentals of type IV hypersensitivity not only in the context of MS, but also in T1DM, Hashimoto's thyroiditis, rheumatoid arthritis, and contact dermatitis in response to poison ivy or the first immunologic metric, discovered in 1896, the tuberculin (PPD) skin test for exposure to mycobacterium tuberculosis.

The immunologic study of MS through EAE has illustrated a broad theme of critically important T cell responses that also typify the nature of Guillain-Barré syndrome, celiac disease, tuberculosis, graft-versus-host disease, and solid organ transplant rejection. This underscores the importance of CD8+ T cell biology and its application to a variety of clinical scenarios.

REGULATORY CD8+ T CELLS IN EAE AND ANIMALS STUDIES

A novel population of Qa-1-restricted CD8+ T cells were described with suppressive ability toward CD4+ T cells in EAE [184]. Murine Qa-1 is known as HLA-E in humans. Mice were immunized or not with 9- or 10-mer peptides derived from the TCR Vβ8 CDR2 chain and then all mice were immunized one week later with MBP for the induction of EAE. Mice vaccinated with the CDR2 region showed less paralytic disease and enjoyed a quicker recovery. CD8-deficient mice were given the same treatment and lacked any regulation of EAE.

TCRαβ+ CD8αα+ T cells lines were generated using the Vβ8 peptides. The clones were negative for CD62L and expressed high levels of CD28 and the NKG2D homodimer and CD94, which pairs with NKG2A, B, C, and E as heterodimers. CD8αα+ clones secreted IFNγ and TNFα and were targeted to kill only Vβ8+CD4+ T cells. The addition of the single 9-aa peptide (AMAPRTLLL) derived from the leader sequence of many MHC class Ia proteins and referred to as the Qa-1 determinant modifier (QDM) in mice, blocked the proliferative response by a representative CD8+ Treg clone. Qa-1 expression by MBP-reactive CD4+ T cells was required for killing by CD8αα+ T cells. Adoptive transfer of CD8αα+ T cells into EAE mice decreased paralytic disease. For the first time, a novel CD8αα+ T cell population was described with regulatory activity in EAE. The role of HLA-E within other subsets of CD8+ T cells remained poorly understood.

Studies by Chen and colleagues have elucidated the role of T cell activation in regulation by other T cells. Qa-1-restricted CD8+ T cells targeted HSP60sp peptide bound to Qa-1 [185]. HSP60 binding to Qa-1 protects target cells from killing-mediated by QDM and NK cells. HSP60 and QDM compete for binding to Qa-1. The relative ratio of each complex in activated T cells may provide the biological basis of regulation. CD8+ T cells selectively down-regulated intermediately activated CD4+ T cells by recognizing HSP60-Qa-1 complex. Intermediately-avidity T cells expressed the highest ratio of HSP60/QDM complexes of peptide-Qa-1. Vaccination with HSP60-loaded DCs protected mice from paralysis of EAE and increased Qa-1-restricted CD8+ T cells

specific for HSP60-Qa-1. T cell activation may role an important role in antigen-processing and expression of HLA class I. The mechanism of relative expression of HSP60/QDM complexes of peptide-Qa-1 remains poorly understood. The avidity model of Qa-1-mediated regulation has important implications based on thymic selection. Self-reactive T cells of high avidity are most likely to undergo programmed cell death in the thymus, thereby enriching the pool of intermediate avidity autoreactive T cells in the periphery. HSP60-Qa-1 complexes may provide a mechanism of peripheral tolerance.

Recent studies in my lab by York and colleagues have demonstrated the potential immune regulatory role of CNS-reactive CD8+ T cells in EAE [106]. Several induced models of EAE generated CD8+ T cells with adoptive immune suppressive activity, including MOG_{35-55}, $PLP_{139-151}$, and MBP_{1-11}. MOG_{35-55}-reactive CD8+ T cells response were prevalent in active EAE mice. Adoptively transferred into recipients, these cells decreased EAE paralysis compared to OVA-reactive CD8+ T cells. When MOG_{35-55}-loaded target cells were co-adoptively transferred into recipient mice, MOG_{35-55}-reactive CD8+ T cells decreased targets *in vivo* compared to OVA-reactive CD8+ T cells and modulated APC function. While HLA-E may have important roles in the presentation of GA and TCR-derived peptides to CD8+ T cells, the role of HLA-E in regulation of EAE by CNS-reactive CD8+ T cells remains poorly understood.

IMMUNOLOGIC BASIS OF MS

Initiation of demyelination in EAE requires high-avidity CD4+ T cells that are specific for myelin antigens, such as MBP, myelin oligodendrocyte

glycoprotein (MOG), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), or others [186][187]. This is not clearly established in humans. Although, autoreactive T cells alone are not sufficient to cause disease in healthy individuals [188]. Autoantigen-specific T cells may function improperly by possessing dual specificity due to the expression of two different beta chains of the TCR [189] [190, 191]. Although, antigen specificity of T cell populations in patients with MS is largely similar to that of healthy individuals [192]. While some myelin epitopes are thought to be more discriminatory in predicting those individuals who are at high risk of MS, no statistically significant differences exist between healthy and MS in the quantity of any bulk T cell population [193] or frequency of certain antigen-specific T cells [194]. In addition to the pre-existing autoantigen-specific TCR repertoire, an unknown, inciting environmental insult is thought to trigger a slightly altered differentiation, migration, and expansion of certain T cell populations in acute MS [195] [196]. Pleocytosis and oligoclonal bands are present in a fraction, but not all, of acute MS patients [197]. T cells have seen their antigen *in* vivo and become activated [198]. T cells cloned directly from MS brain lesions revealed that a significant portion of T cells infiltrating lesions are not specific to putative MS-related encephalitic epitopes and that CNS infiltration may be the result of by-stander activation [199]. Clones derived from the brain, spinal fluid, and blood of MS patients did not proliferate in response to MBP or PLP, in constrast, to prevalent MBP-specific CD4+ T cells found from post-infectious encephalitis brain tissue. CNS resident autoreactive T cells are not exclustive to MS.

IFNγ-producing Th1 and IL-17-producing helper type 17 (Th17) CD4+ T cells are required to infiltrate and propagate CNS lesions. How antigen is initially presented to pathogenic CD4+ T cells is unclear, but probably involves transient, systemic inflammation, leading to acute phase reactants (IL-1, IL-6, and TNFa), which opens the BBB, leading to increase immune cell trafficking into the CNS and spinal fluid compartment [200]. Neuroantigen-specific T cells, as well as other acitivated T cell populations, cross the blood-brain barrier (BBB). High-avidity CD4+ T cells encounter their cognate/specific antigen. Local inflammation leaks myelin antigen into the periphery. The antigen is picked up by macrophages, dendritic cells, B cells, or microglia. The context of the antigen presentation dictates the activation status and reaction of further autoantigen-specific CD4+ T cells encountering their antigen. Having overcome anergy through co-stimulation by APC, activated myelin-reactive CD4+ T cells expand in number probably in a local cervical lymph node. The differentiation of naïve CD4+ T cells into Th1 and Th17 T cells and migration into the CNS allows re-stimulation after the antigen priming by APC. Activated T cells slow to a roll on the cerebrovascular endothelium through binding selections and adhesions molecules, such as VCAM. Adhering T cells express MMP that degrade collagen and fibronection, permitting trafficking across the damaged BBB. MRI studies visualize that the BBB is disrupted typically for a month before resolution. Most often, BBB disruption goes unnoticed during the appearance of “silent lesions” [201]. During this time, Th1 and Th17 cells release cytokines and chemokines in the CNS. Tumor necrosis factor alpha (TNFα) and IFNγ production leads to

further T cell, macrophage, microglia, and other immune cell extravasation to the CNS. Activated IFNγ, TNFα, and IL-1-producing macrophages, autoantibody-producing B cells, complement, and free radical release contribute to demyelination. Immune cell to cell contact-mediated damage plays a major role in the dysfunction of myelin. Myelin breaks down with variability in acute lesions. Oligodendrocytes and neuronal axons degenerate from apoptotic or necrotic processes.

CLONALITY OF T CELLS IN MS

MS is thought to involve an auto-immune responses directed against CNS myelin antigens. Th1 cells are assumed to be the primary immune culprit leading the autoimmune infiltration into the CNS with an important role for macrophages and IFNγ. The exact identification of the target antigen has been debated. A ground-breaking study by Babbe and colleagues reported the use of microdissection to study the clonality of T cells in MS lesions [95]. MS lesion tissue sections were stained frozen for CD4 and CD8, clearly identifying T cells. A micromanipulator separated single T cells from surrounding tissue. As held by the theory of clonal specificity, the feature of the T cells of highest importance was the TCR. As described previously of clonal specificity, only the descendants of a clone share the same TCR. The TCRs of the microdissected lymphocytes were analyzed. For the first time, the analysis revealed that the infiltrating CD8+ T cell represented the descendants of only a few less numerous and different CD8+ T cell clones, which came to dominate the lesion infiltrate. The oligoclonal CD8+ T cells that came to dominate the MS lesion suggested that CD8+ T cells

played a more important role in the disease process than previously appreciated. A different study showed that the same brain-infiltrating CD8+ T cell clone may persist in the CSF and blood for as long at 5 years [102]. It is still unclear if the oligoclonal persistence of CD8+ T cells in the lesion is due to selective recruitment of certain clones or random migration of overly representative clones from the periphery. The way to approach this question would be to isolate cells from the periphery in order compare TCR sequencing analysis to cells form the CNS.

Using peripheral cells, my lab analyzed T cell proliferation using CFSE. The study focused on the antigen targets of CD4+ and CD8+ T cells that mediate the anti-myelin response using a wide spectrum of epitopes [202]. Crawford and colleagues identified HLA-restricted CNS-specific CD4+ and CD8+ T cell populations in untreated MS patients by measuring proliferative responses to 530 serial overlapping peptides spanning the entire sequence of 9 CNS autoantigens, including MBP, PLP, MOG, and 6 others. They assessed T cells proliferative responses, phenotype, and cytokine secretion from patients from various stages of MS. CNS-specific CD4+ T cells were highly prevalent but not significantly altered among healthy persons and all MS patient groups. MS patients' myelin-specific CD4+ T cells did produce increased IFNγ and decreased IL4, IL5, and CCR5 compared to healthy persons' cells. This suggests that MS patients' myelin-specific CD4+ T cells are not quantitatively significantly different than healthy cells, but more differentiated and antigen-experienced. The study revealed an intriguing pattern from the CD8+ T cell responses. Among RRMS

patients, the most common autoreactive CD8+ T cell responses were MOBP-specific. MS patients' myelin-specific CD8+ T cells produced increased IFNγ, IL10, and CXCR3. The functions of CD8+ T cells in the CNS infiltrate remained unclear.

Evidence supported two opposing roles for CD8+ T cells in MS. In the experimental autoimmune encephalomyelitis (EAE) model of MS, using transgenic and wild-type mice, studies have revealed a potential pathogenic and regulatory role. Mice genetically depleted of CD8 T cells showed increased relapses in EAE [156]. Recent studies in EAE in my lab have demonstrated a regulatory role for CNS-specific CD8+ T cells. Earlier human studies had compared either neuroantigen-specific T cells clones' TCR sequences from predominantly the CNS and/or periphery from healthy subject or MS patients. The comprehensive TCR sequencing studies in MS performed by my lab are reviewed here.

Recently, my lab performed a clonal TCR sequence analyses using peripheral neuroantigen-specific CD4+ and CD8+ T cells from leukapheresed untreated RRMS patients and healthy controls [203, 204]. Biegler and colleagues then performed a basic local alignment search tool (BLAST) search with the dominant clone sequences among published TCR data. Previous studies have shown limited sensitivity to detect differences between healthy persons and MS patients when using fluorochrome-conjugated antibodies to Vβ segments [205]. Alternatively, my lab analyzed TCR complementary-determining region (CDR3) Vβ sequences by short-term culture with MBP or PLP, flow-sorting of CFSE low

populations, and an anchored PCR approach. The anchored approach took advantage of a proprietary SMART switching mechanism at the 5' end of the RNA transcript to avoid the requirement of multiple 5' primers for each variable region segment of the Vβ CDR3. Flow cytometric sorting of electronically gated CFSE low cell after stimulation with 530 different overlapping peptides of 9 putative target CNS myelin antigen provided pure populations of myelin-specific CD4 and CD8+ T cells for PCR analysis. Healthy persons showed a more focused colonality of MBP-specific CD8+ T cells compared to untreated MS patients. PLP-specific CD4+ T cells among untreated MS patients showed less focused clonality and increased polyclonal TCR repertoire compared to MBP-specific responses. Upon treatment of MS patients with glatiramer acetate, the CD8+ T cell repertoire evolved into a focused, oligoclonal GA-specific Vβ usage. GA-specific CD4+ T cell responses remained less focused, polyclonal in nature and continually changing after months of GA therapy. Published TCR sequences were then interrogated using a BLAST search. Sequences were compared to that of published sequences to better understand the dynamics of CNS-specific CD4+ and CD8+ T cells. BLASTing MS patient sequences in an open database for similarity revealed a large number of MBP- and PLP-specific CD8+ T cell TCR matches of four or greater amino acids in the CDR3 V-NDN-J hypervariable region. Four matches were found among sequences from micro-dissected CNS-infiltrating CD8+ T cells in MS lesions [95]. One MBP-specific CD8+ T cell clone, representing 12.5% of total clones, isolated from an MS patient in the 2011 Biegler study shared the same LAGQG CDR3 Vβ sequence as one CD8+ T cells

microdissected from an active lesion in the 2000 Babbe study. A similar sequence was also found in the peripheral blood of the 2004 Skulina study which persists for years. An important role for auto-reactive CD8+ T cell clones is highlighted by these studies. Their exact function can not be deduced by their CDR3 Vβ usage, and it remains unclear what suppressor potential these particular CD8+ clones possess. A TCR transgenic mouse using the sequence of interest may reveal its encephalitic or regulatory potential. Biegler and colleages BLAST searches have also highlighted similarities between MS TCR Vβ CDR3 sequences and sequences from non-MS patient controls, including arthritis [206], calcified aortic stenosis [207], and chronic encephalitis of Rasmussen [208]. Commonly in healthy persons, focused oligoclonality of CD8+ T cell TCR Vβ CDR3 usage is higher than that among CD4+ T cells. The degree of clonal distribution, through GA therapy or other antigen exposure, may be an indirect and inverse indicator of regulatory CD8+ T cell function. Reoccurring conserved CD8+ T cell clones may also target pathogenic T cells or antigen-presenting cells. Functional assays are the preferred approach to tackling these and other questions.

TREATMENT

Numerous drugs target the immune processes of MS, which are generally divided into those that are immunomodulatory versus immunosuppressive [120]. Considered here are disease-modifying agents (DMA), rather than therapeutics reserved for the treatment of symptoms, such as spasticity, depression, etc. Prevention of relapse is a major objective of DMA [209]. Immunosuppressive agents are generally only indicated for acute flairs or patients whose disease is

resistant to DMA. Corticosteroids (steroids), type-one interferon beta (IFNβ) (betaseron/Avonex® and extavia/Rebif®), glatiramer acetate (GA/Copaxone®), and natalizumab (Tysabri®) are approved for use in RRMS and progressive disease by the food and drug administration (FDA), while mitoxantrone (Novantrone®) is approved for use in progressive MS. IFNβ and GA both reduce relapse rate by thirty percent [210-218]. Natalizumab and mitoxantrone both reduce relapse rate by seventy percent [219, 220]. Several other generally immunosuppressive agents, while not FDA-approved, have efficacy in MS, including azathioprine, methotrexate, rituximab, anti-CD52 (CAMPATH), mycophenolate mofetil, cladribine, and cyclophosphamide. Heroic efforts to perturb circulating Ig or immune status are occasionally reported with limited efficacy, including intravenous Ig (IVIG), leukophoresis [194], plasmaphoresis, and even bone marrow transplant as salvage therapy of the most extreme cases of terminally progressive MS [221]. While each therapeutic measure carries its own unique set of risks and benefits, the underlying processes and mechanism for many are still under investigation.

CORTICOSTEROIDS

The mainstay treatment of acute exacerbations of MS is steroids or adrenocorticotropic hormone [222]. Steroids are functionally diverse, non-specifically anti-inflammatory and immunosuppressive through binding a cytosolic steroid receptor (Gold, Buttgereit et al. 2001). Leukocyte trafficking and matrix metalloproteinase activity in the CSF of MS patients is also decreased by steroids (Rosenberg, Dencoff et al. 1996). Levels of CD4+, CD8+, CD14+ cell

populations, T-bet expression and signal transducers and activators of transcription protein 1 (STAT1) phosphorylation are all down-regulated by steroid therapy in MS (Frisullo, Nociti et al. 2007). CD8+ T cells have been shown to be less transcriptionally affected by steroid therapy as compared to CD4+ T cells (Li, Leung et al. 2007). Studies in EAE have shown that steroid therapy reduces the frequency of neuroantigen-specific T cells and spinal cord infiltration (McCombe, Nickson et al. 1996). Steroids have limited potential as prophylaxis. While steroids are known to accelerate recovery in MS acute exacerbations and mitigate subsequent neurological deficits, the exact effect of steroids on neuroantigen-specific regulatory T cells is unclear.

GLATIRAMER ACETATE

GA (copolymer-1, Copaxone®) is a synthetic, random length, variable sequence peptide composed of the amino acids alanine, lysine, glutamic acid and tyrosine (4.2:3.4:2.1:1 approximate ratio), designed at the Weizmann Institute in 1971 to mimic myelin basic protein (MBP) through EAE studies [223-228]. Peptides vary from 40 to 100 amino acids in length with an average molecular weight of five to nine kilo-Daltons [229]. Rather than having the intended encephalitic potential of MBP, GA surprisingly protected against encephalitis. GA is now the second most prescribed drug for MS with approximately one in eight MS patients in the US having a long-term prescription for GA [230]. Several mechanisms of action have been proposed for GA [231]. Some reports have suggested that it causes a Th1 to Th2 shift [232] or modulated CD4+ T cell apoptotic process [233]. Highly immunogenic with respect to T cells, GA alters the T cell receptor (TCR)

repertoire and increases suppressor/regulatory T cell (Treg) function. GA induces anti-inflammatory type 2 monocytes and increases forkhead box P3 (FOXP3) protein expression by CD4+ T cells. The effect of GA likely also involves dendritic cells, which are activated and traffic to lymph nodes [234]. Highly-inducible GA-specific CD4+ and CD8+ T cell responses are present in healthy persons, but are deficient in untreated MS patients. GA-specific T cell populations are expanded while myelin-specific T cells are perturbed in a manner still not completely understood. GA may act as an altered peptide ligand, antagonizing MBP 82-100 neuroantigen-specific T cells [235-237]. Over months of therapy, GA induces an extraordinary class of regulatory CD8+ T cells, possessing cytotoxic ability toward CD4+ T cells that display GA in the context of HLA-class I and non-classical HLA-E. This suggests that GA utilizes the cross-presentation pathway of exogenous antigen into HLA class I. Analysis of TCR rearrangements selected for by GA therapy has revealed that GA induces a distinctly focused, oligoclonal CD8+ T cell response in comparison to CD4+ T cells. This argues against the proposed theory of a Th1 differentiation shift toward helper CD4+ T cells type 2 (Th2) [238]. The therapeutically-induced GA-specific CD8+ T cells express both effector and regulatory cytokines, suggesting that cytotoxicity and suppression are related, contrary to prior thought [239]. In the perforin, CD8, or indoleamine-pyrrole 2,3-dioxygenase (IDO) knockout mice, GA treatment has an exacerbating effect on EAE. This suggests that GA activates antigen-presenting cells (APC) and that IDO expression by APC is necessary for the induction of perforin-expressing CD8+ Treg. In addition to

immunomodulation, neurotrophic properties are exhibited by GA, inducing CD4+ T cell production of bone-marrow derived neurotrophic factor (BDNF). This suggests that neurotrophic effects and may explain how GA-specific T cells protect from optic nerve crush injuries [240]. It is unclear which epitopes of the GA mixture contain the most potent immunomodulatory and neurotrophic effects or what surface and intracellular molecules determine how cells transmit these signals.

BETA INTERFERONS

Pharmaceutical grade IFNβ is derived from a naturally-occurring biologic and is the most commonly prescribed drug for MS, with approximately 29 percent of patients in the US having a long-term use prescription. Endogenous IFNβ production by virally infected cells interferes with viral replication and spread. However, exogenous IFNβ administration modulates the immune system through induction of numerous proteins through binding of the IFNα/β receptor. HLA class I expression is increased on all nucleated cells. Professional APCs are activated, and other innate immune processes are activated. The adaptive immune system is indirectly modulated by the increased efficiency of antigen turnover and presentation to both CD4+ and CD8+ T cells. IFNβ decreases expression of matrix metalloproteases (MMP), which are necessary for trafficking across the BBB. The effects of IFNβ on suppressor cells function remain unclear.

NATALIZUMAB

Natalizumab is a monoclonal antibody targeted toward very late antigen-4 (VLA4), preventing T cells, B cells, and macrophages from binding vascular

adhesion molecule (VCAM) and trafficking into the CNS. Although natalizumab exhibits potent efficacy, anti-VLA4 monoclonal antibodies carry an associated, significant risk of opportunistic infection. A rare, but significant, portion of patients taking natalizumab develop progressive multifocal leukoencephalopthy (PML), caused by JC viral infection of CNS tissue. This briefly led to the removal of the drug from the market in the US. Abrogation of immune cell extravasation by natalizumab far outlives its half-life, significantly decreasing leukocytes generally, specifically CD4+ T cells, CD8+ T cells, CD19+ B cells, and CD138+ plasma cells in CSF from natalizumab-treated patients for up to 6 months after discontinuation of natalizumab [241]. Surveillance of CNS tissue is a necessary function of lymphocytes to prevent opportunistic and infectious pathogens. Thus, there is a pressing need for an equally powerful, but less blunt therapy, targeting specific regulatory and pathogenic immune cells in MS [242].

FINGOLIMOD

Fingolimod (Gilenya®) is an orally administered immunomodulatory/immunosuppressive agent, capable of reducing relapses in MS by fifty percent. It sequesters lymphocytes in lymph nodes though binding of sphingosine receptors. Patients who take fingolimod may suffer from increased risk of respiratory infections and cancer. *In vitro* studies and murine models have suggested that fingolimod may inhibit regulatory T cell function. It remains unclear if this effect is selectively targeted toward CD4+CD25+ cells or if fingolimod generally inhibits T cell proliferation.

MITOXANTRONE

Mitoxantrone (MX) is a cytotoxic, generally immunosuppressive agent, used mostly for patients who are poorly responsive to the previously-mentioned immunomodulators. Typical side effects of all chemotherapeautic agents include bone marrow suppression, hair loss, diarrhea, immunodeficiency, nausea, and fatigue. MX, in particular, can cause cardiomyopathy, but is well tolerated in most patients. Some evidence points to an ability of MX to induce regulatory T cells. However, it remains unclear if regulatory T cells are selectively resistant to the effect of or induced by MX.

Nearly all approved and non-approved drugs are still under intense investigation. The most important aspect of these agents is that they work, while exactly how they work is worthy of much on-going research. It is important to note that the animal models of MS are to thank for the discovery of many of these therapeutics.

DEFICIENT CENTRAL TOLERANCE IN MS

The event triggering the cascade of failed tolerance before the encephalitic event of MS is unclear. Most theories develop chronologically from the standpoint of central tolerance, suggesting T cell selection in the thymus is dysfunctional. Peripheral tolerance must then also fail, setting up a swiss cheese model of several regulatory mechanisms allowing a overzealous immune response to slip through. The first critical aspect is discussed here. Initially, certain HLA class II alleles must give rise to extraordinary autoreactive CD4+ T cells in one or more of several proposed ways. High risk HLA alleles preferentially bind to a set of myelin, self peptides in the periphery. This may explain a predisposed heightened ability of T cells to react to myelin antigen in the CNS. Alternatively, high risk

HLA alleles preferentially do not bind a set of myelin, self peptides in the thymus during T cell development. The alternative may explain a deficient negative selection process, allowing myelin-specific T cells to escape thymic selection into the periphery. The other major explanations focus upon polymorphisms of the cytokines and T cell receptor (TCR) genes, having effects on TCR affinities, frequency, and differentiation of autoreactive T cells found within the T cell repertoire. Beyond the considerable complexity of thymic selection and genetic background, other major determinants to the development to autoimmunity include antigen exposure over a lifetime and several peripheral tolerance mechanisms, including many subsets of Treg. Our understanding of the nature of both highly complex immune and nervous systems remains limited in the context of MS.

DEFICIENT PERIPHERAL TOLERANCE IN MS

CD4+ Treg have been shown to be deficient in suppressive ability in MS [243, 244]. The role of CD8+ T cells is less clear with evidence supporting both regulatory and cytotoxic functional profiles [100, 104]. Evidence suggests that CD4+ and CD8+ Tregs are deficient in untreated MS and required for therapeutic induction [245] [104, 246]. While it is evident that naturally-occurring GA-specific CD8+ T cells can be therapeutically expanded and confer a protective effect in MS [104], CNS-specific CD8+ T cells remain somewhat enigmatic. How CNS-specific CD8+ T cells contribute to regulatory activity has yet to be clearly characterized in MS. Functional studies point to the class of C-type lectin-like MHC class Ib-specific inhibitory NK cell receptors expressed by activated

CD4+ T cells and other APC; CD8+ Treg up-regulation of NKG2A and CD94 was implicated during exacerbations [67]. Cross-presentation may play an important role in the generation and priming of CNS-specific CD8+ T cells in MS. Exogenously-endocytosed self-antigen or GA by microglia, macrophages, and CD4+ T cells may be cross-presented in the context of HLA class I. CD4+ T cells may present self-antigen through dendritic-cell (DC)-derived exosome trafficking and membrane exchange. CD8+ T cells may down-regulate CD4+ T cells after they exchange membranes with APCs, which confers antigen-bearing HLA class I to CD4+ to act as a target cells for regulatory CD8+ T cells [247]. Further functional studies are required to uncover CD8+ T_{REG} cellular interactions at play in MS.

CD8+ T CELLS IN HEALTH AND DISEASE

CD8+ T lymphocytes are critical to understanding viral, malignant, and immune-related diseases. Cytotoxic potential toward infected and tumor cells is one of CD8 T cells' most well-appreciated attributes. From precursors originating in the bone marrow, CD8+ T cells arise developmentally during thymic selection. In the thymus, thymocytes are selected which are attracted with moderate avidity toward HLA-class I, while nearly all others undergo apoptosis. Central tolerance mechanisms shape the T cell repertoire and ultimately allows nearly all T cells to be directed away from self and instead toward non-self (pathogens) and abnormal self (cancer). All nucleated cells express HLA-class I, thereby allowing the surveillance by CD8+ T cells of nearly every other cell in the body. Through a process of gene rearrangement, each T cell expresses a unique TCR from every other T cell. Thymic selection diminishes potentially-autoreactive and poorly-functional T cells by putting their newly rearranged pre-TCR genes to the test. The unique TCR of each T cell recognizes a specific antigenic peptide of approximately eight to ten amino acids in length in the context of HLA class I. Potentially-autoreactive thymocytes are selected-against through binding strongly to thymic epithelials cells, which ectopically express a variety of tissue specific antigens, transcriptionally regulated by AIRE [248]. The majority of CD8+ T cells, therefore, that escape thymic selection are MHC-class Ia-restricted and possess high avidity for foreign antigens, such as viruses [249, 250]. However, small numbers of low-avidity autoreactive T cells escape the thymus in healthy humans.

Once in the periphery, naïve T cell stimulation and activation is dictated by a primary set of signals. Antigen serves as the first signal, co-stimulation as the second signal, and cytokines as the third signal. T cells respond to various stimuli through mRNA transcription, protein production, and proliferation potential. T cells exhibit extensive plasticity regulated by the context of their activation, largely the status of the APC. Effector T cells may be converted to suppressors (Ts) through APC and paracrine signals. The inappropriate sequence, quantity, or quality of signals leads to T cell apoptosis or anergy. As CD8+ T cells are also capable of simply killing abnormal immune cells, they wield an important influence on human disease. Therefore, the nature of low-avidity, self-reactive CD8+ T cells remains unclear. Relative to CD4+ T cells in MS, the role of CD8+ is less clear with evidence supporting both regulatory and cytotoxic functional profiles.

Although much attention focused on CD4+CD25+ inhibition of priming or expansion of T cell immunity, considerably less has been known about the role of regulatory CD8+ T cells in feedback regulation. Early work by Antel and others in the 1980s presented evidence for activated suppressor cell dysfunction by CD8+ T cells in MS [93, 126, 251] [252]. During acute exacerbations of MS patients, CD8+ T cells were decreased in the peripherical blood compared to controls. Controlling for numbers, suppressor CD8+ T cells were induced *in vitro* by stimulation with ConA or anti-CD3. Concanavalin A (ConA) or anti-CD3-stimulated CD8+ T cells were shown to be defective at suppressing fresh autologous *ex vivo* responder cells during active disease compared to healthy

persons. On average, MS patient OKT3-induced suppressor cells reduced responder cell proliferation by 30% while healthy controls reduced proliferation by 70%. CD8+ T cell-enriched cultures showed greater suppression than CD4+ T cell-enriched cultures. The study focused attention for the first time on the defect of regulatory potential by CD8+ T cells during untreated progressive MS. While the study pointed to inducible regulatory function of CD8+ T cells, the specificity and *ex vivo* activity of the suppressor cells remained unclear. Other studies suggested that cytolytic activity of CD8+ T cells was selectively preserved while suppressive activity was lost in MS patients. The dominant suppression mediated by CD8+ T cells compared to CD4+ T cells in healthy persons, and its deficit in MS patients highlighted the importance of CD8+ T cells in MS [253]. The subset of CD8 required for suppression and their mechanism remained unknown.

AUTOREACTIVE REGULATORY CD8+ T CELLS

The ability of healthy human MBP-specific CD8+ T cells to regulate autologous MBP-specific CD4+ T cells was later reported [254]. A human CD8+ T cell clone blocked proliferation of autologous CD4+ MBP-specific T cells in a manner that required MHC class I. When CD8+ T cells predominated in culture over the CD4+ T cells, cytolytic ability was observed specifically toward MBP-specific CD4+ T cells, but not herpes simplex virus-specific CD4+ T cells. It remained unclear what, if any, regulatory role autoreactive CD8+ T cells might have in MS. This led to human studies where MS patients were vaccinated with irradiated MBP-specific T cells in the hope that regulatory T cells were induced to deplete circulating MBP-reactive T cells [255]. After the experimental treatment,

regulatory CD8+ T cell lines were generated from recipients and showed *in vitro* suppressive ability and cytotoxicity toward vaccine cells in an HLA-restricted manner. Little clinical effectiveness was observed by Jiang and colleagues and similar studies [256] nor in other human studies involving the use of vaccination with the TCR Vβ 5.2 sequence [257]. The principle of interacting autoreactive regulatory T cell in humans was validated. The effect of other immune-deviating therapies on regulatory CD8+ T cells in MS was still poorly understood.

COPAXONE AND CD8+ T CELLS

My lab then reported that, a FDA-approved treatment for MS, Copaxone induced CD8+ T cells which kill GA-loaded target cells using MHC class I [104]. The mechanism of Copaxone is unknown. Several proposed mechanism have been debated. The composition and effects of Copaxone are reviewed below. Tennakoon and colleagues showed that naturally-occurring GA-reactive T cells are prevalent in healthy persons while GA-reactive T cells are decreased in MS patients. Anti-CD3-stimulated and GA-reactive CD8+ T cells were functionally suppressive toward CD4+ T cell proliferation. CD8+ T cells mediated suppression through cellular contact. Daily treatment with Copaxone for months by MS patients resulted in an increased suppressive function toward CD4+ T cell proliferation that was HLA class I (A, B, and C)-restricted. GA-specific killing of CD4+ T cells was inhibited by anti-HLA-E antibodies in four of five subjects. Tennakoon and colleagues finally generated antigen-reactive T cell lines with myelin antigens, Copaxone, and foreign control antigens. Perhaps most interestingly, GA-specific CD8+ T cell lines were capable of suppressing CNS-

specific CD4+ T cells line proliferation. It remained unclear if autoreactive CD8+ T cells were deviated by Copaxone therapy or if regulatory CD8+ T cells originated from a distinct GA-specific lineage. The composition and structure of Copaxone may have allowed it to promiscuously bind to both non-classical HLA-E and classical HLA class I, whereas the restriction and antigen targets of suppressive autoreactive CD8+ T cells were still unclear. The role of autoreactive CD8+ T cells in MS remained poorly understood.

AUTOREACTIVE CD8+ T CELLS AND MS

Debate continued upon the presumed regulatory role of autoreactive CD8+ T cell in MS. For example, two human studies generated similar experimental results and gathered two opposing interpretations. The first study by Zang and colleagues generated MBP-specific CD8+ T cells lines from healthy persons and MS patients [258]. The MBP-specific CD8+ T cells exhibited cytotoxicity toward MBP-loaded autologous B cells or human HLA class I-expressing monkey kidney cell lines. The second study generated CD8+ T cell clones recognizing autologous myelin-reactive CD4+ T cell clones from the blood and CSF of healthy controls and MS patients during acute exacerbations and remissions [67]. The *in vitro* expanded CD8+ T cells from MS patients killed autologous myelin-specific CD4+ T cell clones with granules only when target cells were activated and expressing HLA-E. During acute exacerbations of MS, serum levels of IL15 were elevated, and the expression of CD94 and NKG2A by CD8+ T cells was up-regulated and therefore decreased cytolytic ability towards CNS-specific CD4+ T cells. *In vitro* IL15 and IFNγ increased CD94/NKG2A expression on CD8+ T

cells and decreased cytotoxicity towards CD4+ T cells. The authors of the first study by Zang and colleagues dismissed any potential role of human autoreactive CD8+ T cells in suppression of immune responses in MS. In the second study, the Correale and colleagues suggested that cytolytic ability of CD8+ T cells toward other immune cells may act as an important tolerance mechanism. The second study also suggests that cytokine levels an important role on the cytotoxic and regulatory ability of CD8+ T cells in MS. Clinical exacerbations may become apparent when physiologic regulatory ability by CNS-specific CD8+ T cells is lost due to increased levels of IL15. The effects of cytokines on regulatory/cytolytic activity of CD8+ T cells may differ according to levels of IL15. The controversy surrounding autoreactive CD8+ T cells in MS remains, as exemplified by these two cellular immunology studies. The mechanism by which CD94/NKG2A expression by myelin-specific CD8+ T cells is increased during acute exacerbations of MS remains poorly understood.

BIOLOGY OF HLA CLASS I MOLECULES

Major histocompatibility complex (MHC) genes, also known as human leukocyte antigen (HLA), have strong associations to autoimmune, infectious, and inflammatory diseases [259]. The murine MHC locus was discovered by George Snell [260]. Jean Dausset discovered HLA on white blood cells [261]. Baruj Benacerraf described the biology of HLA in the immune response [262]. HLA class I molecules present endogenous peptides to CD8+ T cells and natural killer (NK) cells for screening of viral infection and malignant transformation [263]. Both classical (Ia) and non-classical (Ib) HLA class I molecules play a critical

role in immune surveillance and tolerance by acting as ligands for NK cell inhibitory receptors and TCRs of CD8+ T cells. HLA class Ia and Ib molecules are expressed by all nucleated cells and play a critical role in both innate and adaptive immunity. On the cell surface, mature HLA class I contains a heavy chain, β_2 microglobulin light chain, and peptide of eight to ten amino acids. Expression of class I proteins on the cell surface requires endogenous antigen-processing of peptides within the endoplasmic reticulum (ER). Antigen-processing from the cytosol into the ER is transporter associated with antigen processing (TAP)-dependent. TAP-deficient animals also express a limited number of peptides that contain a leader signal sequence [264]. Other regions of proteins outside of the leader signal peptide and some leader-derived peptides processed or recycled into the cytosol in a TAP-dependent manner of antigen-processing and expression in the context of HLA class I [265].

CLASSICAL HLA I

The highly polymorphic, classical HLA class Ia molecules (A, B, and C) are thought to present a great variety of endogenously synthesized peptides (greater than 10^5) to CD8+ T cells. αβ CD8+ T cells recognize virus- or self-derived antigens in the context of HLA-Ia. The TCR-peptide-HLA-Ia clonotypic interaction is the foundation of the adaptive immune response, and certain HLA alleles are highly linked to clinical syndromes in humans. The CDRs of the α and β TCR chains are positioned in close proximity to the antigen resting within the polymorphic regions of the HLA-Ia molecule peptide-binding groove. The CD8

molecule augments the interaction through binding of the constant region of HLA-Ia molecules.

NON-CLASSICAL HLA

The nearly monomorphic, non-classical HLA-Ib molecules (E, F, and G) have been thought to present only a limited number of peptides [266] [267]. HLA-class Ib pathways have been thought to be evolutionarily older and more conserved than the HLA-Ia pathways. HLA-F has been poorly studied. HLA-G is intermediately polymorphic between strictly Ia and Ib molecules [268]. The CD94/NKG2-peptide-HLA-E interaction plays an important role in the innate immune response and discrimination of self/non-self by a small subset of cytotoxic and regulatory T cells that express CD8. The innately germline-encoded CD94/NKG2 receptors engage the peptide and interface with HLA-E in a similar manner by which hyper-variable TCR binds variable peptide-HLA-class Ia. NK and CD8+ T cells express the CD94/NKG2 hetero-receptor which binds peptide-HLA-E, which decreases cytolytic ability toward the HLA-E expressing cell [269]. HLA-E is expressed by nearly all cells with intact antigen-processing machinery. If a virally infected cell has down-regulated HLA-E expression, NK cell cytolytic ability is increased toward the infected cell [270].

PEPTIDE REPERTOIRE OF HLA-E

The predominant peptides that fill HLA-E are derived from the signal sequence of classical HLA class Ia. The predominant peptide is known as the Qa-1 determinant modifier in mice (AMAPRTLLL) and B7sp in humans (VMAPRTVLL). All peptides that bind to HLA-E have a nine amino acid

sequence with methionine second from the N-terminus and leucine at the C-teriminus. Similar to the limited set of peptides presented in HLA-Ia of TAP-deficient animals, HLA-E-presented peptides contain the leader signal sequence of class Ia molecules and similar leader sequences from other proteins [271]. The HLA-E was recently shown to express a leader sequence from heat shock protein (HSP) 60 [272, 273]. The HLA-E-HSP60 complex was no longer recognized by the CD94/NKG2 inhibitory receptor, leading to increased cytolytic ability toward stressed cells. Jiang and colleagues went on to show that HLA-E restricted CD8+ T cells are involved in the development of autoimmune diabetes type 1 diabetes. HSP60-specific, but not B7sp-specific, HLA-restricted CD8+ T cells were able to suppress the GAD-specific CD4+ T cells and MBP-specific T cells [274]. HSP60 may activate CD8+ T cell suppressive activity and B7sp may inhibit their suppressive activity. The role of CD8+ T cells that are inhibited by B7sp-HLA-E complex is poorly understood.

HLA-E RESTRICTED CD8+ T CELLS

In addition to an important role in the innate immune system, HLA-E may serve adaptive immunity as well. Recent evidence suggests that the peptide repertoire that is presented by HLA-E is broader than what was once thought [275]. HLA-E may also act as a back-up antigen-presentation pathway to αβ CD8+ T cells in the case of TAP-deficient tumors. Considerable evidence suggests that HLA-E may act as a ligand for the TCRαβ. In mice and human studies, CD8+ T cells proliferate in response to cytomegalovirus, Epstein-Barr virus, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Salmonella enteric* infection in

a HLA-E restricted manner [276-279]. The mechanism of how foreign antigens replace self peptides during HLA-E antigen-processing is poorly understood. The consequences of innate and adaptive immune cells competing to bind HLA-E are poorly understood. HLA-E may possess dual roles at the interface of innate and adaptive immunity, presenting predominantly conserved leader signal sequences in an inhibiting context and a variable repertoire under conditions of tolerance and infection. The potential regulatory role of HLA-E-restricted CD8+ T cells will be described in the context of EAE and other animal studies.

POTENIAL FOR CELL BASED THERAPIES IN AUTOIMMUNITY AND TRANSPLANT

While Tregs have been recognized as having an essential role in controlling autoimmunity [280] and prolonging transplant survival [281], individualized *ex vivo* immune cell-based therapies have yet to become mainstays of therapy for immune-mediated diseases [282]. While *ex* vivo immune cell-based therapies have reached the clinical trial stage, current protocols have yet to overcome important obstacles [283]. Injected cell populations traffic poorly to lymph nodes, have limited interaction with other immune cells, or are eliminated by the host immune system. The development of dendritic cell and cytotoxic T lymphocyte-based therapies for bone marrow and skin cancers have shown limited success [284-287]. In contrast, the fixation and transfer of donor cells with the crosslinker agent (ECDI) as therapy has shown promise for antigen-specific tolerization of human solid-organ transplantation [288]. Cancer-specific CD8+ T cells co-transferred in conjunction with an autologous hematopoietic transplant confer protection from relapse [289]. *Ex vivo*-expanded Treg suppress autoimmune T1DM in non-obese diabetic (NOD) mice [17] and GvHD after allogeneic bone marrow transplantation in mice [290]. Antigen-specific therapies, if proven effective, would be a dramatic improvement over non-specific immune modulation. Further mechanistic and functional studies are necessary in order to facilitate wide-spread use of *ex vivo* immune cell-based therapies.

SUMMARY OF CD8+ SUPPRESSORS

Much controversy centers upon the role of CD8+ T cells. While most studies of MS and EAE have focused on the role of CD4+ T cells, considerable evidence points to an important role for CD8+ T cells. While it is widely accepted that CD4+ Tregs regulate varying aspects of EAE and MS, their CD8+ counterparts have been left largely understudied and poorly understood. To hasten the advent of novel therapeutic approaches, there is a pressing need for a precise understanding of the immune regulatory and effector roles for CD8+ T cells in MS.

The regulatory T cell (Treg) field has re-emerged since the decline in the early 1990s, following resurgence in the CD4+ Treg domain. In 1990, it was thought that CD8+ T cells could be divided into two distinct categories. Cytotoxic T lymphocytes (CTL) were armed for effector function while suppressor CD8+ T cells (Ts) were for modulating responses, distinguished by CD11b- and CD11b+ subsets respectively [291]. Today, the distinction between cytotoxic and regulatory CD8+ T cell is less clear than ever. Killing of unwarranted autoreactive cells is a reasonable solution. A major barrier to *ex vivo* cell-based therapies is that the precise phenotype of CD8+ Treg eludes comprehension. Collectively, several human and mouse studies point to a heterogeneous CD8+ Treg population with suppressive activity in the steady state and upon activation. Evidence for an important role of naturally-occurring CD8+ Treg can be found in several human diseases and experimental disease models: experimental autoimmune encephalomyelitis, MS [67], experimental autoimmune uveitis [61, 292], inflammatory bowel disease and infectious colitis [72, 293], T1DM [68],

and human immunodeficiency virus (HIV) infection [294]. Several *in vitro* studies have observed CD8+ Treg suppressive effects imparted on B cells [295], CD4+ T cells and CD8+ T cells [71]. Induction of CD8+ Treg *in vivo* have been observed in the pathogenesis of HIV [294] and Mycobacteria [90] and during the therapeutic induction of tolerance for the purposes of transplant [61, 296, 297], T1DM [298, 299], and multiple sclerosis [104]. *In vitro*, CD8+ Treg may be expanded through mitogen or antigen-specific means [46, 300]. While subsets overlap, several putative phenotypes emerge: CD8+IL2R+, CD8+CD28-, CD8+PD-1+, and other activated CD8+ T cells by upregulation of several costimulatory and cytotoxic molecular markers. Besides the patent use of HLA class-I, CD8+ suppression occurs through contact dependent- (CD28, CTLA-4-, CD80, PD-1, and CD86) and independent-mechanisms (TNFα, IFNγR, IFNγ, TGFβ, IL10, CCL4 and IDO). The most well accepted CD8+ T cell regulation occurs by a precisely described mechanism involving HLA-E and the CD94/NKG2A complex [59, 301]. Activated T cells express the non-classical MHC, HLA-E, as a function of T cell receptor (TCR) avidity to their cognate antigen. CD8+ T cells engage HSP60 peptide-HLA-E via CD94 and selectively-down regulate T cells with intermediate TCR affinity for self or foreign peptide. The end result for this peripheral regulatory mechanism is that high TCR affinities for foreign antigens are enriched, while only low affinities are allowed for self-specific TCR. The importance of this regulatory pathway is further illustrated by its perturbed role in MS pathogenesis. During MS exacerbations, CD8+ T cell clones express significantly higher killer-inhibitory receptor CD94/NKG2A,

which IL-15 and IFNγ further increase, and exhibit reduced cytolytic ability towards MBP- and MOG-specific CD4+ T cells [67]. The transcriptional regulation, plasticity, and dynamics of CD8+ T_{REG}s in MS remain unclear throughout different phases of the disease. The precise role of CNS-specific CD8+ T cells in the pathogenesis/regulation of MS is poorly understood.

SUMMARY OF INTRODUCTION

Multiple sclerosis (MS) is the most common disabling neurological disease of young people of unknown etiology and is thought to be an immune-mediated disease. Much of what is known about the immunologic processes that underlie MS derives from work in murine experimental autoimmune encephalomyelitis (EAE), wherein T cells are major mediators of disease. Similarly, MS patients harbor CNS-specific T cell responses and mononuclear CNS infiltrates, and exhibit improvement upon treatment with immunomodulatory drugs. To hasten the advent of novel therapeutic approaches, there is a pressing need for a precise understanding of the immune regulatory and effector roles for T cells in MS.

Previous observations from us and others highlight the importance of CD8+ T cells in MS. MS patients harbor CNS-specific CD8+ T cells displaying both effector and regulatory properties [202]. CNS lesions in MS patients show oligoclonally expanded CD8+ T cells [294]. However, the roles of CD8+ T cells in the pathogenesis and regulation of MS are unclear. Following a course of glatiramer acetate (GA), untreated MS patients exhibit an expansion of CD8+ T cells possessing regulatory activity, which are lacking before treatment as compared to healthy individuals [104].

The regulatory T cell (T_{reg}) field has re-emerged since the decline in the early 1990s. While most studies of MS and EAE have focused on the role of CD4+ T cells, considerable evidence points to an important role for CD8+ T cells. While it is widely accepted that CD4+ T_{reg}s regulate varying aspects of EAE and MS, their CD8+ counterparts have been left understudied largely due to the ongoing surge of CD4+ T_{reg} studies starting in late 1990s. CD4+ and CD8+ regulatory T cells (Treg) are an important part of the immune system that maintain peripheral tolerance in a healthy immune system and may be used to establish therapeutic tolerance to transplants.

HYPOTHESES

Based on prior studies and preliminary evidence, we hypothesized that CNS-specific CD8+ T cells play an important immunomodulatory role in MS and mediate the effects of clinically successful therapy. In the steady state and during therapeutic induction, distinct subsets of CD8+ T cells are proposed to be involved in the down-regulation of pathogenic T cell responses. Specific subsets of CD8+ Treg harbor potent regulatory activity. High CD28+ and low CD62L expression positively correlates with high CD8+ Treg suppressive ability. Autoreactive regulatory CD8+ T cells use contact-dependent means of suppression toward CD4+CD25- T cells.

SPECIFIC AIMS

The biology of CNS-specific CD8+ T cells as well as other CD8+ T_{reg} subsets remained poorly studied in the context of MS immunopathology. We addressed these issues through the following specific aims:

- Specific Aim 1. To assess CD8+ T_{reg} suppressive ability in healthy subjects and in relapsing-remitting MS patients during stable disease and during exacerbations through cross-sectional study.
- Specific Aim 2. To characterize the mechanisms of CD8+ T_{reg} suppression.

Through these aims, we hoped to address the role of self-specific CD8+ Treg in pathology and during therapy as well as provide more fundamental insights into steady-state non-specific CD8+ Treg peripheral tolerance mechanisms. We hoped to pave the way for better therapeutic interventions for transplant, allergy and autoimmunity.

CHAPTER 2: METHODS AND MATERIALS

SUBJECT CHARACTERISTICS

MS patients were recruited and gave written informed consent at the UT Southwestern Clinical Center for Multiple Sclerosis. Table 1 summarizes patient characteristics. 11 treatment-naïve adult clinically definite RRMS patients (McDonald criteria) with quiescent disease were recruited [302]. Exclusion criteria included pregnancy, HIV positivity, active cancer, other autoimmune, immunosuppressive, neurodegenerative conditions, clinical relapse or corticosteroid treatment within last 3 months, any history of disease-modifying immunomodulatory therapy. In addition, 9 treatment-naïve MS patients were recruited during an active acute clinical episode/relapse. 15 healthy subjects were recruited as controls (HC). All studies were approved by the UT Southwestern IRB according to Declaration of Helsinki principles.

Table 1: Summary of Patient Characteristics

	Healthy Controls (HC)	RRMS: Quiescent (MS)	MS: Acute Exacerbation	MS: Exacerbation Follow-up
Number of Subjects	15	11	9	4
Average age, y (Range)	44 (21-65)	40 (23-56)	45 (31-65)	44 (35-53)
Sex (M/F)	5/10	2/9	3/6	2/2
Days from Last Relapse [Mean (Range)]	N/A	599 (90-2920)	8 (2-50)	81 (31-118)

CELL PREPARATION AND BEAD SORTING

PBMC were isolated from whole blood using Ficoll Hypaque (GE Healthcare Biosciences, Pittsburgh, PA) density gradient. Purified CD8+ T-cells were isolated using CD8+ Microbeads positive selection kit (Miltenyi Biotec, Auburn, CA) and AutoMacs separation, according to the manufacturer's instructions. CD8+ enriched populations were >95% CD8+ and <0.1% CD4+ by flow cytometric analysis. "Untouched" CD4+ T-cells were isolated using CD4 negative selection kits (Miltenyi Biotech). CD25+ T-cells were depleted from the purified CD4+ using CD25 Microbeads (Miltenyi Biotec). CD4+CD25- enriched populations were >98% CD4+, <1% CD25+, and <0.1% CD8+ by flow cytometric analysis. CD4+CD25+ enriched populations were >98% CD4+ and <0.1% CD8+. CD25 expression ranged from 40.5-73.8%. The CD4+ and CD8+ T-cell-depleted PBMC population was irradiated with 3000 rads before being used as antigen-presenting cells (APC).

CFSE STAINING

To detect proliferative responses upon antigenic challenge, cells were stained with carboxyfluorescien diacetate succinimidyl ester (CFSE) (Invitrogen Molecular Probes, Eugene, OR), as described previously [245] [100]. Briefly, cells are suspended at 1 x 10^6 cells/mL and incubated for 7 min at 37ºC with 0.25 μM CFSE (Invitrogen), then washed twice with media containing 5% human serum.

CMTPX STAINING

Cell Tracker Red CMTPX (Invitrogen Molecular Probes) was used to stain putative regulatory cells. CD8+ and CD4+CD25+ suppressor cells, or

CD4+CD25- negative control cells were marked with CMTPX, as described previously [106]. Briefly, cells were suspended at 1 x 10^6 cell/mL and incubated 15 min at 37ºC with 700 nM CMTPX, then washed twice with media containing 5% human serum. The longer-wavelength CMTPX exhibits bright red fluorescence that is easily distinguished from that of green fluorescent probes, such as CFSE.

FLOW CYTOMETRY-BASED SUPPRESSION ASSAY CULTURES

Peripheral blood mononuclear cells (PBMC's) from healthy buffy coats and cord blood samples (where indicated) were separated using ficoll gradients. CD4+CD25- and CD4+CD25+ T cell populations were isolated using CD4 negative selection and CD25 positive selection magnetic microbead isolation kits from Miltenyi Biotec. Irradiated CD4 depleted populations were used as antigen presenting cells (APC) in all experiments. FOXP3 expression in CD4+CD25- T cells was induced by activation with soluble or plate bound anti-CD3/anti-CD28 or allostimulation by healthy APC's as indicated. FOXP3 expressing activated cells from varying time points were added back in a suppression assay to test their suppressive activity as described further. 1 x 10^6 CFSE-stained CD4+CD25- T-cells were used as responders in a 1 ml culture. 1 x 10^6 CD4- and CD8- depleted PBMC were irradiated with 3000 rads and used as APC. In replicate cultures, varying ratios of CMTPX-stained suppressors (CD8+ or CD4+ cells where indicated) were added and cultured with various antigenic stimuli for 7 days in complete RPMI 1640 media containing 5% human serum, 100 U/mL Penicillin,

100 μg/mL Streptomycin, and 0.92 mg/mL L-glutamine. Cells were washed and stained for flow cytometry, as described below.

ANTIGENIC STIMULATION

Pools of 15-mer peptides, overlapping by 10, spanning entire neuroantigenic proteins were used, as described previously [100]. These were used at 1 μg/ml final concentration for each peptide and covered myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMGP) and αβ-crystallin (CRAB). In addition, whole bovine MBP (wbMBP) was also used at 20 μg/ml. For control foreign antigens, we utilized pools of known CD4 and CD8 epitopes of CMV (5 and 14 peptides, respectively) as well as whole cytomegalovirus (CMV) (Microbix Biosystems, Ontario, Canada) and tetanus toxoid (TT) (Accurate Chemical & Scientific Corp, Westbury, NY). 1 μg/mL anti-CD3 monoclonal antibody (OKT3) was used for mitogenic stimulation.

OTHER REAGENTS

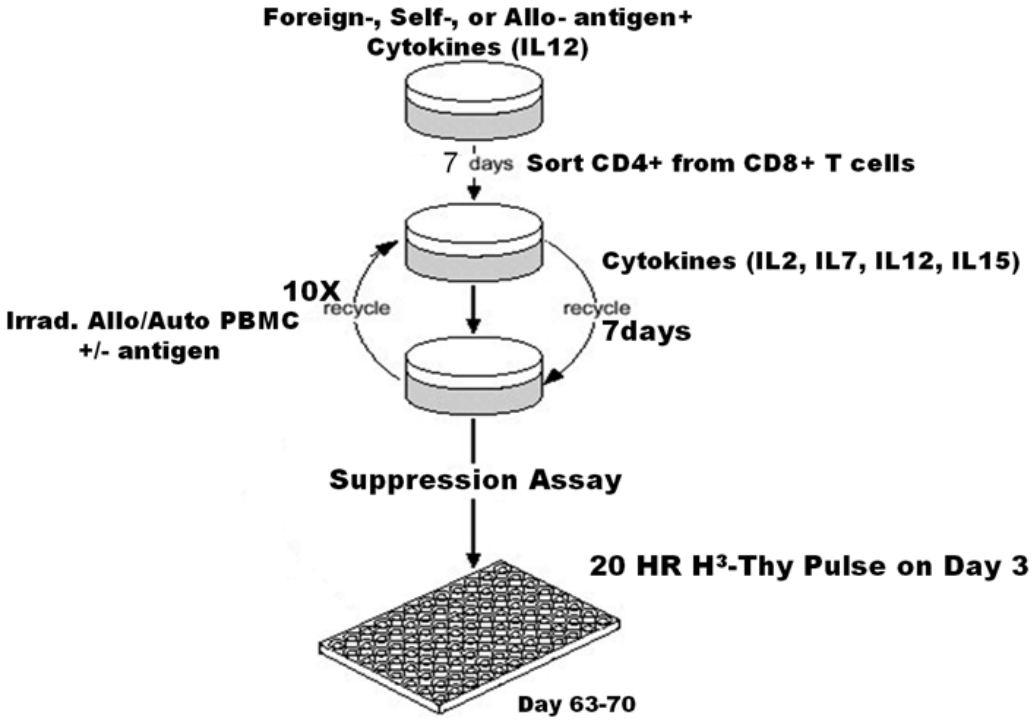
Anti-CD3 (OKT3 Clone) was used at a concentration of 1μg/ml/million cells. Anti-CD28 (BD Biosciences) was used at 1 μg/ml/million cells. Recombinant human IL-2 obtained from Invitrogen. Recombinant human TGFβ, human IL-10, CTLA-4/Fc Chimera, anti-hIL2 (clone 5334), anti-hIL-2Rα (clone 22722), anti-TGFβ123 (clone 1D11) and anti-TGFβ were obtained from R&D Biosytems Inc. CD45RO microbeads were used for CD45RO depletion (depletion greater than 95%) to obtain a CD45RA enriched naïve and CD45RO enriched memory

population. CD4 depleted enriched APC's were stained with the red fluorescent cell membrane dye PKH26 (Sigma) as per the manufacturers protocol.

T CELL LINE GENERATION

We generated neuroantigen- and control antigen-specific CD8+ and CD4+ T-cell lines by bead-sorting CD8+ (or CD4+) T-cells after 1 week of *in vitro* PBMC stimulation, followed by repeated antigen-specific expansion with autologous APC (Figure 9). CD8+ T-cell lines were maintained with 25 IU/mL IL-2 (Peprotech, Rocky Hill, NJ), 10 ng/mL IL-7 (Peprotech), 1 ng/mL IL-12 (Peprotech), and 1 ng/mL IL-15 (Peprotech), as previously described [303, 304].

Figure 9: Growing antigen-specific T cell lines



FLOW CYTOMETRIC ANTIBODY STAINING

On day 7 of *in vitro* stimulation, cells were washed with 0.1% (w/v) sodium azide/phosphate-buffered saline (Mediatech Cellgro). Cells were stained with anti-CD3-PE (BD Biosciences, San Jose, CA), anti-CD4-PECy5.5 (Invitrogen), anti-CD8-Pacific Blue (BD Biosciences), and anti-CD25-APC (BD Biosciences), then resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfiled, PA). Flow cytometric data were acquired on a 4-Laser, 17-color LSRII using FACSDiva software (Becton Dickinson). CFSE was detected in the FITC channel and CMTPX in the PE-Texas red channel on the LSR. Human and mouse FOXP3 staining kits from eBiosciences were used to stain for intracellular FOXP3. PCH101 and 236AE/7 anti-FOXP3-PE, AlexaFluor700 or Pacific Blue were used to stain for human FOXP3.

DATA ANALYSIS

Linear uncompensated data was transferred as FCS 3.0 files and analysed after compensation and transformation using FlowJo version 8.4.1 (TreeStar, Ashland, OR). Using Flowjo software (Treestar), putative Treg (CD4+CD25+, CD8+, and CD4+CD25- as a negative control) were CMTPX(high) and were gated out from flow cytometric analysis of CFSE-stained cells. Similar PKH-26 (Sigma-Aldrich, St. Louis, MO)-labeling techniques have been utilized for the purpose of excluding Treg from proliferative quantitation of CD4+ responder T-cells (Joosten, van Meijgaarden et al. 2007). T-cell activation and proliferation was quantified by the percentage of CD25(high) and CFSE(low) events among gated CD4+ (or CD8+) T-cells. Cut-offs for positive populations were determined by

using either fluorescence minus one (FMO) staining for polychromatic flow cytometry, no stimulus background CFSE staining, or isotype control staining, as appropriate [305]. A "positive" T-cell response to antigen was defined as having (1) a response index (RI) greater than or equal to 1.5 and (2) a %CD25+CFSElow response of the antigen-stimulated cells at least 1% greater than the %CD25+CFSElow response of the cells in the no antigen tube. Response index (RI) was the stimulated cells' %CD25+CFSElow divided by 100-%CD25+CFSElow divided by the unstimulated cells' %CD25+CFSElow divided by 100-%CD25+CFSElow. If these criteria were unmet, absence of T-cell response was indicated. For suppression assays, % response was calculated by normalizing the 'responder only' proliferation to 100%. %Suppression was 100 minus %response. CMTPX(high)CD8+ cells were analyzed for CD25+ expression. Stimulation index of the CD8 response was defined as the percentage of CD25+ cells with antigenic stimulus divided by percentage with no antigen.

3H THYMIDINE BASED ASSAYS

Assays were performed in triplicate in 96-well plates using antigen-specific T-cell lines. 1x105 CD4+ line cells were cultured with 1x105 irradiated autologous PBMC in a total volume of 200 μl/well, with or without indicated antigens. $1x10^5$ CD8+ line cells were added to the cultures as suppressors. The cultures were pulsed with 3H-thymidine on day 3 and harvested after 20 hours to measure proliferation in CPM, as previously described [100, 245]. ΔCPM was calculated by subtracting background proliferation in the absence of antigen.

STATISTICAL ANALYSES

Statistical tests were performed using Prism 5 (Graphpad Software, La Jolla, CA). Correlation regression and t tests were used to compute a two-tailed P value assuming a 95% confidence interval. P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values 0.01 to 0.05, 0.001 to 0.01, and <0.001 were significant with "*", "**", and "***" notated respectively. R squared values were computed from non-transformed raw data with the use of non-linear regression, assuming a semi-log X line model (days since start of last relapse is plotted on a logarithmic X axis)

STANDARDIZATION OF MIXED LYMPHOCYTE REACTION (MLR)

Autologous reaction: $1x10^6$ CD4+CD25(-) responder and $1x10^6$ T-cell depleted PBMC target cells from the same donor were co-cultured in mL H5. These acted as background control, and were also useful to determine the self-reactivity and pseudo-reactivity of responders in absence of true antigenic stimulus. Alloreaction: Target APC cells from another random donor (HLA-mismatched) were used to activate responders. Responder cell proliferation in MLRs was determined by CFSE. APC were irradiated (30Gy). Proliferation and activation in MLR were compared against autologous control on day 7 of culture

MLR-BASED SUPPRESSION ASSAY

Suppression of alloreactive CD4 stimulation was carried out in culture with varying CD4 to suppressor ratios including the following: 1:0.125, 1:0.25, 1:0.5, and 1:1. CD8 suppressors autologous to CD4 responders or the APC were used, as well as a third party donor. Donor origin is annotated in Figure 59 and Table 2. Similar to the previously described flow-based suppression assay, on day 7, cells

are stained with florescent antibodies and fixed with 1% paraformaldehyde. Suppression was determined by comparison to CD4 response in the absence of suppressors.

MICE

C57BL/6 (B6) female mice were purchased from Taconic (Hudson, NY) and the UT Southwestern Mouse Breeding Core Facility (Dallas, TX). SJL/J female mice were purchased from National Cancer Institute (Bethesda, MD). All mice were housed and bred in the UT Southwestern Medical Center Animal Resource Center and used according to approved IACUC protocols.

MOUSE IMMUNIZATION

Six to 8 week-old C57BL/6 mice were immunized subcutaneously at two injection sites with 200 μg MOG35–55 (MEVGWYRSPFSRVVHLYRNGK, UT Southwestern Protein Chemistry Technology Center) emulsified in CFA supplemented with 4 mg/ml Mycobacterium tuberculosis (MTB, H37Ra, Difco). Ovalbumin peptide 323–339 (OVA323–339, ISQAVHAAHAEINEAGR) was used as a peptide control. On days 0 and 2 post-immunization, 250 ng of pertussis toxin (PTX, List Biological Laboratories) was administered intraperitoneally in 100 μl of phosphate buffered saline (PBS).

MURINE FLOW-BASED SUPPRESSION ASSAY

Splenocytes from immunized mice were harvested. CD8+ cells were magnetically separated using a negative selection protocol (Miltenyi Biotech, Germany) to recover "untouched" CD8+ cells (>90%). Irradiated splenocytes from naïve or immunized mice were used as APC at a ratio of 1:1 (CD4+ T cells:APC). Cells

were harvested on glass fibers mats and counted using a Betaplate counter (Wallac, Gaithersburg, MD). Proliferation assays were performed using a carboxyfluorescein succinimidyl ester (CFSE)-dilution assay as described previously. Splenocytes were harvested and used either in bulk proliferation assays or as a source for CD8+ cells, which were isolated using a negative selection magnetic bead protocol. Bulk splenocytes or "untouched" CD8+ cells were suspended at 1×10^6 cells/ml in PBS and incubated at 37 °C for 7 min with 0.25 μM CFSE, followed by addition of serum and two PBS washes. Subsequently, CD8+ cells were suspended at 0.5–1 × 106/ml of media. On day 5, cells were washed with staining buffer and labeled with phycoerythrin (PE)-conjugated-anti-CD8 and allophycocyanin (APC)-conjugated-anti-CD4 antibodies (Caltag/Invitrogen, Carlsbad, CA). After incubation for 30 minutes at 4 °C, cells were washed and fixed in 1% paraformaldehyde (PFA, Electron Microscopy Sciences, Hattfield, PA). Flow cytometric data were acquired on BD LSR II flow cytometer using FACSDiva software. For analysis, FlowJo (Treestar, Ashland, OR) software was used to gate on lymphocytes and further on the CD4+ CD8− or CD8+ CD4− T cell subsets.

SHRNA KNOCKDOWN OF ACTIVATED AND NATURAL FOXP3

Human FOXP3 specific lentiviral particles and ShRNAs were obtained from Santa Cruz biotechnology (CA, USA). CD4+CD25- (Treg depleted) cells were thawed and infected with FOXP3 shRNA lentivirus or control (scrambled) shRNA with polybrene (Sigma-Aldrich Life Sciences, St. Louis, MO) for 24 hours, then washed and resuspended with fresh autologous APC. After an

additional 24 hours, CD4+ cells were stimulated with anti-CD3 plus puromycin selective antibiotic, concentration determined per standard curve killing assay. After 48 hours, CD4+ cells were washed and resuspended with more APC, anti-CD3, and puromycin. After 48 hours, CD4+ cells were intra-cellularly stained with FOXP3 or set up in 7 day flow-based suppression assay with autologous responders as described earlier.

CHAPTER 3: RESULTS

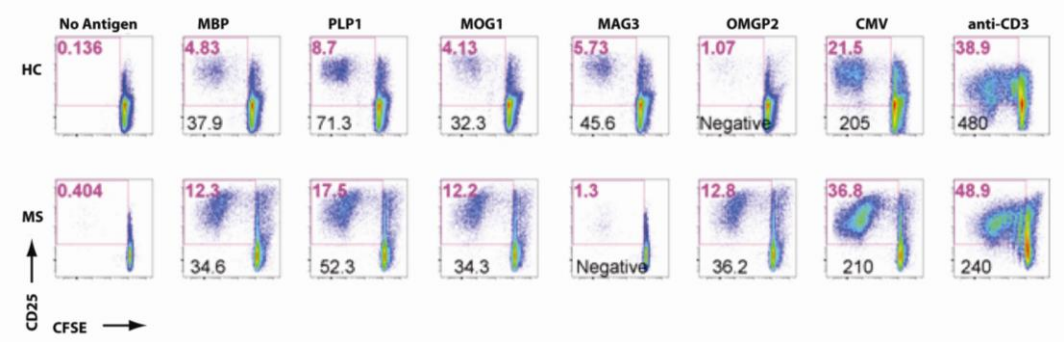
CD8+ T CELLS IN MULTIPLE SCLEROSIS

MULTIPLE SCLEROSIS PATIENTS AND HEALTHY CONTROL SUBJECTS SHARE SIMILAR T CELL RESPONSES

Most prior studies comparing CNS-specific T-cell responses between MS patients and healthy subjects have employed proliferation assays using bulk PBMC. Using CFSE-based flow cytometric proliferation assays, my lab has shown a high prevalence of CD4+ and CD8+ T-cell responses to neuroantigens in both healthy subjects and MS patients, with some functional differences [202]. In the current study, I performed CFSE assays using magnetically purified CD4+CD25- and CD8+ cells. I observed that, similar to bulk PBMC, purified populations of CD4+CD25- and CD8+ T-cells from treatment-naïve MS patients (MS) and healthy control subjects (HC) showed similar responses to neuroantigens, foreign antigens and mitogenic (anti-CD3) stimulation (

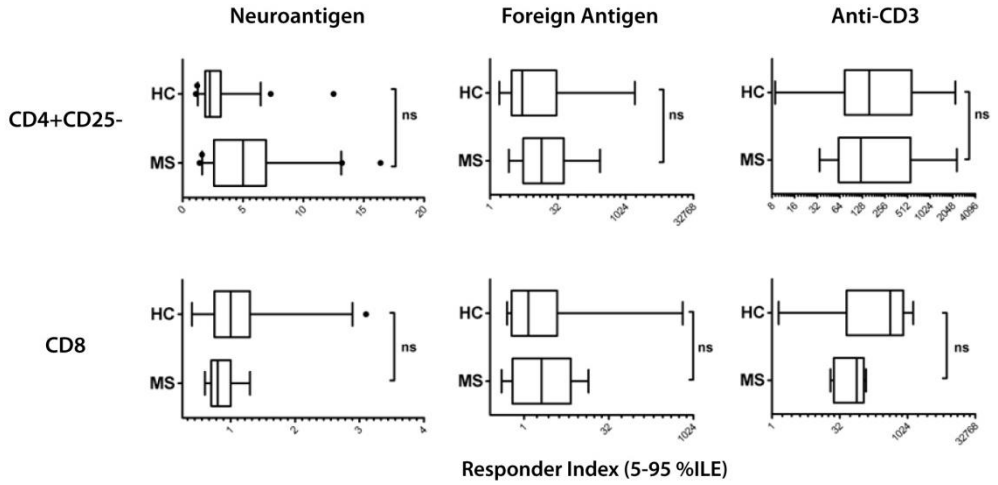
Figure 10-Figure 11). Figure 10 shows examples of CD4+ T cell proliferation from representative HC and MS subjects, whereas Figure 11 shows cumulative data from 15 HC and 11 MS, representing 50 and 37 detectable CNS-specific CD4 responses and 25 and 13 CD8 responses, respectively.

Figure 10: Multiple sclerosis patients and healthy control subjects share similar T-cell responses



CFSE-based proliferation assays were performed on purified CD4+CD25- or CD8+ T-cells from 15 HC and 11 MS patients. Representative responses from CD4+ T-cells from a single HC (top row) and single MS patient (bottom row) are shown, with CFSE on X-axis and CD25 on the Y-axis. Various stimuli are indicated above each column. The numbers in red toward the top of each dotplot indicate the %CD25+/CFSE(low) (activated/proliferating) cells, representing the response. Numbers in black toward the bottom represent the response index (RI), calculated based on background proliferation in the absence of any stimulus. "Negative" represents lack of a response, based on criteria described in the methods.

Figure 11: Multiple sclerosis patients and healthy control subjects share similar T-cell responses

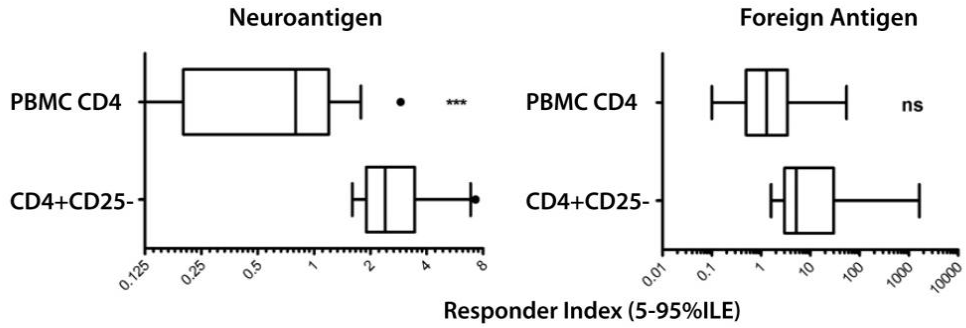


Cumulative results from 15 HC and 11 MS patients (9 neuroantigenic responders) are shown as RI for both CD4 responses (top row) and CD8 responses (bottom row), stimulated with neuroantigens, foreign antigens or anti-CD3 (as indicated). These results represent 85 and 60 positive assays with neuroantigens performed on HC and MS, respectively. P values >0.05 were not significant with "ns" notated where applied in figures.

I also performed parallel assays using bulk PBMC versus purified CD4+CD25- T-cells [i.e., in the absence of CD8+ T-cells and CD4+CD25+ regulatory T-cells], predominantly using HC PBMC. I observed that depletion of CD8+ T-cells and CD25+ cells, resulted in a significant increase in CD4+ T-cell responses to neuroantigens, but not to control foreign antigens like CMV or TT (

Figure 12). This suggested that CNS-specific CD8+ T-cells may possess immune suppressive ability. We therefore set about designing a novel suppression assay.

Figure 12: Depletion of CD8+ T cells and CD25+ cells increased CD4+ T-cell responses to neuroantigens, but not to control foreign antigens



From 9 HC, CFSE-based proliferation assays were performed on both bulk PBMC as well as sorted CD4+CD25- T-cells. Cumulative results from gated CD4 responses from each condition are shown as RI. *** indicates significant elevation of neuroantigen-specific responses ($p<0.001$), whereas foreign antigen-specific responses were not significantly different (ns).

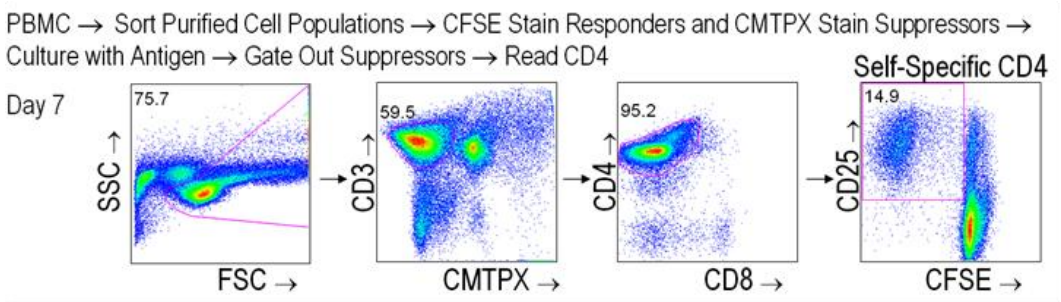
NOVEL FLOW-CYTOMETRY-BASED SUPPRESSION ASSAY

The assay system utilized in my studies took advantage a proliferation dye (CFSE), a cellular tracking dye (CMTPX) and overlapping antigenic peptide pools to monitor neuroantigen-specific CD8+ T-cell suppressive ability (Figure 13, Figure 14, Figure 15). This assay has excellent sensitivity and specificity for detecting functional antigen-specific suppressive ability, by allowing the exclusion of suppressor populations from the analysis. Moreover, it allows an unbiased characterization of T-cell suppressive ability without limited range of HLA haplotype or epitopes. Finally, the assay also enables separate concurrent characterization of CD4+ and CD8+ T cell responses within the same culture. Thus, this unique approach allowed me to discover and quantify this novel autoregulatory function of CNS-specific CD8+ T-cells and activated regulatory T cell populations described below.

I hypothesized that CNS-specific CD8+ T cells may possess regulatory function. To test this hypothesis, I first took advantage of a sensitive flow cytometry-based suppression assay [90] to measure suppressive ability of bulk autologous CD8+ T-cells (Figure 14A). This assay measured the proliferation and activation of CFSE-stained CD4+CD25- responder T-cells. Putative suppressor cells were stained with a tracker dye, CMTPX [306], allowing their exclusion from the analysis. CMTPX-stained CD4+CD25+ (positive control), CD8+ or CD4+CD25- (negative control) T-cells were added in increasing numbers and their effect on responder proliferation was quantified, by normalizing to the RI of CD4+CD25- T-cells (treated as 100% proliferation or 0% suppression).

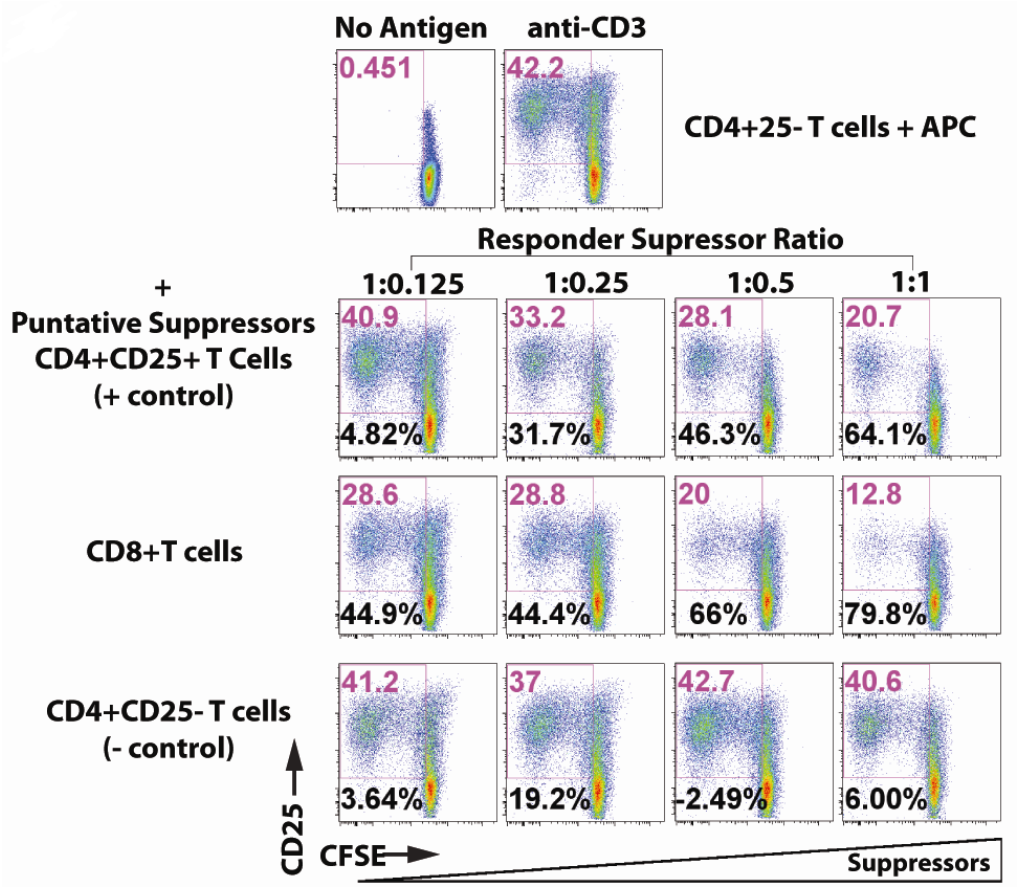
Representative dotplots of anti-CD3-stimulated assays are shown in Figure 14, with cumulative % proliferation shown in Figure 15 and % suppression from a single responder to suppressor ratio in Figure 16. Using anti-CD3 stimulation, we observed consistent suppressive activity in the CD4+CD25+ and CD8+ populations, while CMTPX-stained CD4+CD25- T-cells did not significantly dampen pan-stimulated CD4+CD25- T-cells (negative control). Interestingly, non-fractionated CD8+ T-cells showed greater suppressive capacity than CD4+CD25+ T-cells, a fraction known to contain regulatory T-cells.

Figure 13: Basic design and gating strategy of flow-based suppression assay



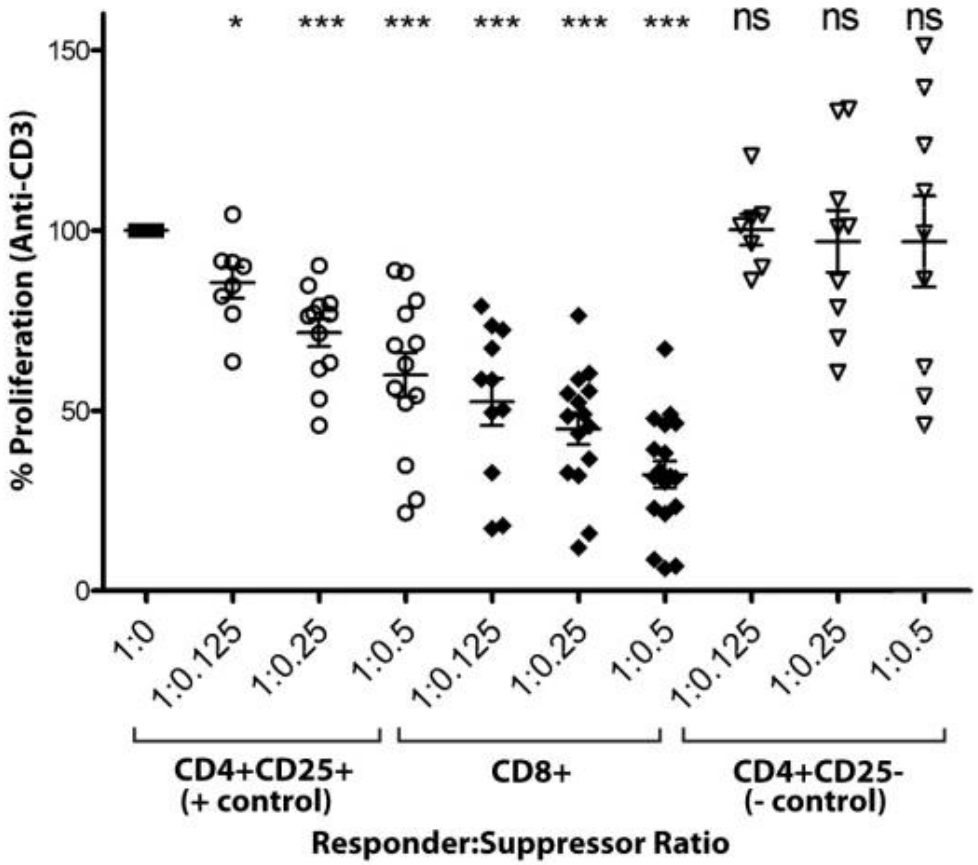
PBMC were magnetically-sorted into CD4+CD25- and CD8+ cells, and then stained with CFSE and CMTPX respectively. Co-cultured with CD8+ suppressors, CD4+ responders were cultured in RPMI containing 10% human serum for seven days with PLP antigen and autologous APC. All cells were stained for FACS analysis, and representative pseudo-color plots were gated on CD4+ T cells.

Figure 14: Flow-based suppression Assay is validated: CD4+CD25+ Tregs and CD8+ T-cells suppress anti-CD3-stimulated CD4+ T-cells, but not CD4+CD25- cells



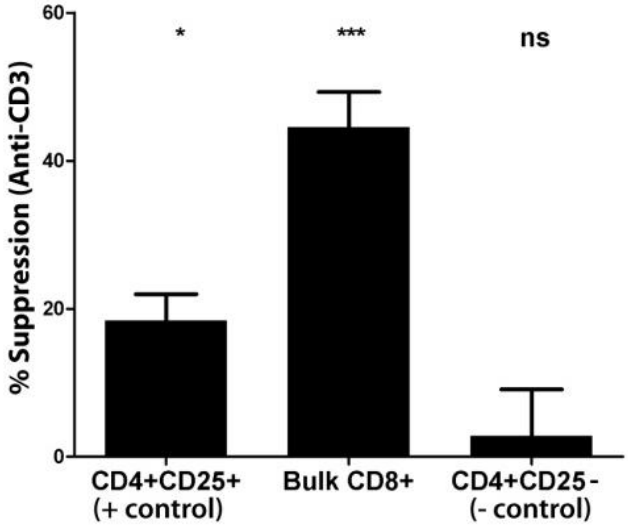
CFSE-stained healthy ex vivo purified CD4+CD25- T-cells were used as responders in anti-CD3-stimulated suppression assays. Dotplots from a single representative experiment demonstrate CFSE on the X-axis and CD25 expression on Y-axis. Indicated in red at the top of each dot plot is the gated percentage of CD25+/CFSE-low cells (activated and proliferating), representing the "response". Indicated in black in the lower left is the calculated %suppression, based on normalizing to the anti-CD3-mediated response in the absence of suppressors (top row). Indicated to the left of the bottom three rows are the CMTPX-stained cell populations used as suppressors at the indicated ratios over each column. The results are representative of 15 flow-based suppression assays from 15 healthy controls.

Figure 15: Flow-based suppression Assay is validated: CD4+CD25+ Tregs and CD8+ T-cells suppress anti-CD3-stimulated CD4+ T-cells, but not CD4+CD25- cells



Cumulative results from suppression assays from 15 healthy controls are displayed as percent proliferative response normalized to the response without suppressors (defined as 100%), indicated as 1:0. Open circles represent the response in the presence of increasing numbers of CD4+CD25+ T-cells (positive controls), closed diamonds for bulk CD8+ T-cells and open triangles for CD4+CD25- T-cells (negative controls). P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values 0.01 to 0.05, and <0.001 were significant with "*" and "***" notated respectively.

Figure 16: Anti-CD3-stimulated and neuroantigen-specific CD8+ T-cells suppress CD4+ T-cells

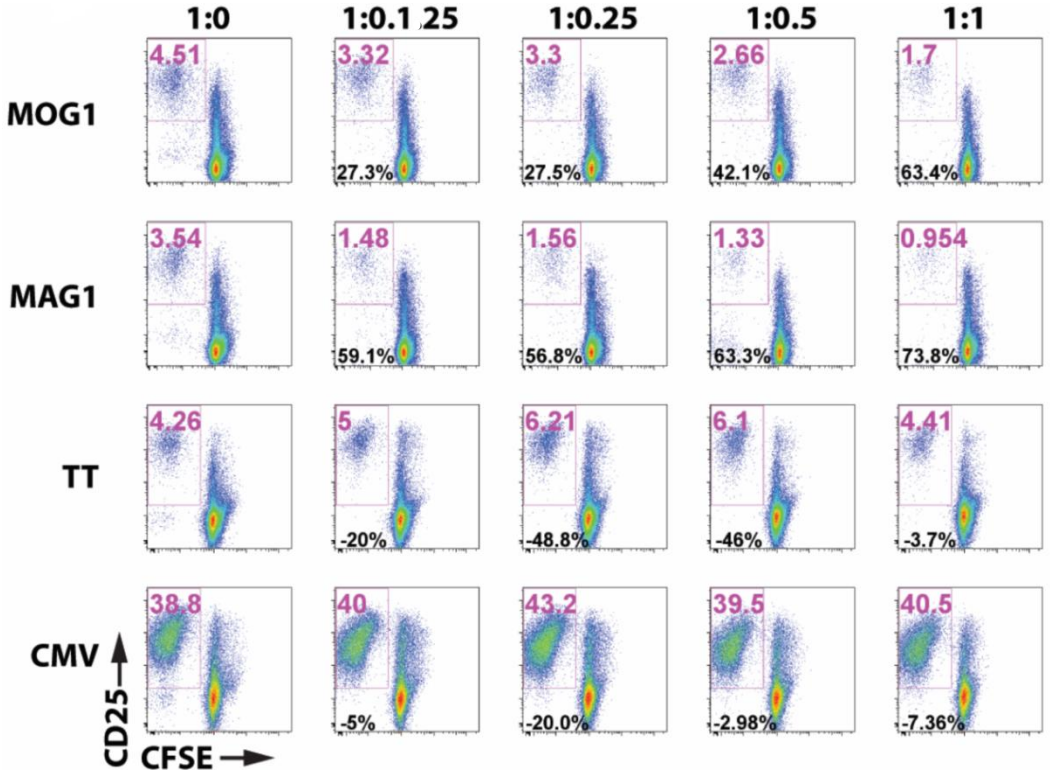


Results from Figure 15 are represented as % suppression at a single responder: suppressor ratio (1:0.25). P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values 0.01 to 0.05, and <0.001 were significant with "*", and "***" notated respectively.

CD8+ T-CELLS SPECIFIC FOR CNS AUTOANTIGENS, BUT NOT THOSE SPECIFIC FOR CONTROL FOREIGN ANTIGENS, SUPPRESS CD4+ T-CELL PROLIFERATION

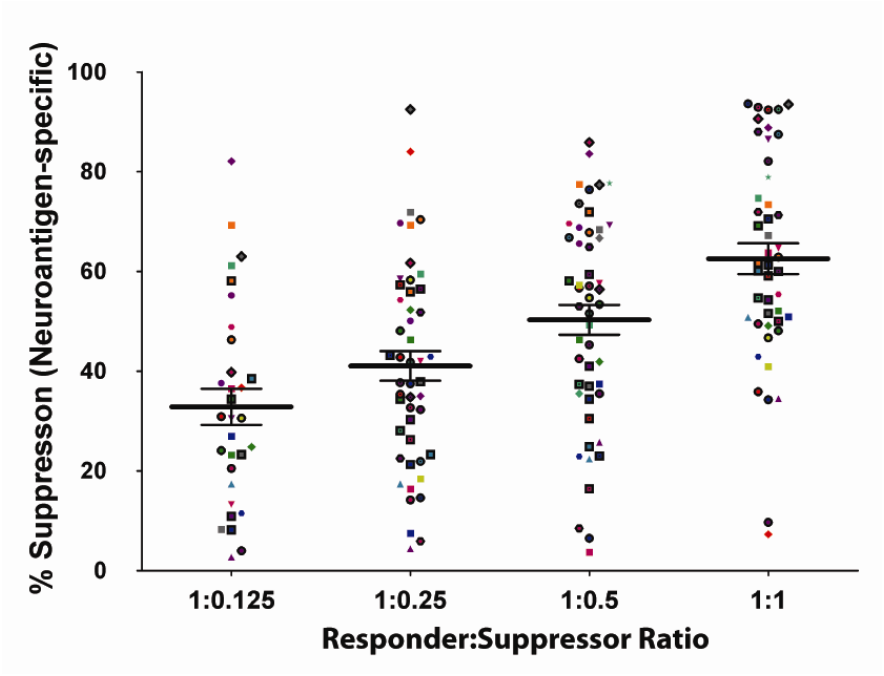
I then sought to evaluate the suppressive ability of CD8+ T-cells in cultures stimulated with specific antigens by conducting suppression assays using a panel of CNS and control antigens. Positive CD4 T-cell responses to specific antigens were screened with MBP, MOG, PLP, MAG, OMGP, CMV, and TT and suppression was quantified. Figure 17 shows representative responses from one HC, where the addition of increasing numbers of CD8+ T-cells suppressed the proliferation and activation of neuroantigen-stimulated responses, in contrast to foreign-antigen-stimulated responses. Figure 18 shows cumulative data from 15 HC, demonstrating consistent dose-dependent suppression in neuroantigen-stimulated cultures (left), contrasting with lack of consistent suppression in response to foreign antigens (right), which in many cases led to enhanced proliferation of the responders [denoted as "negative suppression"]. This suggested that neuroantigen-specific CD8+ T-cells obtained *ex vivo* possessed immune suppressive ability, whereas foreign antigen-specific ones did not show consistent suppression.

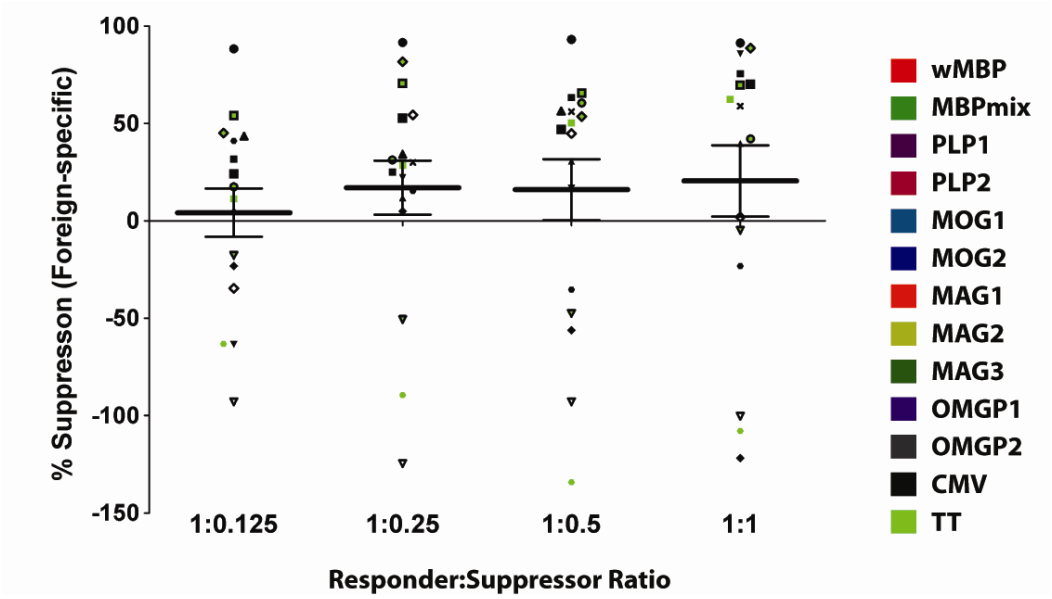
Figure 17: Neuroantigen-specific CD8+ T-cells suppress CD4+ T-cells



Representative dotplots from a single subject demonstrate CD8-mediated suppression assays in the presence of neuroantigens (MOG1, MAG1) and foreign antigen (TT, CMV). The left column represents CD4+CD25- responders only, where positive responses were selected to evaluate suppression. The right three columns contain increasing numbers of CMTPX-stained bulk CD8+ T-cells, with % proliferation and % suppression indicated.

Figure 18: Neuroantigen-specific CD8+ T-cells suppress CD4+ T-cells



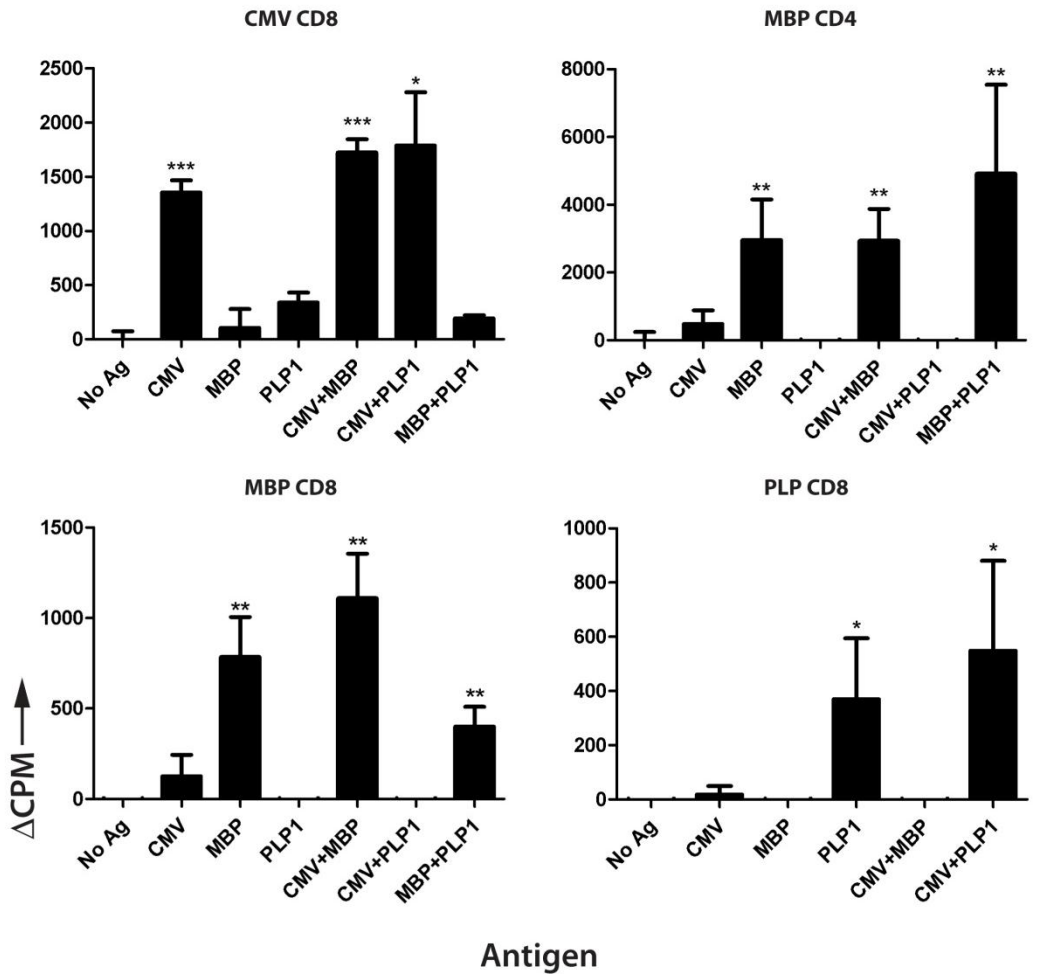


Cumulative results are shown from 67 suppression assays. Data points represent neuroantigen- (top) and foreign antigen-(bottom) specific % suppression. Each of 15 subjects is indicated by a different shape. Neuroantigen or foreign antigen used in the suppression assay is indicated by the color legend at right (for some proteins, multiple pools were made to limit the number of peptides in each pool, as described previously [202].

CNS-SPECIFIC CD8+ T-CELLS REQUIRE STIMULATION WITH COGNATE ANTIGEN FOR SUPPRESSIVE ACTIVITY

In the experiments above, the antigens added to the bulk culture presumably stimulated both the CD4+ T-cells and CD8+ T-cells. Thus, it was possible only to test the effect of neuroantigen-stimulated CD8+ T-cells on CD4+ T-cells stimulated by the same antigenic peptides. To ascertain that these results were based on cognate antigen-specific recognition, I generated over thirty-four CD4+ and CD8+ T-cell lines, using PBMC from 8 HC. Specificity was confirmed by ^{3}H-thymidine uptake, showing reactivity to the intended antigen but not to other CNS or foreign antigens (Figure 19).

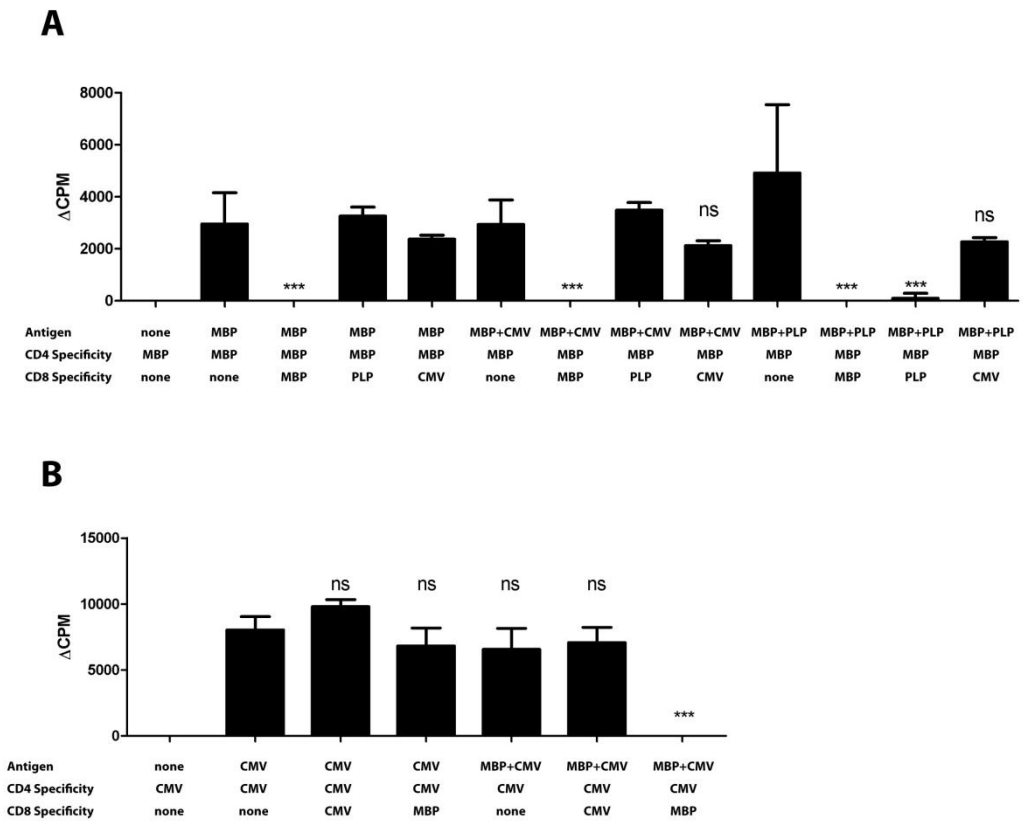
Figure 19: Antigenic specificity of T cell lines



CD4+ and CD8+ T cell lines of multiple specificities were grown from PBMC, as described in methods. Antigenic specificity was confirmed using 3H-Thymidine assays, before using these lines in suppression assays (Figure 14). These bar graphs show ΔCPM (counts per minute, background subtracted) of representative lines, confirming response to the desired antigen (*) but not other antigens. These results are representative of 32 T cell lines derived from 8 HC. P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values 0.01 to 0.05, 0.001 to 0.01, and <0.001 were significant with "*", "**", and "***" notated respectively.

Using these lines, I performed autologous ^{3}H-thymidine-based suppression assays, culturing CNS- or foreign antigen-specific CD4+ T-cells alone or in the presence of CNS- or foreign antigen-specific CD8+ T-cells in various combinations. These cultures contained APC with antigens that would stimulate just the CD4+ T-cells or both CD4+ and CD8+ T-cells (Figure 20). Figure 20A shows a single MBP-specific CD4+ T-cell line, cultured with autologous MBP-, PLP- or CMV-specific CD8+ T-cells. When cultured in the absence of any CD8+ T-cells, the MBP-specific CD4+ T-cells showed a similar proliferative response to stimulation by MBP, MBP+CMV or MBP+PLP. The addition of MBP-specific CD8+ T-cells to the MBP-stimulated cultures resulted in robust suppression of the response. Importantly, the addition of PLP- or CMV-specific CD8+ T-cells did not affect cultures stimulated only by MBP. However, when PLP antigen was added, the PLP-specific CD8+ T-cells suppressed MBP-specific CD4 proliferation. Most interestingly, this was not true of CMV-specific CD8+ T-cells. In cultures containing CMV peptides and CMV-specific CD8+ T-cells, no significant suppressive effect was exerted. Figure 20B demonstrates an example of a CMV-specific CD4+ T-cell line, in combination with autologous CMV-specific or MBP-specific CD8+ T-cells. Again, in contrast to CMV-specific CD8+ T-cells, MBP-specific CD8+ T-cells had a suppressive effect in the presence of their cognate antigen. Thus, similar to bulk cultures (Figure 17, Figure 18), antigen-specific lines confirmed that neuroantigen-specific CD8+ T-cells had robust immune suppressive properties compared to foreign antigen-specific CD8+ T-cells and required the presence of cognate antigen.

Figure 20: Activated neuroantigen-specific CD8+ T-cells suppress CD4+ T-cells

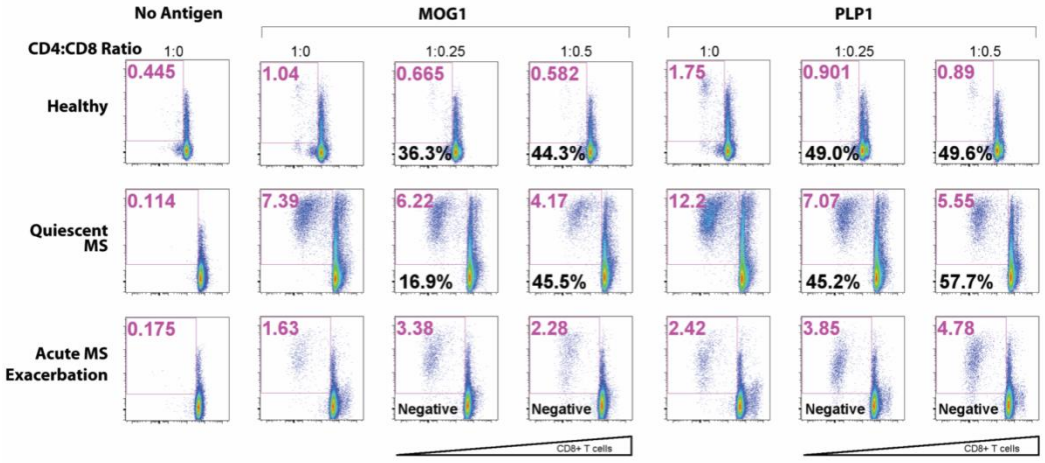


Responder CD4+ T-cell lines were cultured with APC and indicated antigens in the presence or absence of the indicated CD8+ T-cell lines. ^{3}H-Thymidine-based proliferation assays were performed. Panel A shows ΔCPM (background subtracted) from a single MBP-specific CD4+ line and Panel B shows a CMV-specific CD4+ line. The results are representative of 8 independent assays, each repeated twice, with lines obtained from 8 different HC. Further illustration provided in Figure 42. P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values <0.001 were significant with "***" notated respectively.

CNS-SPECIFIC SUPPRESSIVE ABILITY IS SIGNIFICANTLY DIMINISHED DURING ACUTE EXACERBATION OF MS AND RECOVERS DURING REMISSION

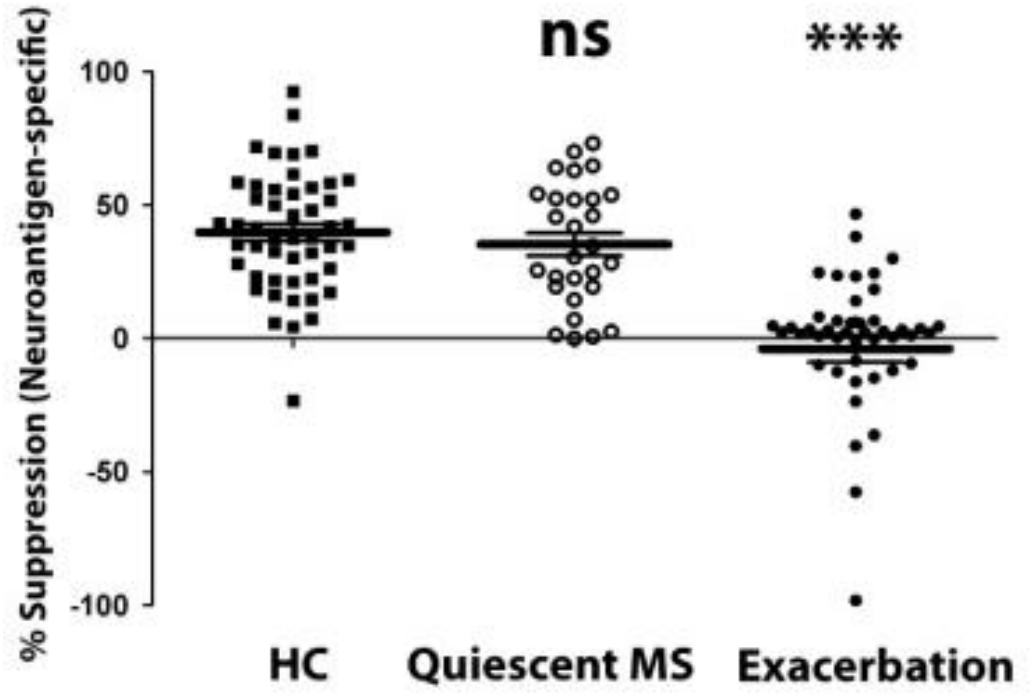
I then postulated that neuroantigen-specific CD8+ T-cell suppressive ability may be relevant in MS and may influence the clinical disease course. Several studies by others and my lab have demonstrated a global deficit in regulatory CD4+CD25+ [243, 244, 307] or CD8+ T-cell function in MS [104, 126, 308]. To test the possibility that CNS-specific suppressive ability has a bearing on MS clinical presentation, I compared flow-based suppression assays on PBMC from 15 HC, 11 treatment-naive RRMS patients (quiescent MS) and 9 treatment-naïve MS patients during an acute exacerbation (Figure 21, Figure 22, Figure 23). CD8+ T-cells from HC and quiescent MS patients showed similar neuroantigen-specific suppressive ability (Figure 21, Figure 22). Interestingly, CD8+ T-cells obtained during an acute clinical episode showed significantly lower neuroantigen-specific suppressor ability, whether viewed as suppression stimulated by independent multiple antigens (Figure 22) or as a mean neuroantigen-specific suppression per subject (Figure 23). This corroborated with a global CD8 suppressor deficit, demonstrated in anti-CD3-stimulated suppressor assays (Figure 24), whereas none of the patients showed significant foreign antigen-specific CD8+ suppressor ability (Figure 25).

Figure 21: Neuroantigen-specific suppressive ability is deficient during acute MS exacerbation



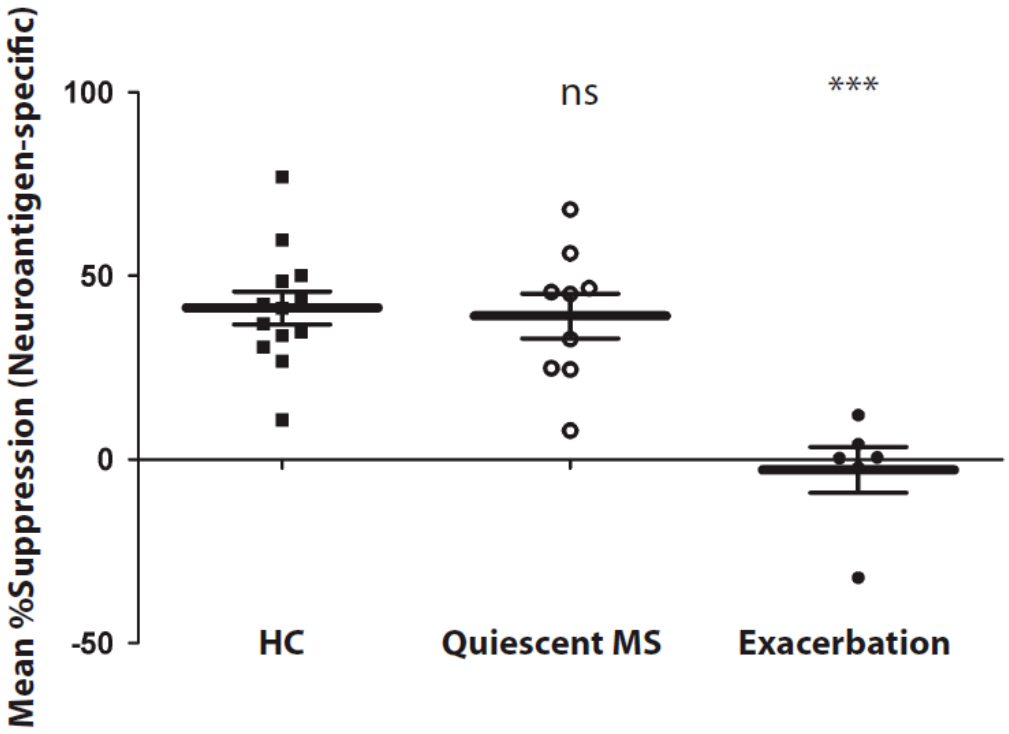
Ex vivo-purified, CFSE-stained CD4+CD25- T-cells from HC, quiescent MS patients or MS patients suffering from an acute exacerbation were used as responders in autologous suppression assays. Dotplots display CFSE vs. CD25 from representative subjects responding to two neuroantigens (MOG-pool 1 and PLP-pool 1) in the absence of suppressor cells (1:0) or with CD8+ T-cells added at indicated ratios. Red numbers at the top of each dotplot represent proliferative response, whereas the black numbers represent the calculated %suppression. This is representative 15 HC, 11 quiescent MS patients (9 responders) and 9 acute MS exacerbation patients (6 responders), equivalent to 50, 47, and 37 flow-based suppression assays, respectively.

Figure 22: Neuroantigen-specific suppressive ability is deficient during acute MS exacerbation



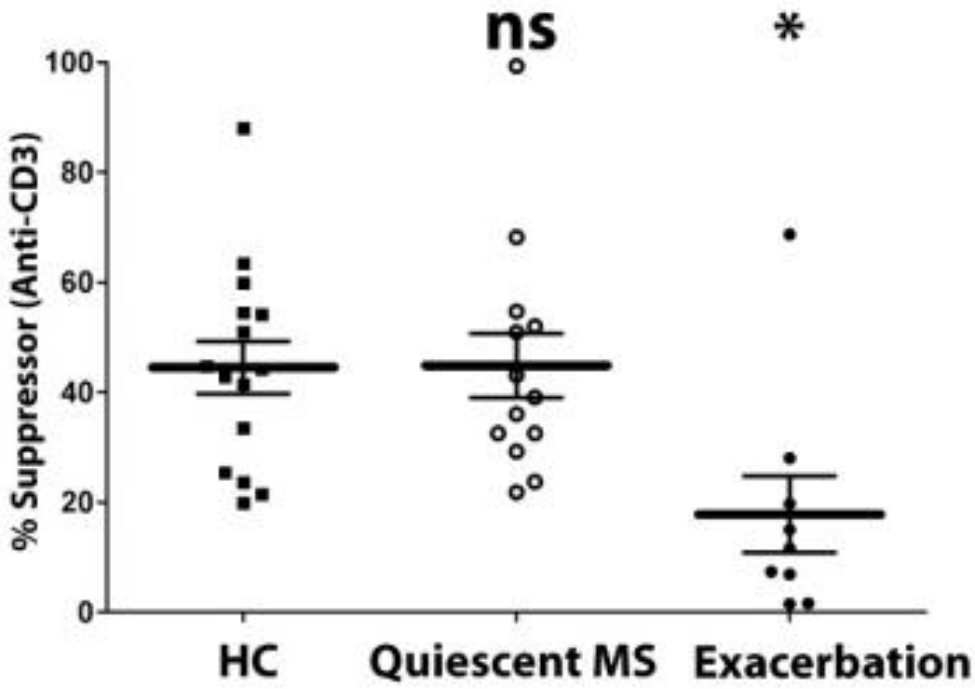
Panels show cumulative % suppression data at the 1:0.25 responder:suppressor ratio from assays containing neuroantigens, foreign antigens or anti-CD3, as indicated. P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values <0.001 were significant with "***" notated respectively.

Figure 23: Average neuroantigen-specific suppressive ability is deficient during acute MS exacerbation



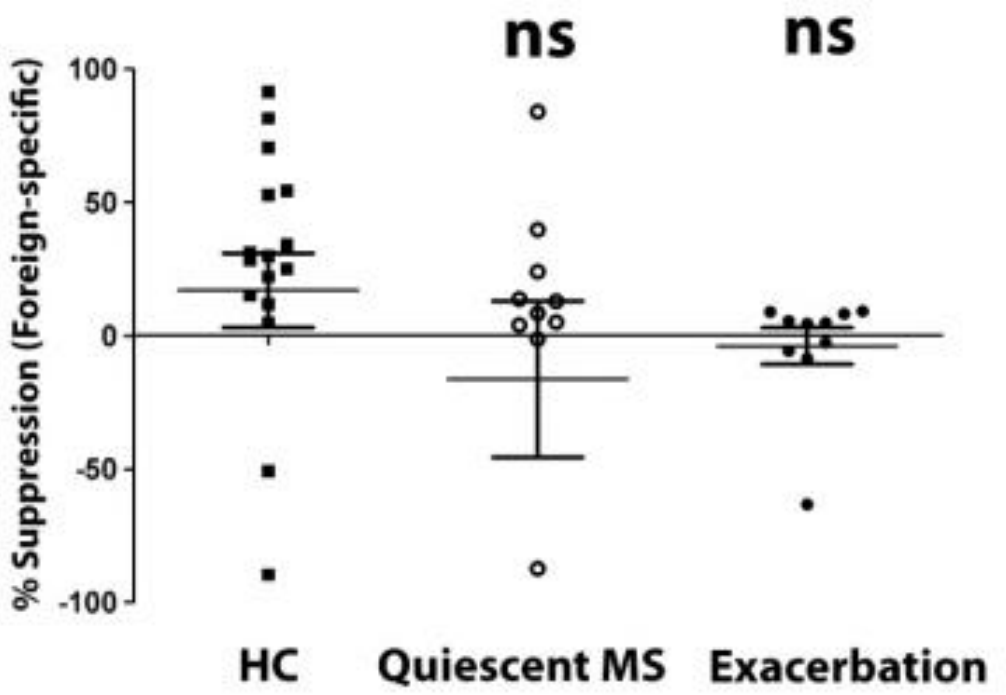
Data from Figure 22 were re-evaluated to obtain a single "mean neuroantigen-specific suppression" per subject, by averaging the % suppression against various neuroantigens. Each dot represents the mean neuroantigen-specific CD8 suppression per subject in HC, quiescent MS patients and acute MS exacerbation patients, as indicated (ns=not significant, compared to HC; *** = $p<0.001$, compared to either HC or quiescent MS). P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values <0.001 were significant with "***" notated respectively.

Figure 24: Global CD8+ T cell suppressive ability is deficient during acute MS exacerbation



Panels show cumulative % suppression data at the 1:0.25 responder:suppressor ratio from assays containing anti-CD3. P values >0.05 were not significant with "ns" notated where applied in figures. Likewise P values 0.01 to 0.05 were significant with "*" notated respectively.

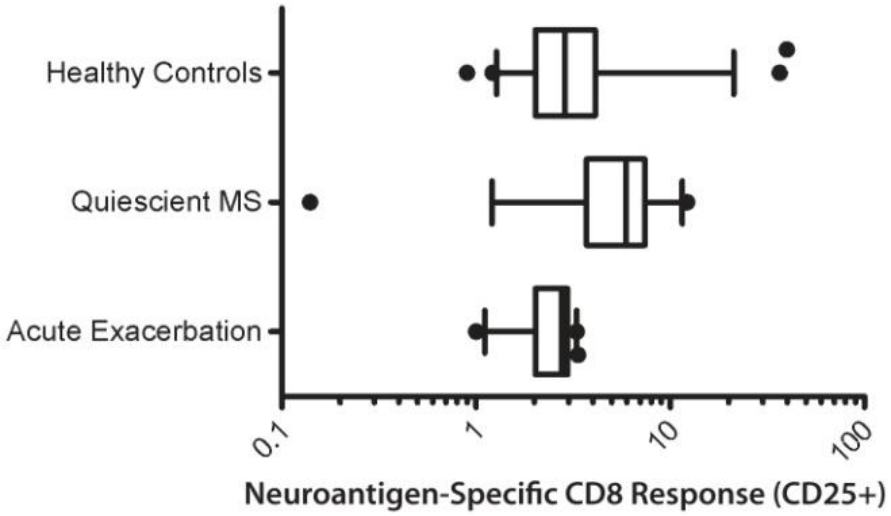
Figure 25: Foreign-specific suppressive ability is unchanged during acute MS exacerbation



Panels show cumulative % suppression data at the 1:0.25 responder:suppressor ratio from assays containing foreign antigens. P values >0.05 were not significant with "ns" notated where applied in figures.

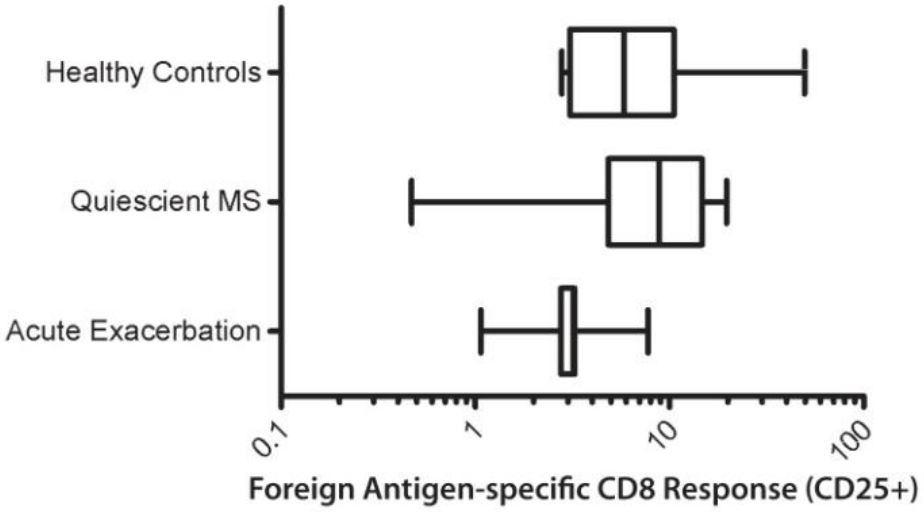
To address whether the lack of suppression could be explained by major changes in T-cell subsets, I first evaluated CD4:CD8 ratios across various cohorts and found no significant differences between any of the cohorts, especially between quiescent MS (1.75 ± 0.69) vs. acute exacerbation (2.12 ± 0.72). I further evaluated whether there may be an absence of CNS-specific CD8 reactivity in the peripheral blood during acute exacerbation or enhanced activation or proliferation of CNS-specific CD8 cells in the suppression assays. Using CMTPX as a cell tracker, I was able to specifically evaluate CD8 T-cell activation. While CMTPX is not optimal for use as a proliferation dye, I could evaluate total CD25 expression by the CMTPX-stained CD8 cells in these cultures. I found that, albeit slightly diminished, CNS-specific CD8 reactivity was detectable even during acute exacerbation (Figure 26, Figure 27, Figure 28), suggesting that these responses may be functionally different rather than simply quantitatively suppressed.

Figure 26: During suppression assays, MS and healthy controls share similar CD8+ T cell activation to neuroantigen



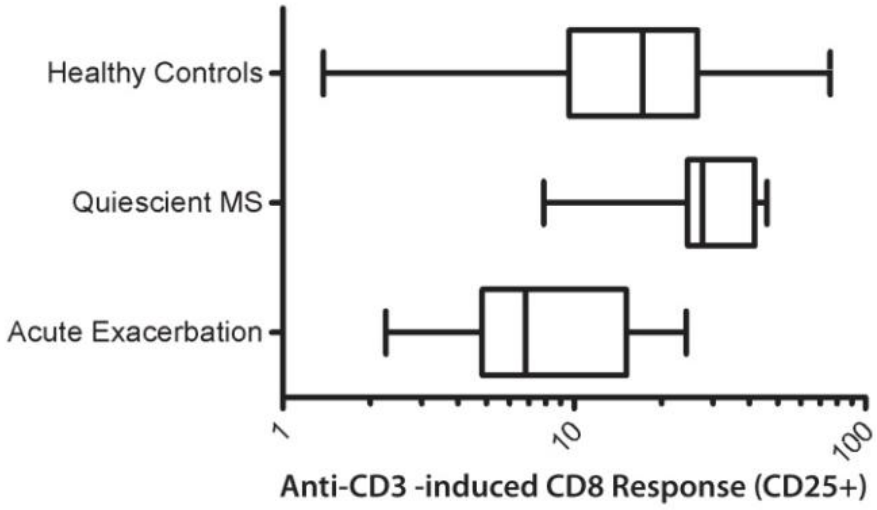
CMTPX-stained CD8+ T cells from suppression assays were evaluated for activation in the presence of neuroantigens by comparing their CD25 expression to that in the absence of antigen. Cumulative stimulation indices (percent CD25 with stimulus divided by percent CD25 with no stimulus) are shown from 50 neuroantigenic responses from 15 healthy controls, 29 responses from 11 quiescent MS patients, and 47 responses from 9 acute exacerbation patients. P values >0.05 were not significant with "ns" notated where applied in figures.

Figure 27: During suppression assays, MS and healthy controls share similar CD8+ T cell activation to foreign antigen



CMTPX-stained CD8+ T cells from suppression assays were evaluated for activation in the presence of foreign antigen (CMV, TT) by comparing their CD25 expression to that in the absence of antigen. Cumulative stimulation indices (percent CD25 with stimulus divided by percent CD25 with no stimulus) are shown from 17 foreign antigen responses from 15 healthy controls, 13 responses from 11 quiescent MS patients, and 11 responses from 9 acute exacerbation patients. P values >0.05 were not significant with "ns" notated where applied in figures.

Figure 28: During suppression assays, MS and healthy controls share similar CD8+ T cell activation to anti-CD3

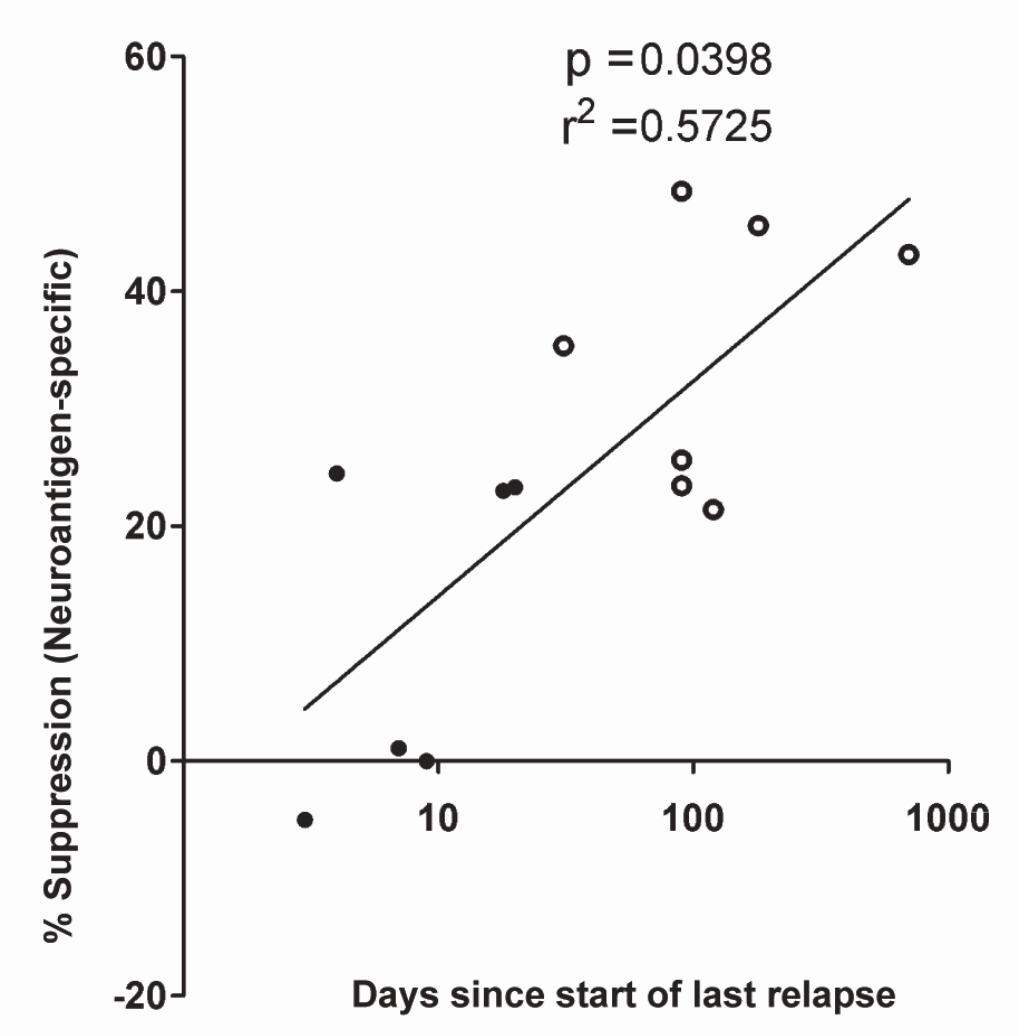


CMTPX-stained CD8+ T cells from suppression assays were evaluated for activation in the presence of anti-CD3 by comparing their CD25 expression to that in the absence of anti-CD3. Cumulative stimulation indices (percent CD25 with stimulus divided by percent CD25 with no stimulus) are shown from 15 anti-CD3 responses from 15 healthy controls, 11 responses from 11 quiescent MS patients, and 9 responses from 9 acute MS exacerbation patients. P values >0.05 were not significant with "ns" notated where applied in figures.

NEUROANTIGEN-SPECIFIC CD8+ T CELL SUPPRESSIVE ABILITY CORRELATES WITH DAYS SINCE LAST RELAPSE

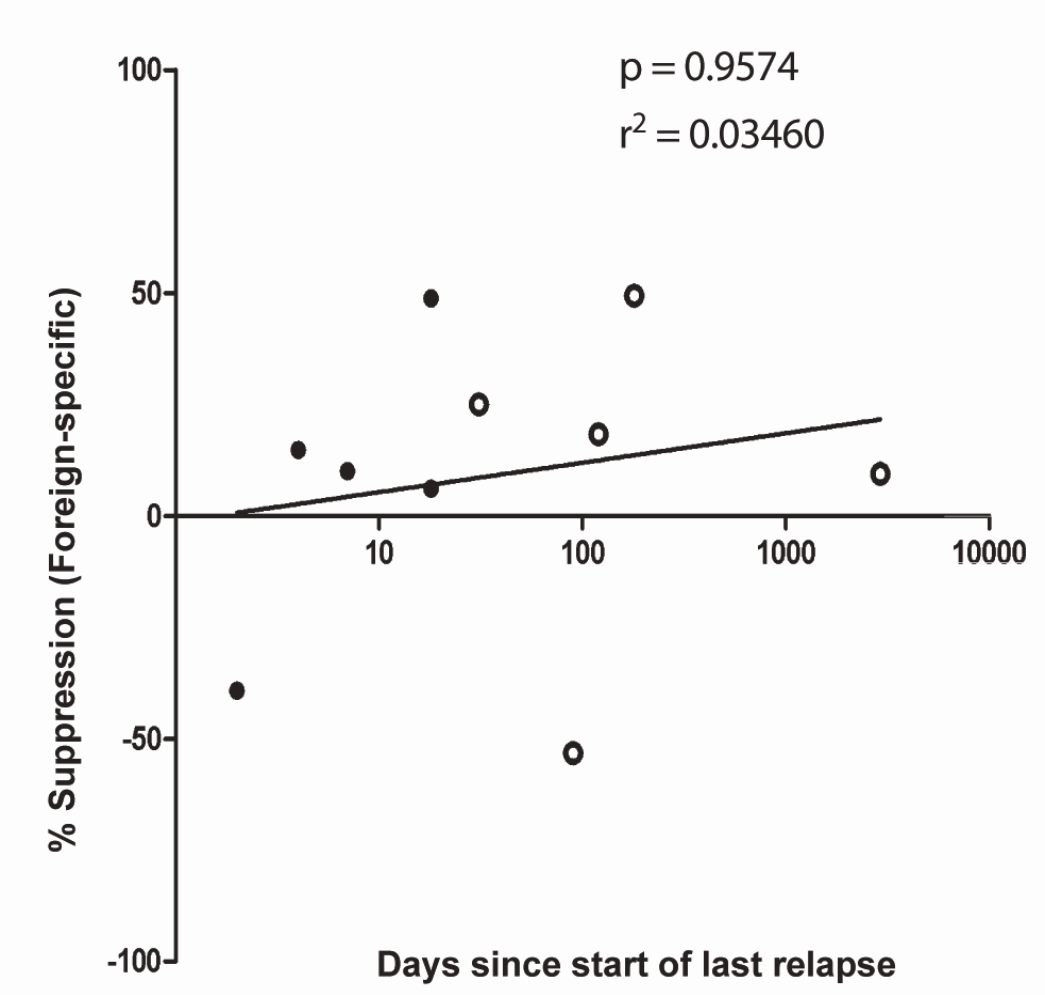
To gauge the clinical relevance of these findings further, I asked whether there was any correlation between CD8 suppressor ability and the distance from an acute clinical episode. I found that the duration from the latest clinical episode correlated significantly ($p = 0.0398$) with CNS-specific CD8 suppression (Figure 29), but not with foreign-specific ($p = 0.9574$) (Figure 30) or global (anti-CD3-mediated) suppression ($p = 0.4373$) (Figure 31). In contrast to foreign-specific and anti-CD3-induced CD8 suppression, most of the neuroantigen-specific CD8+ suppressive function, plotted versus time since last relapse, could be explained by the regression line demonstrated in Figure 29. This suggested that correction of the CNS-specific CD8 suppressor deficit would correlate with recovery from an acute relapse.

Figure 29: Neuroantigen-specific CD8+ T-cell suppressive-ability correlates with days since last relapse



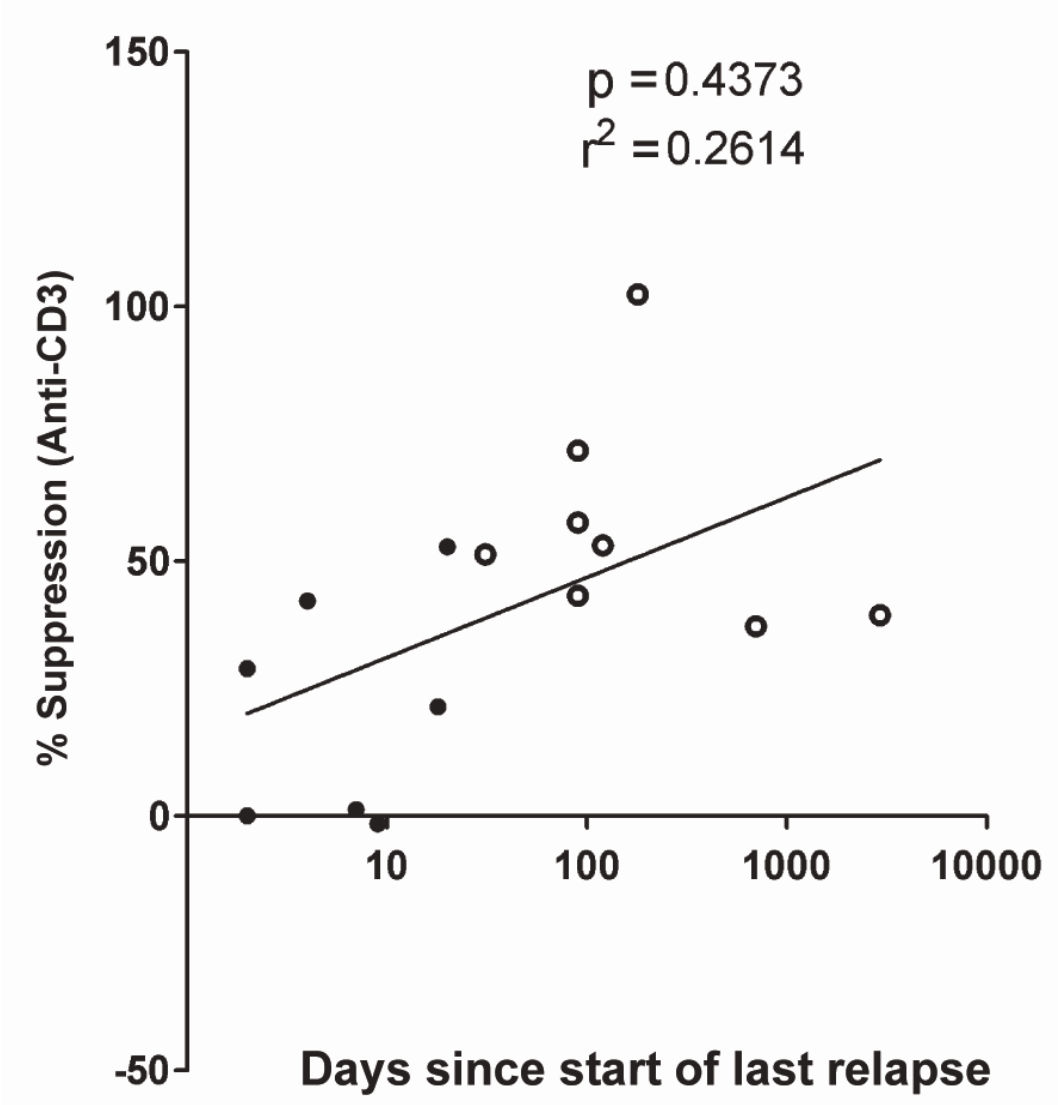
Each dot represents one of 13 MS patients. Dots represent average CD8+ T-cell suppressive ability of individual MS patients in the presence of neuroantigens. Closed and open circles are acute MS exacerbation and quiescent MS patients, respectively. R squared values are shown for nonlinear regression assuming a semi-log X line model. P values are shown for correlation analysis.

Figure 30: Foreign antigen-specific CD8+ T-cell suppressive-ability does not correlate with days since last relapse



Dots represent average CD8+ T-cell suppressive ability of individual MS patients in the presence of foreign antigens. Closed and open circles are acute MS exacerbation and quiescent MS patients, respectively. R squared values are shown for nonlinear regression assuming a semi-log X line model. P values are shown for correlation analysis.

Figure 31: Global CD8+ T-cell suppressive-ability does not strongly correlate with days since last relapse



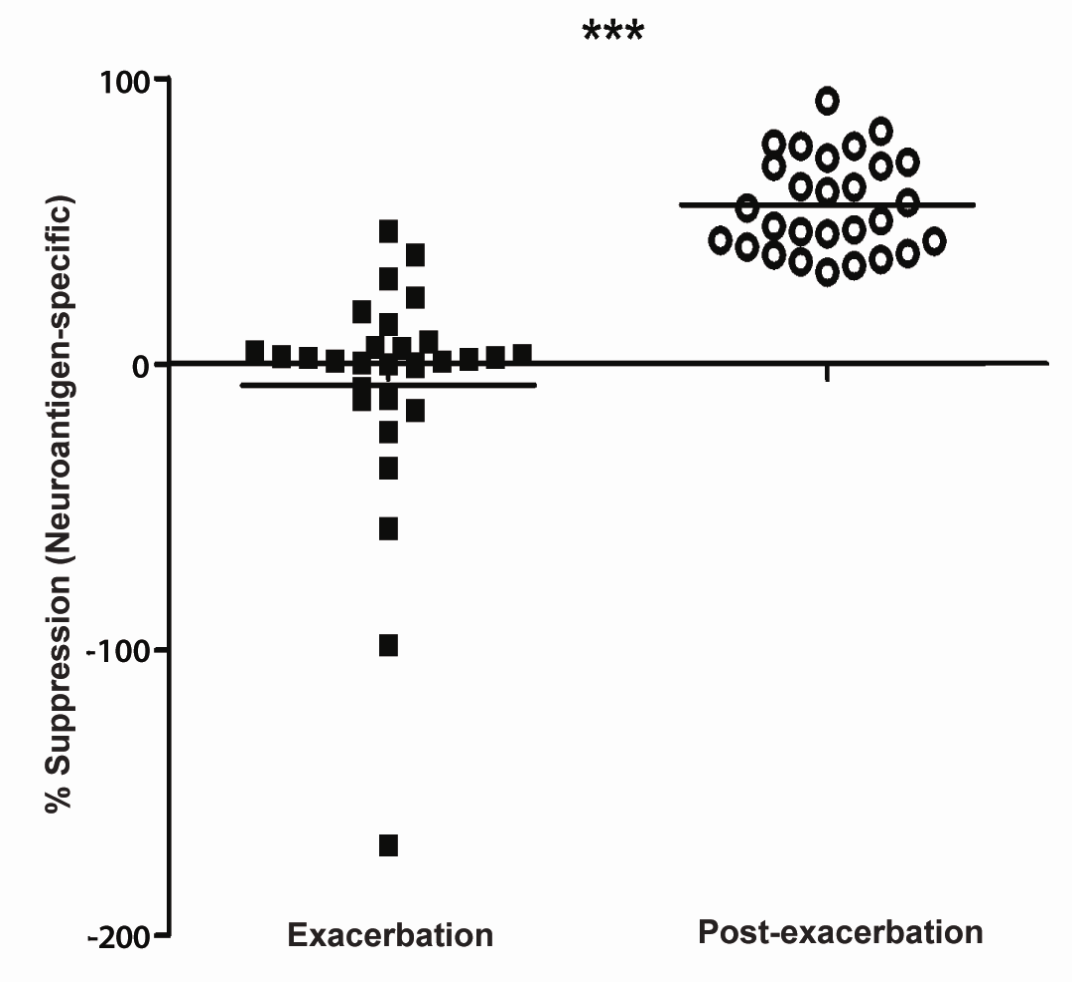
Dots represent average CD8+ T-cell suppressive ability of individual MS patients in the presence anti-CD3. Closed and open circles are acute MS exacerbation and quiescent MS patients, respectively. R squared values are shown for nonlinear regression assuming a semi-log X line model. P values are shown for correlation analysis.

NEUROANTIGEN-SPECIFIC CD8+ T CELL SUPPRESSIVE ABILITY CORRELATES WITH DAYS SINCE LAST RELAPSE AND RECOVERY

To test this hypothesis prospectively, I re-evaluated a subset of the subjects longitudinally, after their disease had become clinically quiescent either with or without immunomodulatory therapy (Figure 32, Figure 33). We observed a robust and significant recovery of the CNS-specific CD8 suppressive ability, whether viewed as suppression against multiple neuroantigens (Figure 32) or as mean suppression per subject (Figure 33). Again, foreign-specific CD8 suppression showed no changes over time (Figure 34), whereas there was some recovery of anti-CD3-based suppression (Figure 35). In one MS patient, I was able to perform the CNS-specific suppression assay before an exacerbation, shortly afterwards, and once again afterwards later (Figure 36). Compared to before the exacerbation, I observed an increase in the number of CNS-specific CD4+ T cell responses shortly after the exacerbation, represented by the number of data points at day -84 and +18, respectively. The number of CNS-specific CD4+ T cell responses in this one patient remained relative constant shortly after the exacerbation compared to approximately three months later, represented by the number of data points at day +18 and +118, respectively. Interestingly, the ability of the CD8+ T cells to suppress CNS-specific CD4+ T cell responses was marked improved 118 days after the exacerbation compared to the only 18 days after the exacerbation. This suggested that the immunological changes taking place during an exacerbation involve both a transient decrease in the CD8+ T cells to suppress neuroantigen-specific CD4+ T cells responses *and* a transient

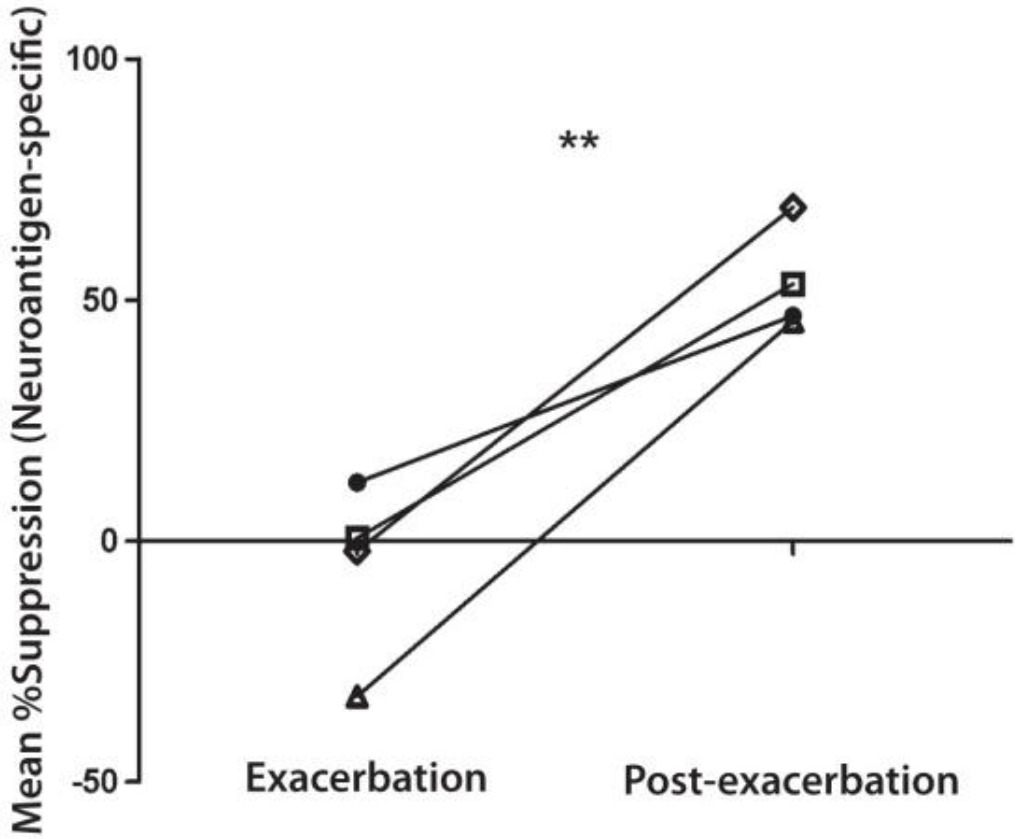
increase in numbers of CNS-specific CD4+ T cells and/or their resistance to suppressive ability of CD8+ T cells.

Figure 32: Neuroantigen-specific CD8+ T-cell suppressive-ability correlates with days since last relapse and recovery



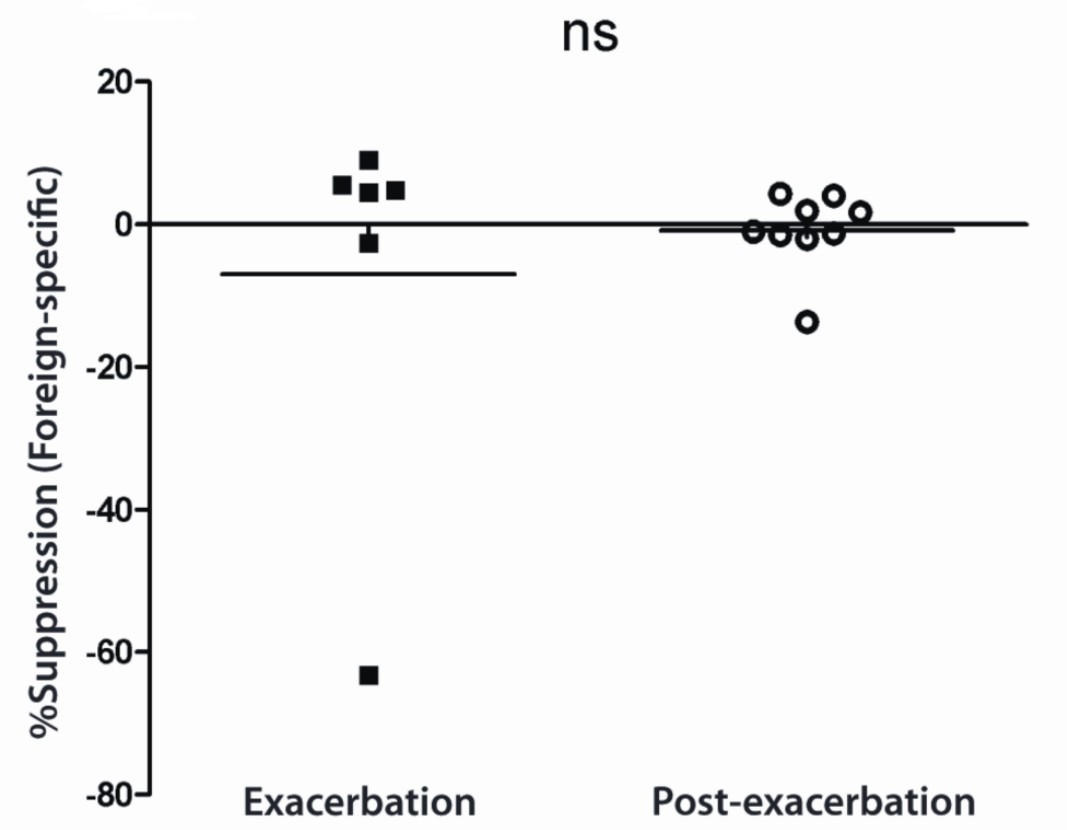
Dots represent neuroantigen-specific suppression assays performed longitudinally during exacerbation and after a quiescent clinical state as reached. Closed squares and open circles represent patients who averaged 12 and 81 days since start of last relapse, respectively. P values <0.001 were significant with "***" notated.

Figure 33: Mean neuroantigen-specific CD8+ T-cell suppression decreases transiently during acute MS exacerbation



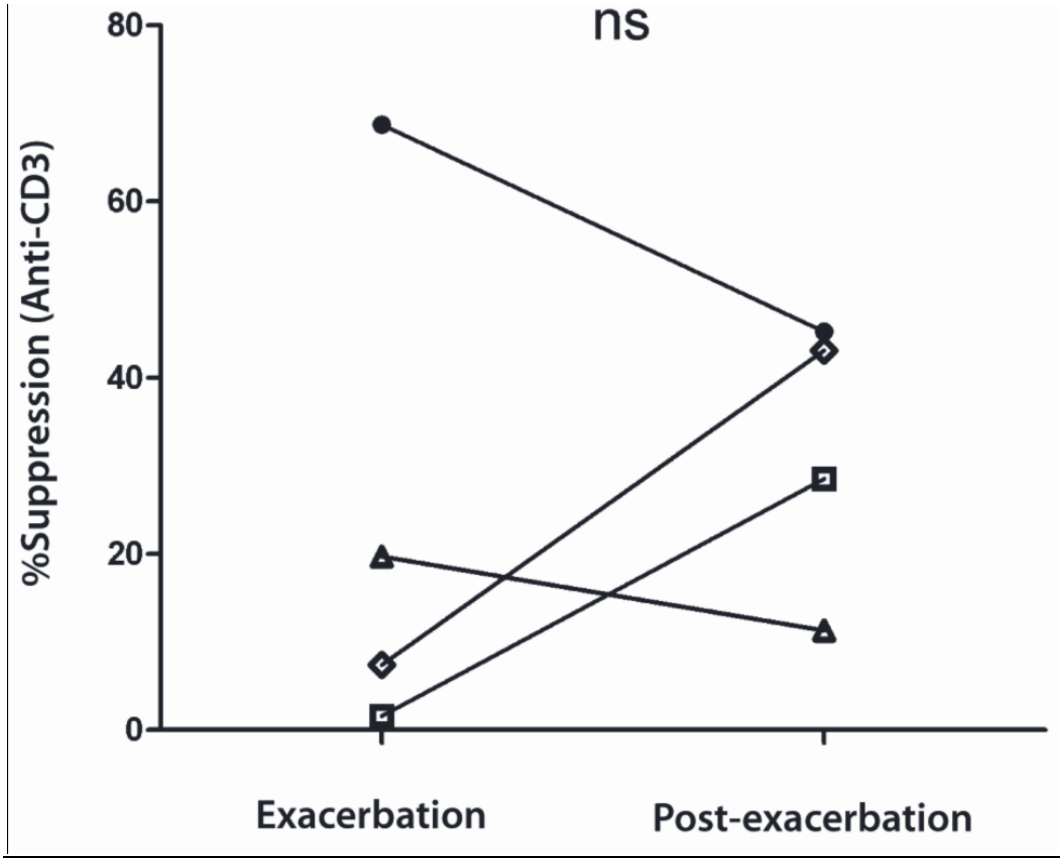
Data from Figure 32 were re-evaluated to obtain a single "mean neuroantigen-specific suppression" per subject, by averaging the % suppression against various neuroantigens. Each dot represents the mean neuroantigen-specific CD8 suppression per patient during an exacerbation and in a longitudinal specimen collected after quiescent state was established either with or without immunomodulatory therapy. Dot shape and lines indicate paired longitudinal values (**$p<0.01$). At follow-up, the four patients averaged 81.3 days since start of last relapse. At post-exacerbation, one patient was treatment naïve through out (closed circle), one was on Copaxone for 3 months (open triangle), one was on IFN-beta (open square) and one stopped IFN-beta therapy after two doses (open diamond). P values 0.001 to 0.01 were significant with "**" notated respectively.

Figure 34: Foreign antigen-specific CD8+ T-cell suppressive-ability does not correlate with days since last relapse and recovery



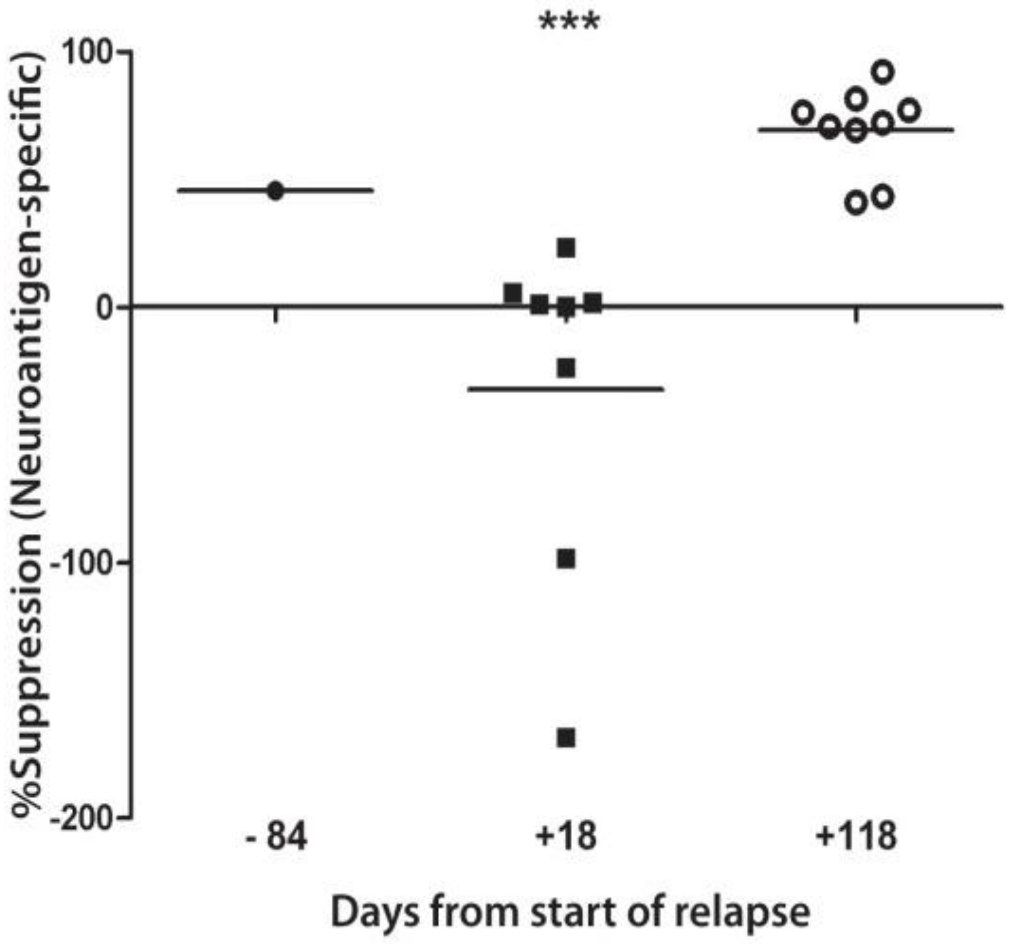
Foreign-specific CD8 suppression from four acute MS exacerbation patients during and post-exacerbation is shown. P values >0.05 were not significant with "ns" notated where applied in figures.

Figure 35: Global CD8+ T-cell suppressive-ability does not correlate with days since last relapse and recovery



Longitudinal anti-CD3-stimulated CD8+ T cell suppressive ability is depicted. Each dot represents the mean global CD8 suppression per patient during an exacerbation and in a longitudinal specimen collected after quiescent state was established either with or without immunomodulatory therapy. At follow-up, the four patients averaged 81.3 days since start of last relapse. At post-exacerbation, one patient was treatment naïve through out (closed circle), one was on Copaxone for 3 months (open triangle), one was on IFN-beta (open square) and one stopped IFN-beta therapy after two doses (open diamond). P values >0.05 were not significant with "ns" notated where applied in figures.

Figure 36: Neuroantigen-specific CD8+ T-cell suppression decreases transiently during acute MS exacerbation

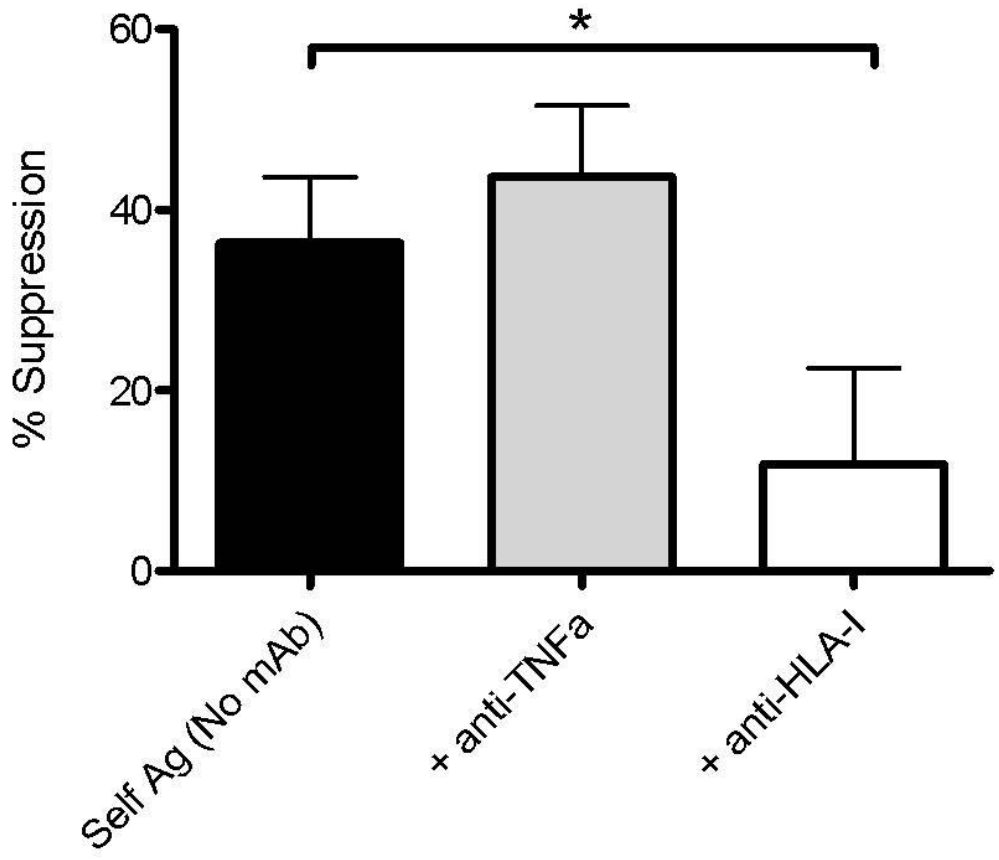


These data represent neuroantigen-specific suppression assays performed longitudinally from a single MS patient, who was evaluated at a quiescent stage of disease before and after an acute exacerbation. P values <0.001 were significant with "***" notated, comparing +18 to +118 days post-exacerbation.

NEUROANTIGEN-SPECIFIC CD8 SUPPRESSION REQUIRES HLA CLASS I AND INTERFERON GAMMA

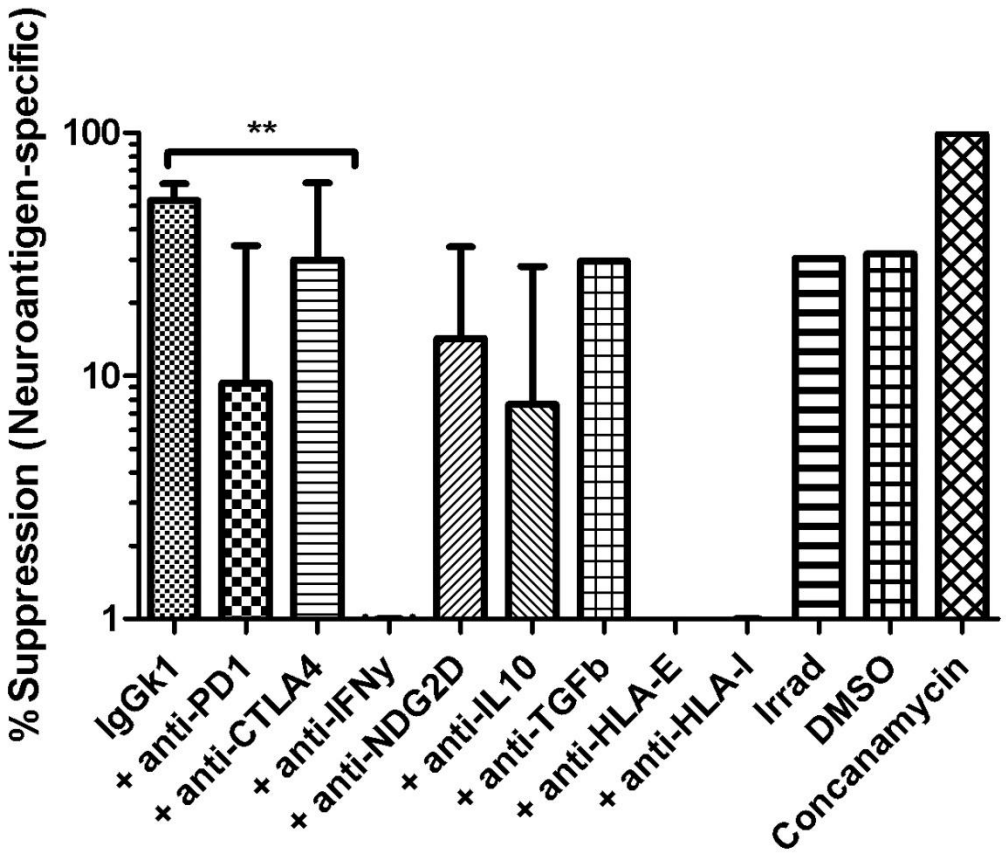
To test this hypothesis, I conducted CNS-specific suppression assay with the addition of blocking antibodies (Figure 37). I observed a decrease in neuroantigen-specific suppressive ability by CD8+ T cells upon the addition of a commercially available cocktail of anti-HLA (classical) class I antibodies. This suggested that at least some suppressive CNS-specific CD8+ T cells were HLA class I (A, B, or C) restricted. I then repeated similar experiments with additional blocking antibodies (Figure 38). I observed a large decrease in neuroantigen-specific suppressive ability by CD8+ T cells upon the addition of anti-IFNγ or anti-HLA-E antibodies. I observed a smaller, yet significant, decrease upon the addition of anti-PD-1, anti-NKG2D, and anti-IL10 antibodies. This suggested that neuroantigen-specific suppression by CD8+ T cells may involve both classical and non-classical antigen-presentation pathways. The suppressive mechanism of CNS-specific CD8+ T cells may involve both contact-dependent and –independent processes.

Figure 37: CNS-Specific CD8+ T cells require HLA class I, not TNF, in order to suppress CNS-specific CD4+ T cells



Ex vivo PBMC were used in myelin self antigen-specific flow-based suppression assays +/- anti-TNF or –HLA class I antibodies. P values 0.01 to 0.05 were significant with "*" notated respectively.

Figure 38: Neuroantigen-specific suppression by CD8+ T cells was dependent upon HLA class I, IFNγ, with possible partial involvement by NKG2D, PD-1, and IL10

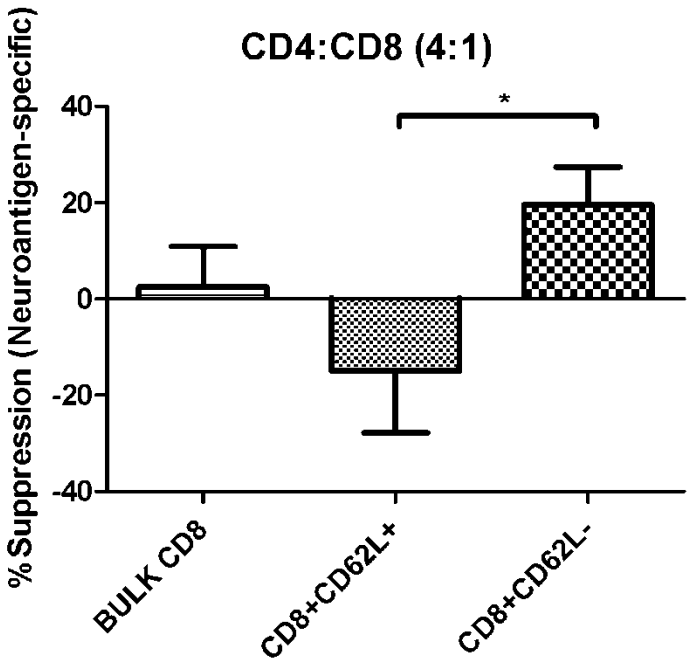


Ex vivo PBMC were used in myelin self antigen-specific flow-based suppression assays in the presence of antibodies or CD8+ T cells were exposed to physical and chemical stimuli prior to suppression assay. P values 0.001 to 0.01 were significant with "**" notated respectively.

ANTIGEN-SPECIFIC SUPPRESSION IS HARBORED IN THE CD8+CD62L- SUBSET

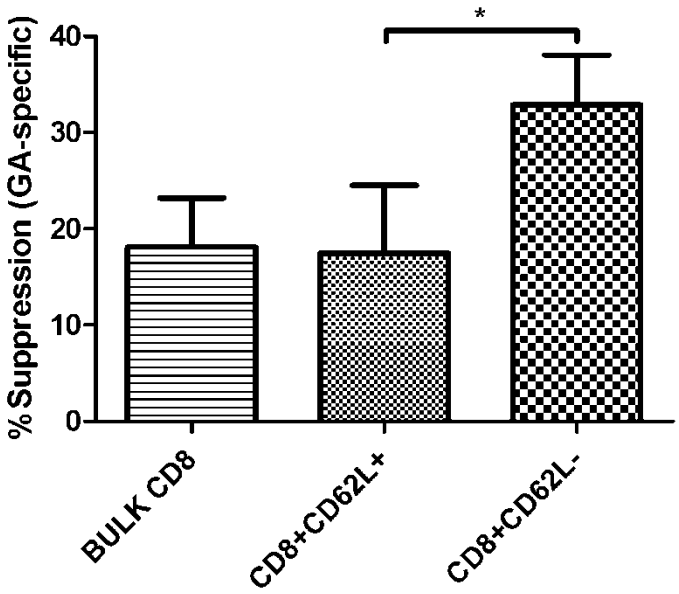
To test this hypothesis, I conducted a flow-based suppression assay using bulk CD8+ T cells from healthy subjects and their magnetically sorted CD62L subsets as suppressor cells (Figure 39, Figure 40). I observed in suppression assays, using neuroantigen and Copaxone as the stimulus, that the CD8+CD62L- subsets possessed increased mean suppressive ability compared to bulk CD8+ T cell and the CD8+CD62L+ subset. This suggested that CNS- and GA-specific suppressive ability by CD8+ T cells may share similar mechanisms and that CD8+ cells having the most suppressive ability may include terminally differentiated or exhausted CD8+ T cells.

Figure 39: CNS-specific suppression is harbored in the CD8+CD62L(-) subset



Representative of flow-based suppression assays from 5 healthy controls. P values 0.01 to 0.05 were significant with "*" notated respectively.

Figure 40: GA-specific suppression is harbored in the CD8+CD62L(-) subset

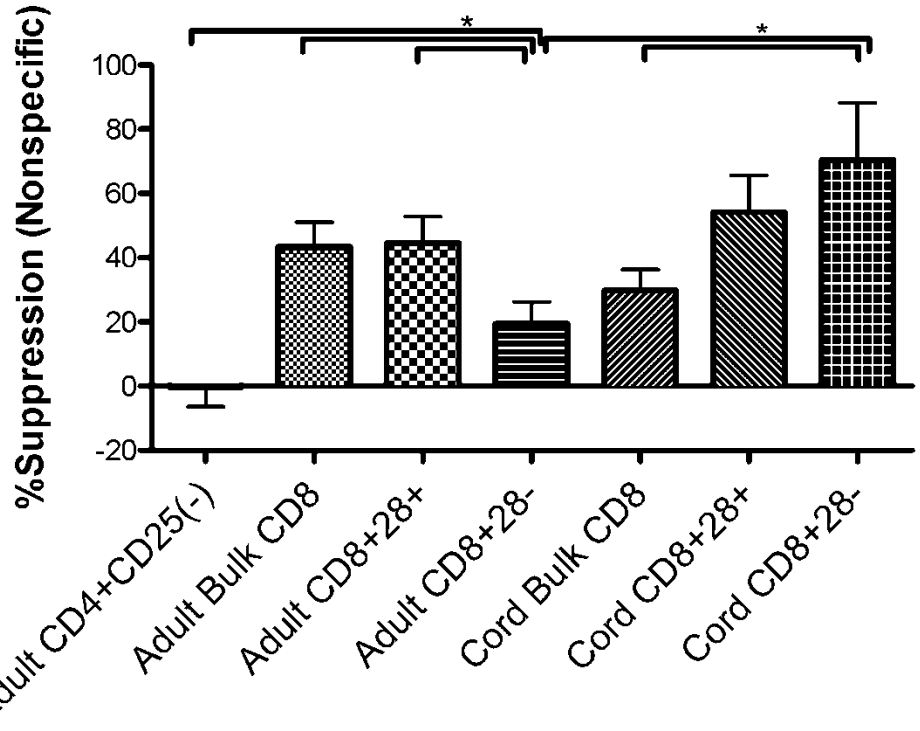


Representative of flow-based suppression assays from 2 healthy controls. P values 0.01 to 0.05 were significant with "*" notated respectively.

GLOBAL SUPPRESSIVE ABILITY IN ADULTS IS HARBORED IN THE CD8+CD28+ SUBSET

To test this hypothesis, I conducted a flow-based suppression assay using bulk CD8+ T cells and their magnetically sorted CD28 subsets, taken from both adult PBMCs and neonatal cord blood, as suppressor cells (Figure 41). I observed in suppression assays, using anti-CD3 as the stimulus, that the CD8+CD28+ subsets from adults possessed increased suppressive ability compared to CD8+CD28- cells. Contrarily, CD8+CD28- cells from cord blood possessed increased suppressive ability compared to bulk CD8+ cells from cord blood. This suggested that the cells responsible for global, non-specific, CD8+ T cell suppressive ability may change over time due to age-related developmental factors, antigen-exposure, and other unknown factors.

Figure 41: Global CD8 suppressive ability is harbored in the CD28+ subset in adults, in contrast to neonates. CD8+ subset harboring non-specific suppression changes over a lifetime



Representative of flow-based suppression assays from 9 healthy adults and 5 neonates. Ex vivo bulk CD8+ T cells and magnetically sorted subsets from adults and cord blood were put into flow-based suppression assays with autologous anti-CD3 stimulated CD4+CD25(-) T cells for 7 days. Autologous CD4+CD25(-) T cells were used as a negative control for suppression. P values 0.01 to 0.05 were significant with "*" notated respectively.

CHAPTER 4: DISCUSSION

DEFICIENT CNS-SPECIFIC CD8 TREG IN MS ACUTE EXACERBATIONS

To my knowledge, these studies were the first evidence that *human* autoreactive CNS-specific CD8+ T-cells play an immune regulatory role, in contrast to foreign-antigen-specific effectors. Moreover, my studies showed a clear clinical relevance for this regulatory role, in that suppressive activity is greatly diminished during *relapses* of MS and recovered as the patients enter remission. Classically, CNS-targeted, MHC Class I-restricted CD8+ T-cells were thought to have a pathogenic role in disease, with reports demonstrating *in vitro* cytotoxic killing of oligodendrocytes [94, 103]. However, my studies identified an unexpected and novel immune regulatory role in both HC and quiescent MS patients, corroborating studies in EAE, where CNS-specific CD8+ T-cells inhibited disease, whereas control antigen-specific ones did not [306]. Sporadic, acute exacerbations are characteristic of the relapsing-remitting form of MS. While MS suppressor cell dysfunction has been recognized for decades, the role of CNS-specific CD8+ T-cells remains elusive in the context of accumulating disability, axon trans-section, and gliosis which are characteristic of secondary progressive MS [93, 309]. It appears that CNS-specific regulatory ability is directly or indirectly involved in the mechanism of MS clinical phase changes from remission to relapse. It still remains unclear whether underlying pathology of chronic progressive MS exhibit similar deficient suppressor CD8+ T-cell activity [307].

IMMUNOLOGIC EPIDEMIOLOGY

Human studies are often hampered by the lack of implicit causation. Epidemiology is the study of disease in populations, and my study of MS patients resembled a case/control study. I reasoned that acute exacerbations of MS vary in frequency, severity, length, and type, and I sought to test if CNS-specific CD8+ T cell suppressive ability correlated with clinical phases of disease. I selected subjects based on their disease status and investigated a potential exposure that both groups (cases and controls) may have encountered. It would be faster and more cost-effective than first determining exposure in a random population and observing prospectively the occurrence of a rather rare disease, MS. I selected individuals with or without disease, MS, and then examined the exposure of all groups to an immunologic parameter, neuroantigen-specific CD8+ T cell suppressive ability. From the ratio of exposure to un-exposure in the cases and controls, an odds ratio was determined for the points of this discussion. Odds ratios is defined as the number of cases exposed/cases un-exposed over controls exposed/controls unexposed, or in other words, the likelihood that those with disease were more likely to have been exposed. To determine the odds ratio of exposure between quiescent and acute MS, I first selected MS patients based on the time since their last acute exacerbation, measured their exposure by an *in vitro* assay, and defined the threshold of exposure level to suppressive ability. If one assumes that less than 5% CNS-specific suppressive ability is the definition of the exposure, five of six MS patients during an acute exacerbation and one of nine quiescent MS patients were exposed to deficient CNS-specific suppressive ability, according to **Figure 23**. Thus, MS patients suffering from an acute exacerbation

were 40 times more likely than quiescent MS patients to have been exposed to less than 5% CNS-specific suppressive activity. Total MS patients were 10.5 times more likely than healthy persons to have been exposed to less than 10% CNS-specific ability, and quiescent MS patients were 1.5 times more likely than healthy persons to have been exposed to less than 10% CNS-specific suppressive activity. Clearly, larger odds ratios garner the most interest and statistical support. While the published title of my study was not, "Assuming a less than 5% CNS-specific suppression ability defines a risk exposure, MS patients during an acute exacerbation were 40 times more likely to be deficient than quiescent MS patients," I considered its accuracy before I quickly dismissed it based on its non-intuitive nature. I have focused on the novel observation and biology of CNS-specific CD8+ T cell biology for neglect of the most accurate interpretation of its epidemiological meaning. In other words, I have risked a slight inaccuracy in the published title in order to convey immunologic meaning, while minimizing bias.

One wonders, "how has it come to be that MS patients during an acute exacerbation were 40 times more likely to be deficient than quiescent MS patients?" The suppressor cells could be in the CNS and not in the blood, possibly explaining why I saw less suppressive ability in peripheral CD8+ T cell compartment. As mentioned in the results, to address whether the lack of suppression could be explained by major changes in T-cell subsets, I first evaluated CD4:CD8 ratios across various cohorts and found no significant differences between any of the cohorts, especially between quiescent MS (1.75 ± 0.69) vs. acute exacerbation (2.12 ± 0.72). I further evaluated whether there may

be an absence of CNS-specific CD8 reactivity in the peripheral blood during acute exacerbation or enhanced activation or proliferation of CNS-specific CD8 cells in the suppression assays. Using CMTPX as a cell tracker, I was able to specifically evaluate CD8 T-cell activation. While CMTPX is not optimal for use as a proliferation dye, I could evaluate total CD25 expression by the CMTPX-stained CD8 cells in these cultures. I found that, albeit slightly diminished ($p > 0.05$), CNS-specific CD8 reactivity was detectable even during acute exacerbation (**Figure 26**), suggesting that these responses may be functionally different rather than simply quantitatively suppressed. "How does one assign the clinical significance of less than 5% CNS-specific suppression ability?" Like any experimental parameter, it is only clear how the range of healthy individuals compares to other cohorts. In my study, all normal individuals tested had an average greater than 5% reduction in neuroantigen-specific CD4+ activation and proliferation at the 1:0.25 responder:suppressor ratio when CD8+ T cells were added to the culture for 7 days (**Figure 23**).

LIMITATIONS AND MINIMIZING ERROR

All case/control studies are susceptible to selection and recall bias. I have considered and sought to minimize other possible sources of systemic error within my study of MS patients. Test subjects were likely to be representative of the actual MS population in the United States. In selecting treatment naïve MS patients, the study may be skewed to an early phase of MS, representing a smaller fraction of the overall patient population. The Center for Multiple Sclerosis at University of Texas Southwestern Medical Center is an American neurology

practice, where aggressive treatment is acceptable, leaving few untreated patients. Test subjects and their care-takers properly documented symptoms. Sampling was random in that a component of selection was unpredictable, outside of study inclusion and exclusion criteria.

Sampling bias may be perceived from the relatively small study size. However, large differences between relatively small clusters of data points allowed for a very small chance of incorrectly rejecting the null hypothesis. This suggests that the study possesses statistical power to provide the conclusions I have derived. I lacked the statistical power to make any conclusions about the differential treatments taken by the four MS patients' post-acute exacerbation follow-up and lacked clinical scores by measure of expanded disability status scale (EDSS) or Cambridge multiple sclerosis basic score (CAMBS). Prior studies have used CAMBS in IFNy and TNFa level correlation analyses to fatigue [310]. While CAMBS allows statistical analysis of the otherwise qualitative, retrospective, and descriptive, it is not prospective or predictive of outcome. In some respects, it would have been ideal if EDSS assessments could have been performed routinely on our patient pool. However, EDSS is not possible to perform routinely at a large, busy neurology practice, and it was simply not done on our cohort. EDSS has been shown to correlate with CAMBS disability. Use of the less cumbersome CAMBS may allow future statistical analysis of immunologic parameters.

AUTOREGULATORY T CELLS

In the field of immunology, scientists are only beginning to understand the role of autoreactive, regulatory ("autoregulatory") T-cells in autoimmune disease [58]. There has been some evidence that autoantigen-specific CD8+ T-cells may have immune regulatory properties in diabetes models [68, 311]. Thus, chronic stimulation of CD8+ T-cells with low TCR avidity may induce regulatory function [311], perhaps explaining the therapeutic generation of antigen-specific, cytotoxic immune suppressor CD8+ T-cells following chronic copolymer-based therapy of MS [104, 245] (**Figure 43**). This may also explain the difference between the roles of foreign-antigen-specific CD8+ T-cells vs. autoreactive ones that tend to bear lower avidity TCR, presumably following thymic deletion of higher avidity responders. In contrast to Qa1/HLA-E-restricted suppressor CD8+ T-cells that recognize immune cell-derived peptides, autoregulatory CD8+ T-cells are stimulated by the same tissue antigens that are targets of destructive effector cells, thereby creating an autoregulatory tolerance loop.

IMMUNE THERAPY USING CD8+ T CELLS

This novel concept also unveils a potential strategy for immune therapy. While using autoreactive CD8+ T-cells as therapy may seem unorthodox, this is principally similar to generating autoantigen-reactive CD4+CD25+FOXP3+ T_{reg}s for adoptive immunotherapy. Other forms of autoreactive CD4+ T_{reg}s (Tr1, Th3) have also shown promise in animal models. CD8+ T-cells, representing an underappreciated arm of peripheral immune tolerance, afford an attractive form of adoptive immunotherapy, especially in the context of clinical relapses. In that regard, my lab has shown recently that CNS-specific CD8+ T-cells can inhibit

ongoing EAE [306], dependent on cytotoxic and immune modulatory mechanisms. The phenotypic characteristics of regulatory CD8+ T-cells are not definite and, depending on the model, may range from a CD28(-) [37-43], γδ+ [45], CD25+ [46], CD122+ [47], CD103+ [49, 50], PD-1+ [53] or FOXP3+ [54, 56, 71, 312], among others [313]. In which context neuroantigen-specific CD8+ T-cells regulate, and how, is still unclear. Our preliminary studies reveal autoregulatory CNS-specific suppressor activity in multiple such subsets, with the common features being cytokine- and contact-dependent processes (including cytotoxicity) and an absolute requirement for HLA-Class I (**Figure 37, Figure 38**). Detailed dissection of the characteristics and mechanisms of these cells will be an important pursuit to develop a therapeutic approach.

To summarize, our studies demonstrate a novel, clinically relevant role for neuroantigen-specific CD8+ T-cells, revealing a potential pathway of intrinsic immune regulation that may have implications for the therapy of human MS and other immune-mediated disorders.

FUNDAMENTAL DIFFERENCES BETWEEN AUTOREACTIVE AND FOREIGN SPECIFIC T CELLS

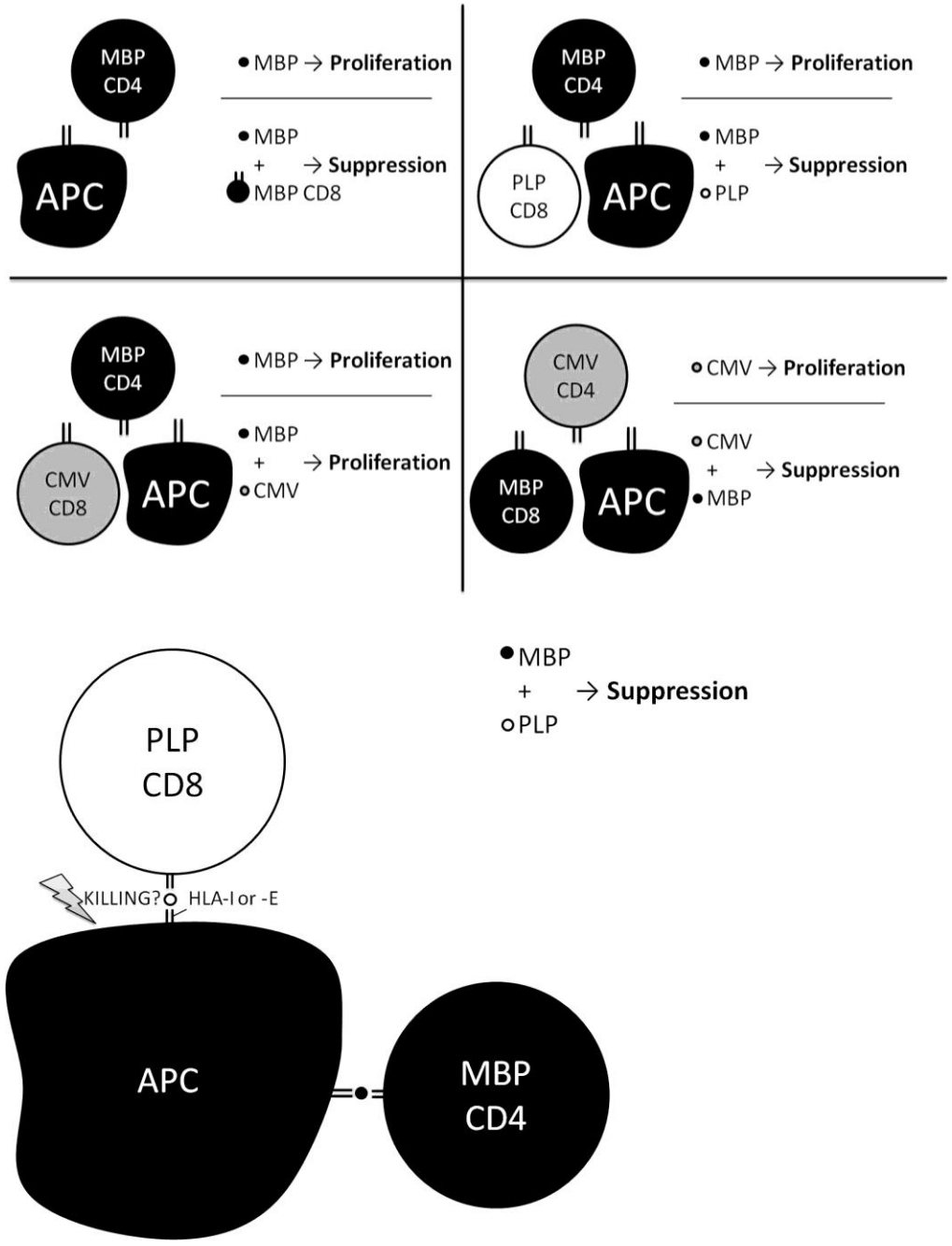
The avidity of the TCR may explain the differences in their suppressive ability. In several experiments, I attempted to isolate pure CNS- and CMV-specific T cells though stimulating CFSE-stained PBMC for 7 days and then *flow-sorting* the CFSElow population. Using the *flow-sorted* cells as suppressors, I set up 7 day suppression assays with fresh autologous responders stimulated with peptide or anti-CD3. I acquired the results with the HTS on the LSR and

analyzed the %CD25+CFSElow of the CD4+ responders. When I used peptides (MBP or CMV) as the stimulus, the result was mostly uninterruptable as I could not effectively gate out the suppressor cells without the use of CMTPX. The antigen-specific assay lacked the sensitivity to detect suppression of any sorted populations. When I used anti-CD3 as the stimulus, interesting differences were found between the flow-sorted populations. Anti-CD3 is a monoclonal antibody that bypasses the TCR and binds directly to the CD3 signalling complex in order to polyclonally-stimulate T cells. Using anti-CD3 as the stimulus, both the MOG-specific and CMV-specific CD8+ T cells were able to suppress anti-CD3 stimulated CD4+ responders (data not shown). This result was unexpected as CMV-specific CD8+ T cells, in previous experiments (**Figure 20, Figure 42**) with peptide, had undetectable suppressive ability. I hypothesized that this might be explained by the biology of anti-CD3. The phenomenon of CNS-specific suppression by CD8+ T cells is likely TCR-dependent. Circumventing the engagement of the TCR eliminated the CNS-specificity of CD8+ T cell suppression. Alternatively, foreign-specific suppression by CD8+ T cells may be appreciable, just undetectable by my peptide-stimulated suppression assays (**Figure 20**). Using flow-sorted cells, foreign-specific CD8+ T cells possessed suppressive ability that was indistinguishable quantitatively from that of CNS-specific T cells (data not shown). Perhaps lowering the amount of anti-CD3 may reveal differences between the sorted, antigen-specific populations. Additional experiments are required to resolve these questions. In summary, my conclusions

are that CNS-specific suppressive ability requires engagement of the TCR; anti-CD3 activated CD8+ T cells suppress, regardless of antigen-specificity.

The use of anti-CD3 in suppression assays with sorted-antigen specific T cell lines must be repeated with proper controls before any conclusions can be made. It remains unclear if CMV-specific CD8+ T cells really prevent suppression or if the suppression assays lack the sensitivity to measure it. The anti-CD3 stimulated global suppression assays are thought to polyclonally activate CD8+ suppressor cells, which mediate suppression through soluble factors. Other studies have suggested that TNFα is required for the induction of regulatory CD8+ T cells, and that suppression occurs through an IL10-dependent mechanism.

Figure 42: Activated neuroantigen-specific CD8+ T-cells suppress CD4+ T-cells



Proposed model illustration based on Figure 20. Responder CD4+ T-cell lines were cultured with APC and indicated antigens in the presence or absence of the indicated CD8+ T-cell lines.

PATHOGENIC ROLE FOR INFILTRATING CD8+ T CELLS IN MS

Although subsets CD4+ T lymphocytes (Th1 and Th17) have been established as important mediators of demyelination, neuronal damage, and astrocyte/microglia activation in MS, CD8+ T cells have remained a subject of intense study and debate. CD8+ cells are oligoclonally expanded and in greater amount than CD4+ T cells in the MS lesion. Their close apposition to MHC class I-expressing cells could allow CD8+ T cells to recognize and target neurons, oligodendrocytes, astrocytes, microglia, CD4+ T cells, and other CD8+ T cells. The mechanisms dictating the acquisition by CD8+ T cells of a pathogenic phenotype/function are poorly understood. During exacerbations of MS, CD8+ T cells may down-regulate inhibitory NK cell antigen receptors (NKGR/CD94) [67]. Where other studies have observed this evidence and interpreted a pathogenic potential of CD8+ T cells [258], others have interpreted that a regulatory CD8+ T cell has become dysfunctional [67]. Both interpretations are correct, insofar that all studies would conclude that dysfunctional regulatory cells are pathogenic, and pathogenic cells are deregulated.

Many human studies have documented the cytotoxic ability of CD8+ T cells from MS to target each many cells types *in vitro*. Most human studies stress the potential of CD8+ T cells to target either neurons, oligodendrocytes, and microglia *or* CD4+ T cells and other CD8+ T cells, the balance being the determining facter whether to call *all* CD8+ T cells in MS as regulatory or pathogenic. Many human studies have demonstrated the proximity of CD8+ T cells and CNS target cells in MS lesions. In human studies, associations, not

causations, are the strongest conclusions, as described earlier in the discussion of epidemiology. Based on numerous studies upon regulatory CD8+ T cells, their apparent heterogeneity may serve as an example of a greater heterogeneity within bulk CD8+ T cells. Naïve, memory, effector, or exhausted CD8+ T cells may display certain aspects of a regulatory CD8+ T cells transiently. If CD8+ T cell differentiation shows any similarity to that of CD4+ T cell biology, one would expect dynamic and plastic differentiation among the forms and functions of CD8+ T cells. For now, the master regulator transcription factors for CD8+ T cell biology remain poorly understood. The fine line between appropriate and detrimental T cell activation involves essential inhibitory signals provided by the cellular environment. Infiltrating CD8+ T cells may be the result of missed inhibitory signals or a response to missed signals by other immune cells. A combination of animal and human studies about MS, as performed in my lab, are likely the best way forward to avoid false assumptions derived from limited approaches.

MODEL

CNS-specific CD8+ T cells are able to suppress CNS-specific CD4+ T cells. While my experiments have shown that MS exacerbations are correlated with a deficiency of CNS-specific suppressive ability, this does not prove that MS is simply caused by a lack of CD8+ T cell suppression. CNS-specific suppressive ability by CD8+ T cell is one factor among many that are strongly correlated to MS. Still, this work may present an opportunity to explore in further studies drugs or cell-based therapies that may target CD8+ T cell in order to potentiate

their suppressive function. It remains unclear if a lack of suppression during exacerbations is the fault of CD8+ suppressors or CD4+ cells being unreceptive to inhibition. Future longitudinal studies may better address these questions.

CNS-specific CD8+ T cells are thought to be activated through the recognition of CNS peptide in the context of classical or non-classical HLA class I on APC or CD4+ T cells. CD4+ T cells may undergo membrane exchange with APC in order to acquire cross-presented antigen in the context of HLA-class I. If CD8+ T cells are activated by APC, suppression may occur through direct killing of APC or indirectly through the selection of tolerogenic APC acting on CD4+ T cells. IFNγ may be required for the activation or induction of regulatory CD8+ T cells. Alternatively, IFNγ may be required for their suppressive mechanism either by acting upon APC or CD4+ T cells. APC may be activated by IFNγ to up-regulate MHC class I or express IDO. IFNγ may deviate CD4+ T cells toward Th1 away from Th17 or Th2 differentiation. Future experiments will interrogate these and other models of CNS-specific regulatory CD8+ T cell function.

FUTURE DIRECTIONS

While much is known about CD4+ Treg, CD8+ Treg remain understudied and poorly understood. Using adult human peripheral blood and cord blood, we made several observations regarding CD4+ and CD8+ Treg. Prior members of my lab have made the following observations, which form part of the basis of my hypothesis. I developed a flow cytometry-based suppression assay, which utilizes CMPTX, CFSE dilution, and CD25 expression (Figure 13). Upon removal of both CD8- and CD25-expressing Treg, CD4+CD25- cells from both healthy persons and those with untreated autoimmunity harbor similar autoreactive T cell responses to self antigens (Figure 10). When activated by cognate antigen, self antigen-specific CD8+ Treg suppress self-specific CD4+CD25- T cells (Figure 20). Foreign, control antigen-specific CD8+ Treg are incapable of suppressing control-specific CD4+CD25(-) T cells. Suppression of CD4+CD25(-) T cells by self-specific CD8+ Treg requires HLA class I, but not TNFalpha (Figure 37). The global CD8+ Treg phenotype (mitogen stimulated) possessing the most suppressive ability changes over a lifetime (Figure 41). The role of regulatory and alloantigen-specific T cell biology in autoimmunity and transplantation has been examined in some detail through my work and that of others from my lab. My data provide evidence that CD8+ Treg possess potent immune suppressive ability. However, the biology of autoantigen- and alloantigen-specific CD8+ Treg remains poorly studied in the context of human disease, as does the mechanisms of global CD8-mediated suppression. To that end, pre-clinical research probing the mechanistic nature of suppression is outlined here. Pending the outcome of

these studies, insight into the role CD8+ Treg play in the settings of transplant and autoimmunity may yield ground-breaking cell-based therapies or new drug targets.

My lab and I are currently pursuing both the mechanism of CD8+ T cell regulation and its earliest detectable effects even hours after various therapeutic measures in MS. The mechanism of action of Copaxone, an immune-modulatory agent, is poorly understood. As part of my study, I evaluated a subset of the subjects longitudinally, after their disease had become clinically quiescent either with or without immunomodulatory therapy (Figure 33, Figure 35). At post-exacerbation, one patient was treatment naïve through out, one was on Copaxone for 3 months, one was on IFN-beta and one stopped IFN-beta therapy after two doses. At follow-up, I observed in all patients an increase in CNS-specific, and, in three of four, an increase in global- CD8+ T cell suppressive ability. At follow-up, the four patients averaged 81.3 days since start of last relapse. This suggests that the first injections of GA or IFNβ may have immediate immunologic consequences on APC or T cells populations that set the foundations for sustained immune regulatory responses, preventing or delaying further relapses. My lab has illustrated that GA treatment induces and restores CD8+ T cell responses in MS. The question is when.

Supporting the work of others, I have observed using flow-based suppression assays that MS patients have deficient global CD4+ and CD8+ Treg function (Figure 60). One wonders when MS therapies take effect to start improving immune-regulatory parameters. In other words, do drugs used in MS

have early effects on T cells or APCs or both? In particular, I am interested if GA immediately impacts global- or CNS-specific CD8+ T cell suppressive ability within hours of injection. Perhaps GA enhances global- or CNS-specific suppressive ability earlier or to a greater extent than IFNβ or fingolimod or others. Do some therapeutics induce transient activation-induced regulatory CD4+ T cells? If intrinsic CD4+ or CD8+ regulatory T cell function is augmented or peaked at a particular time, this could have important therapeutic implications for dosing regimes or experimental adoptive transfer protocols. Perhaps these studies will support the notion of a drug vacation after GA has made its most potent immune regulatory response in the early treatment phase.

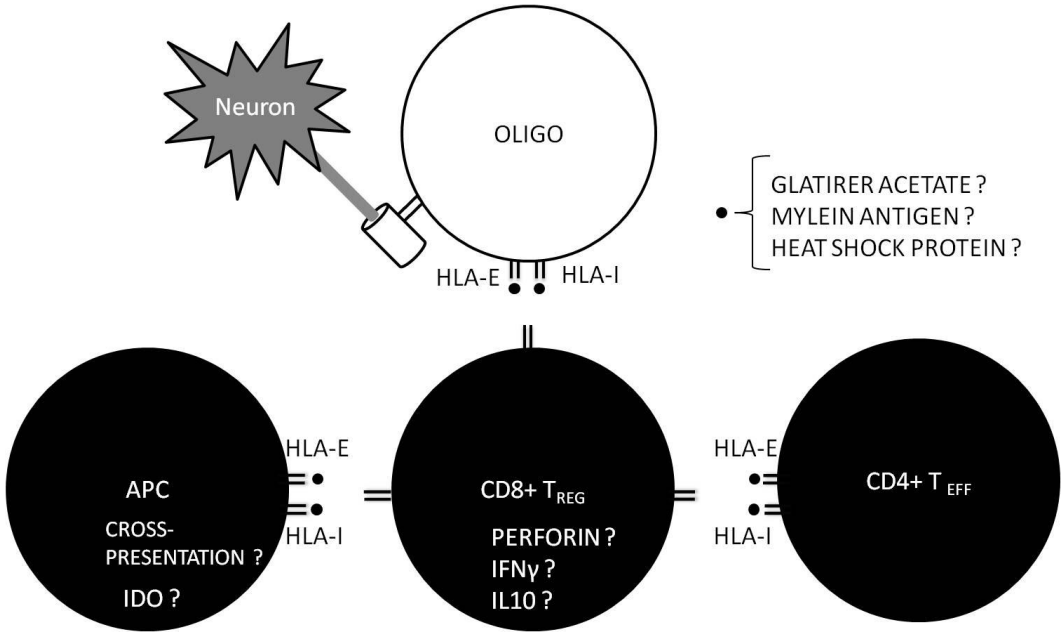
The mechanisms of regulatory CD8+ T cells are poorly understood. I have observed increased regulatory T cell function within certain subsets and blocked suppressive activity through the addition of neutralizing antibodies (Figure 38, Figure 39). HLA-E may act as a ligand for the TCRαβ, and CD8+ T cells proliferate in response to self antigen-, cytomegalovirus-, Epstein-Barr virus-, *Listeria monocytogenes*-, *Mycobacterium tuberculosis*-, and *Salmonella enteric* –derived peptides presented in the context of HLA-E [276-279]. My lab and I are interested in better understanding the role of cytotoxic molecules and non-classical HLA class I molecules in antigen-specific CD8+ regulatory T cell function. Perhaps CD4+ T cell or APC antigen-processing may allow for CNS peptides to be presented in context of HLA-E, creating a potential target for cytotoxic/regulatory CD8+ T cells. If the mechanisms behind regulatory T cells

can be better understood, new strategic targets for therapeutics may be uncovered in the context of allergy, asthma, transplant, and autoimmunity.

PROPOSED MODEL OF CNS-SPECIFIC REGULATORY T CELLS DYSFUNCTION DURING ACUTE EXACERBATION OF MS

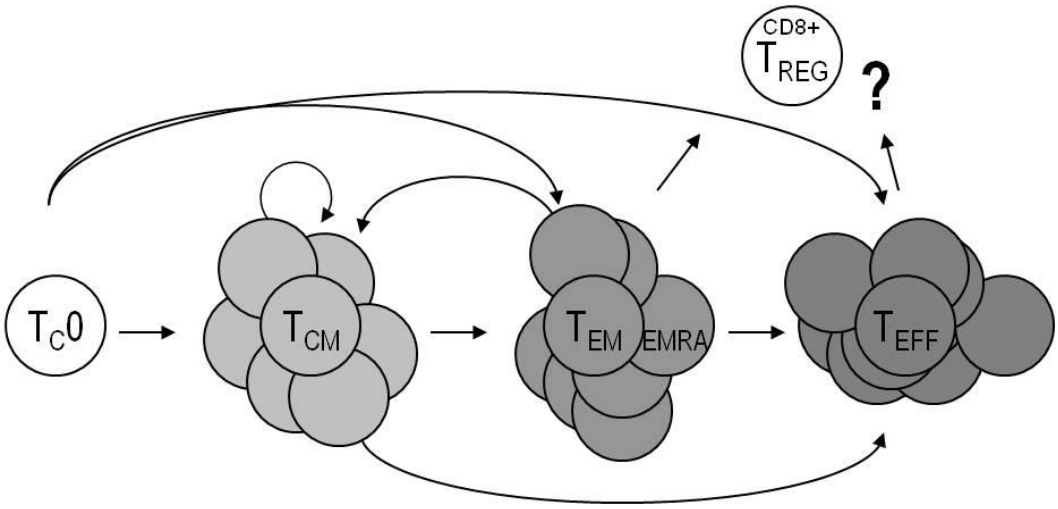
In the center of my proposed model are HLA class I-restricted, CNS-targeted CD8+ T cells (**Figure 43**). I have presented evidence for their immune regulatory role in both HC and quiescent MS patients. CNS-specific regulatory ability is directly or indirectly involved in the mechanism of MS clinical phase change. During the steady-state and remission in MS, CNS-specific regulatory ability has a measurable suppressive effect toward known pathogenic cells, CNS-specific CD4+ T cells. Suppressive action may be mediated through soluble and cytotoxic factors after an initial signal received via classical and non-classical HLA class I. Based on my studies with CD8+ T cell subsets, regulatory CD8+ T cells may be largely derived from more terminally differentiated or exhausted CD8+ T cells (**Figure 44**).

Figure 43: Model of how CNS-specific regulatory ability is directly or indirectly involved in the mechanism of MS clinical phase change



HLA class I-restricted, CNS-targeted CD8+ T cells have an immune regulatory role in both HC and quiescent MS patients. Acute exacerbations of MS may include decreased intrinsic suppressive ability and/or decreased CD4+T cell sensitivity to suppressor T cell function.

Figure 44: CD8+ cells having the most suppressive ability may include terminally differentiated or exhausted CD8+ T cells



CD8+ T cell differentiation is poorly understood. The relationship between effector and memory cell generation and the regulatory CD8+ T cell lineage is less clear. This model is based on Figure 39, Figure 40, and Figure 41.

NOVEL FLOW-CYTOMETRY-BASED SUPPRESSION ASSAY

Regulation of immunity is a fundamental concept in immunology. Many cellular phenotypes possess suppressor activity, including but not limited to the CD4+CD25+ regulatory T cells, CD4+ Tr1 cells, CD4+ Th3 cells, CD8+CD28-, HLA-E-restricted CD8+ T cells, CD8+TCRαα+ cells, among others. Mechanisms of suppression vary per model, subset, and condition, and many are left unresolved. Understanding the biology of human Treg is critical to the understanding of published and unpublished clinical data from intervention studies with suppressor cells. My method of measuring T cells suppressive ability, taking advantage of proliferation dyes and flow cytometry, will likely enable further studies in the fields allergy, transplant, and autoimmunity.

APPENDIX I

IMMUNE RESPONSES IN GENERAL

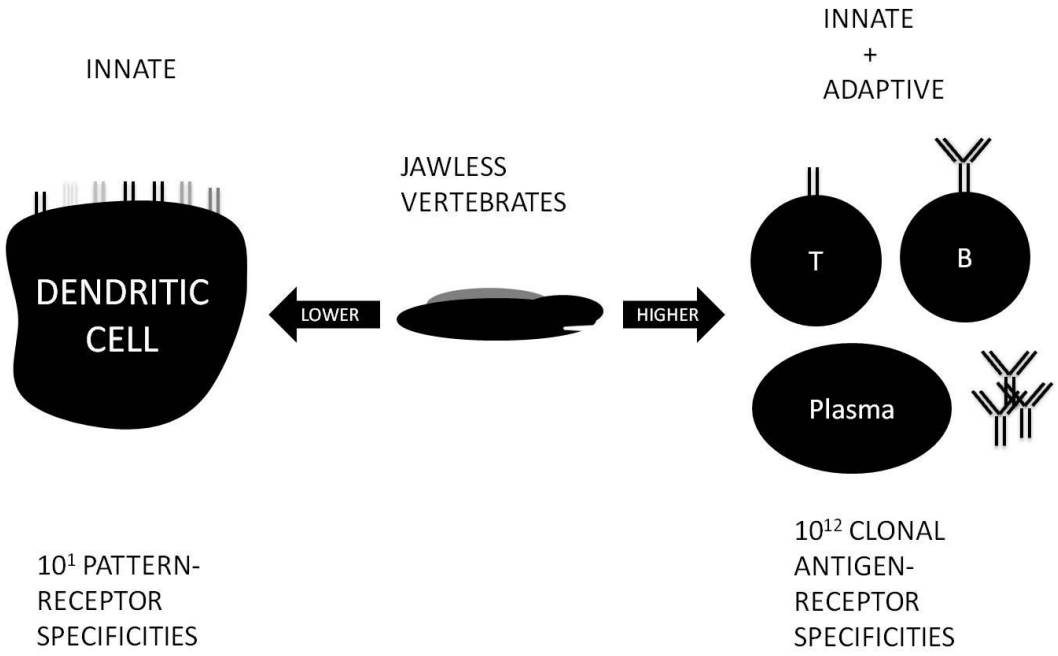
The healthy human body mounts immune responses against cancer-causing cells, microbes, parasites, and allergens through innate and adaptive (learned) mechanisms [314]. The innate immune system acts as first line defense against pathogen-associated patterns, broadly supporting the adaptive immune system. Effective adaptive immune responses involve the differentiation of antigen-specific T lymphocytes (T cells) and B lymphocytes (B cells). T cells express the antigen-receptor, known as the T cell receptor (TCR) and CD3 signaling complex, and are classified into two major groups, known as helper T (Th) cells and cytotoxic T (Tc) cells. The antigen-receptors of lymphocytes are responsible for the memory, diversity, and specificity of adaptive immunity. B cells express on the cell surface the membrane bound antigen-receptor, known as the B cell receptor (BCR), and secret a form of the antigen-receptor, known as immunoglobulin (Ig) or antibodies.

INNATE IMMUNITY

The common theme of the innate immune system is pathogen-associated molecular patterns (PAMP) engaging pattern recognition receptors (PRR), such as the Toll-like receptors (TLR). In other words, the innate immune system is pre-programmed per each cell type of each species toward a limited set of common antigens. For example, human dendritic cells express a repertoire of approximately 10^1 TLRs which are encoded in the host DNA, in order to recognize and attack highly-conserved motifs of bacteria, viruses, and fungi.

Albeit limited as a collection of variability, the innate immune system makes up for its limited range with consistency, dependability, and few-adverse effects while targeting the most common microbial attributes. Many innate immune mechanisms are shared broadly throughout the eukaryotic phylogenetic tree. Despite a decreased number of genes among most invertebrates, the innate immune system provides high function per burden of genetic load. The adaptive immune system is not shared by organisms lower than jawless vertebrates, which lack the recombinase-activating gene (RAG) [315].

Figure 45: The adaptive immune system is not shared by organisms lower than jawless vertebrates, which lack the recombinase-activating gene



ADAPTIVE IMMUNITY

The common theme of the adaptive immune system is lymphocytes bearing antigen-receptors generated by somatic recombination (SR). SR rearranges antigen-receptor gene segments in lymphocytes. The concept of clonal specificity of adaptive immunity describes how each lymphocyte expresses many of one type of antigen-receptor with affinity for one antigen. Adaptive immune responses are mounted by clonal expansion. The lymphocyte bearing the antigen-receptor of interest expands to form a population of lymphocytes bearing the same antigen-receptor. Therefore, each individual of a species has a unique representation of antigen-receptors among its pool of lymphocytes dictated by pathogen exposure and vaccinations. The total number of circulating lymphocytes and relative prevalences of antigen-receptors makes up the unique repertoire of the individual member of the species. The total number of possible antigen-receptor specificities per individual is estimated to be 10^{12} by taking into consideration the combined variability from the MHC and SR of the antigen-receptor [316]. Compared to the innate immune system, the adaptive immune system improves recognition and speed of repeated attack toward highly variable and unique motifs of pathogens, increasing with each encounter. The expanded range, customized specialization, and advanced memory of the adaptive immune system do not come without usage cost. DNA recombination carries the potential to introduce double strand breaks, chromosomal translocations, inversions, and other aberrations. Because of the chance of somatic recombination, many randomly generated antigen-receptor specificities are directed toward self. If left

unchecked, self antigens may be misclassified as foreign, causing disease. In other words, the biological complexity required to increase the diversity of immunity carries with it the potential to produce autoreactive immune responses. Major histocompatiability complex (MHC) is critical to promoting self tolerance and acts as the antigen-receptor ligand for T cells. Distinction between self and foreign antigen is paramount to the adaptive immune system.

T LYMPHOCYTES

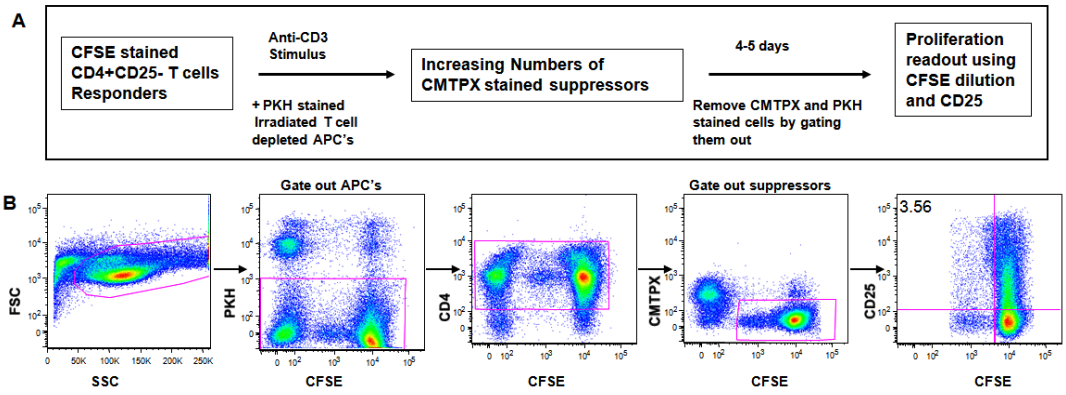
The two major groups of T cells are distinguished by the co-receptor to the antigen-receptor, known as TCR, expressed on the cell surface. The CD4 molecule is expressed by Th cells. The CD8 molecule is expressed by Tc cells. Th cells direct cell-mediated adaptive immune responses through the activation of macrophages and Tc cells in order to eliminate intracellular pathogens, such as viruses and intracellular bacteria, and neoplastic (cancer) cells. Th cells direct humor-mediated adaptive immune responses through the activation of antibody-producing B cells in order to eliminate extracellular threats. When the mechanisms of innate and adaptive immune systems fail, the human body may be at risk of prolonged illness due to malignant cancer, opportunistic infections, allergies, or autoimmunity. While progress has been made in recent years to understand the innate and adaptive immune system, may aspects of immunology remain poorly understood. For this reason, investigators rely heavily on model systems to elucidate immune mechanisms that may translate into better treatments for people affected by illnesses that are immune-regulated and immune-deficiencies. During Th and Tc cell development and activation, loss in tolerance

to self antigens may result in organ-specific autoimmune disease. A demyelinating disease of the CNS, multiple sclerosis, is thought to be immune-regulated. Prior studies have predominantly focused on models of autoreactive Th cells in autoimmunity, leaving Tc cells understudied and poorly understood.

SUPPRESSIVE PROPERTY OF ACTIVATED CD4+CD25- T CELLS REVEALED BY A NOVEL FLOW CYTOMETRIC SUPPRESSION ASSAY

Prior studies by Pillai and colleagues in my lab showed that activated, dividing CD4+CD25- cells expressed FOXP3 under all the conditions tested. Suppressive property of activated T cells is still a matter of great controversy even after years of intensive study. While FOXP3 is currently the best marker to date of a regulatory T cell, it may co-incidently be a marker of activation. We reasoned that functional suppressive activity ought to supersede FOXP3 expression as the best measure of regulatory T cell function. To address the controversy, we developed a novel flow based suppression based on differential staining of cell populations, described in detail previously (Figure 46). Briefly, the CD4+CD25- responders were stained with the green dye CFSE; the suppressors (induced Tregs or natural Tregs) with the dye CMTPX, and the APC's with the dye PKH.

Figure 46: Novel flow cytometry based suppression assay uses differential staining of sorted populations to isolate and measure responder proliferation



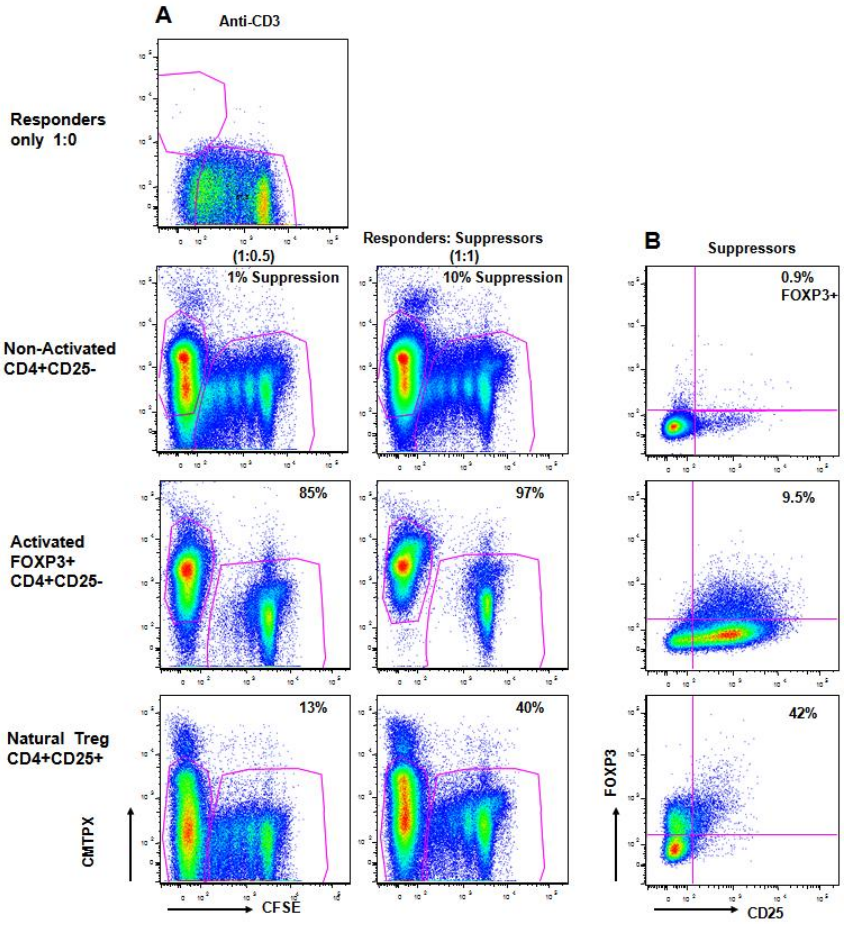
Assay design was representative of several variants designed and performed in collaboration with Dr. Vinodh Pillai (A) Schematic representation of the suppression assay set up. 0.5 million (M) CFSE stained CD4+CD25- T cells were used as responders in every tube. 0.5M CD4- T cells were stained with the red dye PKH, irradiated with 3500 rads were used as APC's. Varying ratios of CMTPX stained suppressors to responders (1:16 to 1:1) were added together along with APC's and antiCD3 and cultured for 5 days. (B) Gating strategy used to isolate and measure responder proliferation. After 5 days of culture cells were stained using CD4 PeCy5.5 and CD25 APC. Flow cytometric data was acquired on a BD LSRII. Proliferation of CD4+ responders was measured using CFSE dilution and CD25 expression after gating out the PKH stained APC's and CMTPX stained suppressors. % Suppression is calculated considering the 'Responder only' proliferation as 100%.

We first tested this suppression assay in *ex vivo* natural Tregs and found that it robustly detects suppressive activity. We then showed that, using this suppression assay, the suppressive activity of activated CD4+CD25- cells was comparable or in most cases increased as evaluated against natural Tregs. Activation-induced suppressive ability was observed when different methods of activation were used: mixed lymphocyte reaction, anti-CD3 (Figure 47), staphylococcus enterotoxin B, PMA/ionomycin.

SUPPRESSIVE ACTIVITY CORRELATES WITH ACTIVATION STATUS AND CD25 EXPRESSION

Another important question in the field is whether the suppressive activity is directly related to FOXP3 expression. Hence, we addressed that question by using the reliable and reproducible flow based suppression assay and correlating it with FOXP3 and CD25 expression. We found that suppressive activity was strongly correlated with activation, CD25 and FOX3 expression (Figure 47). The non-activated CD4+CD25- T cells were not able to suppress the proliferation of the responders. It was also found at very late time points after activation when FOXP3 expression is really low, suppression also decreases (data not shown).

Figure 47: Naturally-occurring CD4+CD25+ Tregs and activated FOXP3-expressing ex vivo CD4+CD25- T cells suppressed while non-activated ex vivo CD4+CD25-FOXP3- did not suppress autologous ex vivo CD4+CD25- responders

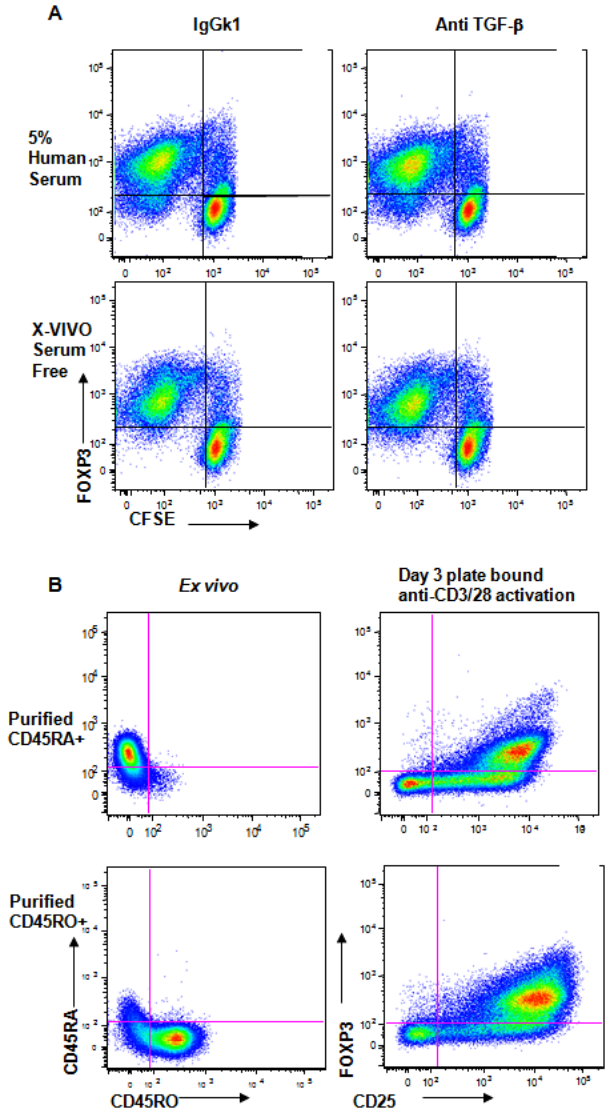


(A) CD4+CD25-FOXP3- T cells from healthy volunteers were activated with anti-CD3 for 5 days to induce FOXP3 expression and the suppressive ability of the activated cells was tested using the suppression assay. The dot plots show gated CD4+ T cells. While the number at top right corner shows the % suppression calculated considering the dilution of CFSE in the top row responders only (1:0) as 100% proliferation. The second row shows CMTPX stained non-activated CD4+CD25- along with CFSE-stained CD4+CD25- responders. The third row shows that the activated CD4+CD25- T cells possess robust suppressive activity. As a positive control the suppressive ability of naturally-occurring CD4+CD25+ T cells are shown in the bottom row. Our novel strategy of gating out APC's and suppressors using PKH and CMTPX showed the suppressive property of activated T cells. (B) FOXP3 and CD25+ expression are shown of cells used in suppression assay: non activated CD4+CD25- T cells, 5 day activated CD4+CD25- T cells, and naturally CD4+CD25+T cells. In the upper right hand corner is the %FOXP3+.

FOXP3 EXPRESSION IN ACTIVATED CD4+CD25- T CELLS IS NOT DUE TO TGF-B PRESENT IN HUMAN SERUM OR THAT PRODUCED BY ACTIVATED T CELLS OR APC

It has been suggested that FOXP3 expression in activated CD4+CD25- T cells is either due to TGFβ present in serum or TGFβ produced by activated T cells or APC's in cultures [317, 318]. It has also been suggested that not all T cells can express FOXP3 on activation and the staining pattern seen is because the most commonly used FOXP3 antibody PCH101 can non-specifically stain activated T cells [319]. CD4+CD25-FOXP3- T cells were activated both in the presence and absence of serum containing media to compare their FOXP3 expression levels. X-vivo 15 (Cambrex Biosience, Walkersville, MD, USA) is a serum-free culture media which does not contain TGFβ. The FOXP3 expression patterns under both conditions were essentially similar (Figure 48). In fact there was higher FOXP3 expression under serum free condition.

Figure 48: FOXP3 expression by activated T cells is not due to presence of TGFβ in serum or that secreted by activated T cells



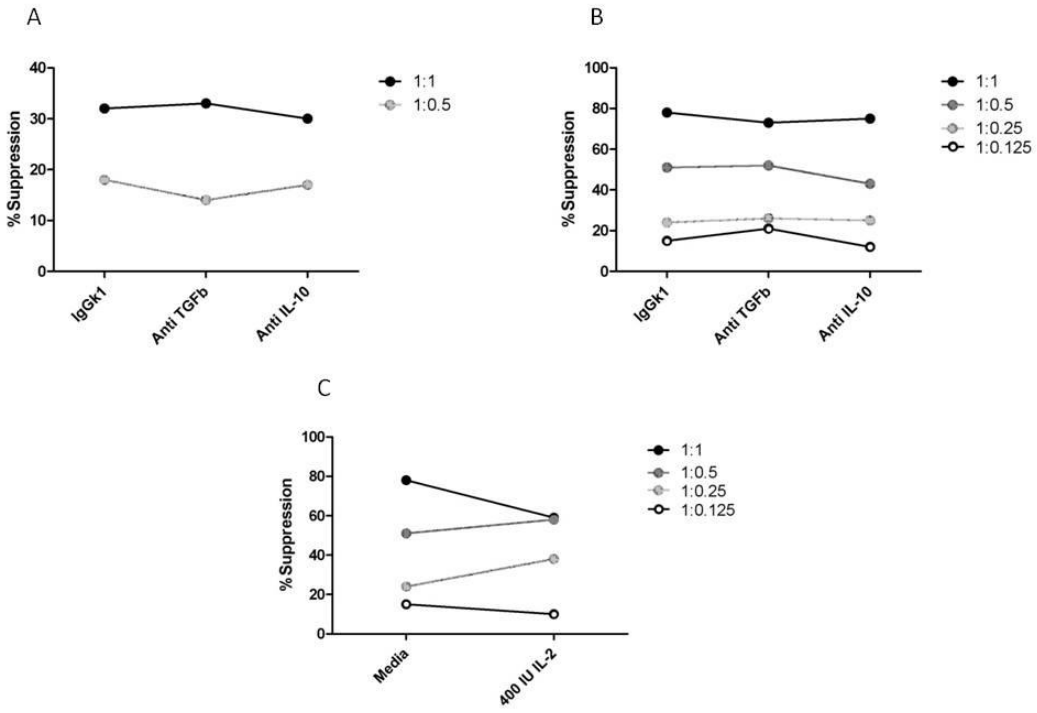
(A) CFSE-stained CD4+CD25- T cells stimulated with an allostimulus for 6 days in 5% human serum-containing media and X-vivo serum-free media both in the presence and absence of anti-TGFβ. (B) Bead sorted CD4+CD25-CD45RA+ naïve and CD4+CD25-CD45RO+ memory T cells were activated by plate bound anti-CD3 plus anti-CD28 for 3 days in X-vivo serum-free media.

SUPPRESSIVE PROPERTY OF ACTIVATED T CELLS IS CONTACT DEPENDENT AND IS NOT DEPENDENT UPON IL-2 CONSUMPTION, COMPETITION FOR APC OR NUTRIENTS IN MEDIA

It has been long been suggested that activated T cells do not suppress and whatever suppression is detected is probably artificial because of competition for IL-2, APCs or nutrients in media. These questions were addressed using the novel flow based suppression assay. Suppression assays were performed as described earlier but now in the presence of varying doses of recombinant human IL-2, excess APCs and media. We found that suppressive activity of activated T cells was still robust in all of the above conditions (Figure 49). Suppression of natural T_{reg}s was not changed by addition of anti-TGF-β or anti-IL-10. Similar to natural T_{reg}s, the suppression of activated T cells was not affected by anti-TGF-β or anti-IL-10. Addition of high dose IL-2 did not make a difference in the suppressive property of activated T cells. This suggests that the suppressive property is an active property of activated T cells and not a pseudo phenomenon due to passive factors. The suppressive activity of activated T cells was contact dependent, similar to that of nTregs (Figure 50). Cell free supernatants from MLR activated or anti-CD3 activated cultures were not able to suppress CD4+CD25- T cells further suggestive the suppressive activity is not a soluble factor (data not shown). Interestingly, high dose IL-2 was able to abrogate the suppressive activity of natural Tregs in parallel experiments suggesting there may be differences in the suppressive activity of nTregs and activation-induced Tregs. This might be a crucial difference between the natural T_{reg}s and activated T cells because

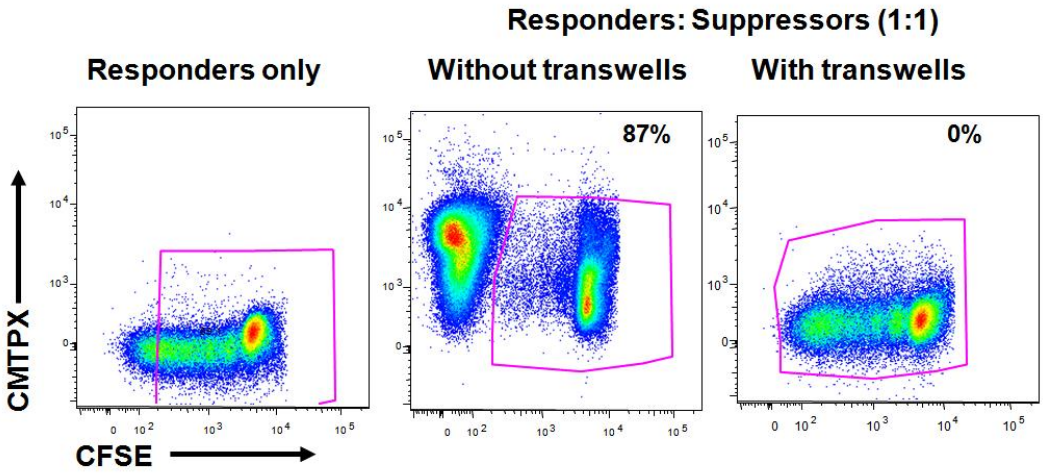
suppression of natural T_{reg}s is known to be abrogated by addition of high dose IL-2.

Figure 49: Compared to nTreg, suppressive activity of activated CD4+CD25- T cells was increased and not affected by addition of anti-TGFβ or anti-IL-10 or high dose IL-2



Experiments were representative of several designed and performed in collaboration with Dr. Vinodh Pillai. The effect of anti-TGF-β, anti-IL-10 and high dose IL-2 on the suppressive activity of natural T_{reg}s (A) and activated T cells (B and C) was examined using the flow based suppression assay. In all graphs the y axis shows the % suppression calculated considering responders only proliferation as 100% while the x axis shows the different conditions.

Figure 50: Suppressive activity of activated CD4+CD25- T cells is contact dependent

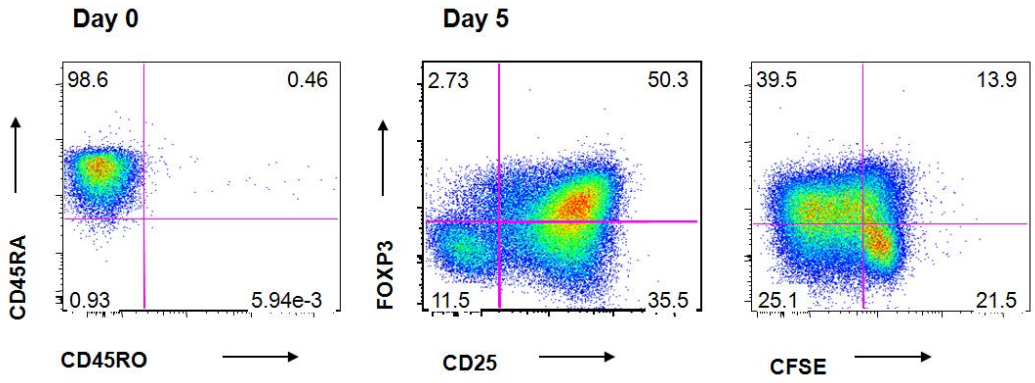


Suppression assays using 3 day anti-CD3 activated CD4+CD25- T cells were set up as described previously. In addition, 0.4 micron transwells were used to separate the responders from the suppressors to determine if contact is necessary for suppression. APC's and anti-CD3 were added both above and below the transwells to ensure activation of both responders and suppressors. CFSE vs CMTPX dot plots from such suppression assays are shown here. Suppressors and responders were mixed in a 1:1 ratio in the absence and presence of transwells. The numbers in the dot plots indicate the % suppression in each of those co-cultures. Presence of the transwell abrogates the suppressive activity of activated CD4+CD25- T cells suggesting that contact is necessary for suppression. Combined with results from figure 3, this suggests that similar to the suppressive activity of natural T_{reg}s, suppressive activity of activated CD4+CD25- T cells is also mediated by a membrane bound or membrane transferred contact dependent factor.

ANTIGEN NAÏVE AND MEMORY T CELLS EXPRESS FOXP3 UPON ACTIVATION AND ACQUIRE SUPPRESSIVE PROPERTIES

Most T cells in adults are antigen experienced cells and our question may be better addressed in antigen naive T cells. Other studies have observed FOXP3 induction in naïve T cells under APC free conditions using plate bound anti-CD3/28 [319]. Memory cells in the CD4+CD25- population may also show inducible FOXP3 expression. In our hands, naïve CD45RA+ cells and memory CD45RO+ cells expressed FOXP3 upon activation using both plate bound anti-CD3 and serum free x-vivo media (Figure 51). Hence, we investigated the FOXP3 expression in CD4+CD25- T cells in cord blood cells. FOXP3 was still expressed even when a nearly antigen naïve populations under plate bound conditions were used (Figure 11). Anti TGF-β was also added to neutralize any TGF-β, making no difference to the FOXP3 expression pattern. This suggests that FOXP3 expression by activated naïve T cells cannot be solely attributed to the presence of TGF-β in serum or that produced by other activated T cells. Other factors are more likely to influence induced FOXP3 expression.

Figure 51: Cord blood naïve T cells express FOX3 upon activation by plate bound anti-CD3/anti CD28 in serum free X-vivo media



Bead sorted CFSE stained CD4+CD25-CD45RA+ naïve (99% pure) from human cord blood were activated by plate bound anti-CD3+anti-CD28 for 5 days in serum free X-vivo media. Most activated T cells can be seen to express FOXP3 on activation suggesting that this phenomenon is not unique to adult humans who have a predominance of memory T cells in their blood.

Activation of antigen-naïve CD4+CD25- T cells from cord blood induced FOXP3 expression and suppressive properties in those cells (data not shown). CD4+CD25-CD45RA+ T cells and CD4+CD25-CD45RO+ T cells each showed suppressive activity upon activation (data not shown). Activated cord blood $FOXP3^{+}$ T-cells exhibit robust and highly reproducible suppressive activity even at very low suppressor to responder ratios. Similar to natural regulatory T-cells, their suppression is contact dependent and anti-TGFβ and anti-IL-10 independent. Suppression is not due to IL-2 consumption since addition of high dose exogenous IL-2 did not make any difference to suppression. In conclusion, we showed the suppressive nature of activated, FOXP3-expressing T-cells and introduced an assay system that will be highly useful in dissecting the biology of induced regulatory T-cells.

EFFECT OF KNOCKING OUT FOXP3 EXPRESSION IN HUMAN T CELLS

We then tried to knock out FOXP3 by RNA interference (RNAi). Vinodh was able to achieve maximal knockdown FOXP3 expression in activated T cells by pulsing CD4+CD25- cells with FOXP3 specific short interfering RNA (siRNA) before activation (data not shown). We were not able to perform suppression assays with siRNA treated cells since the duration of siRNA action was only 36-48 hrs, and our flow based suppression assays is 5-7 days of culture. Vinodh and I also attempted to demonstrate shRNA-mediated knockdown of both activated and natural FOXP3 as described earlier. These experiments were largely inconclusive as polybrene and/or infection of the primary lymphocytes with control scrambled shRNA virus altered their activation status and/or suppressive ability (Figure 52, Figure 53, Figure 54).

Figure 52: Treg-depleted CD4+CD25- T cells were resistant to puromycin treatment after shRNA-lentivirus infection

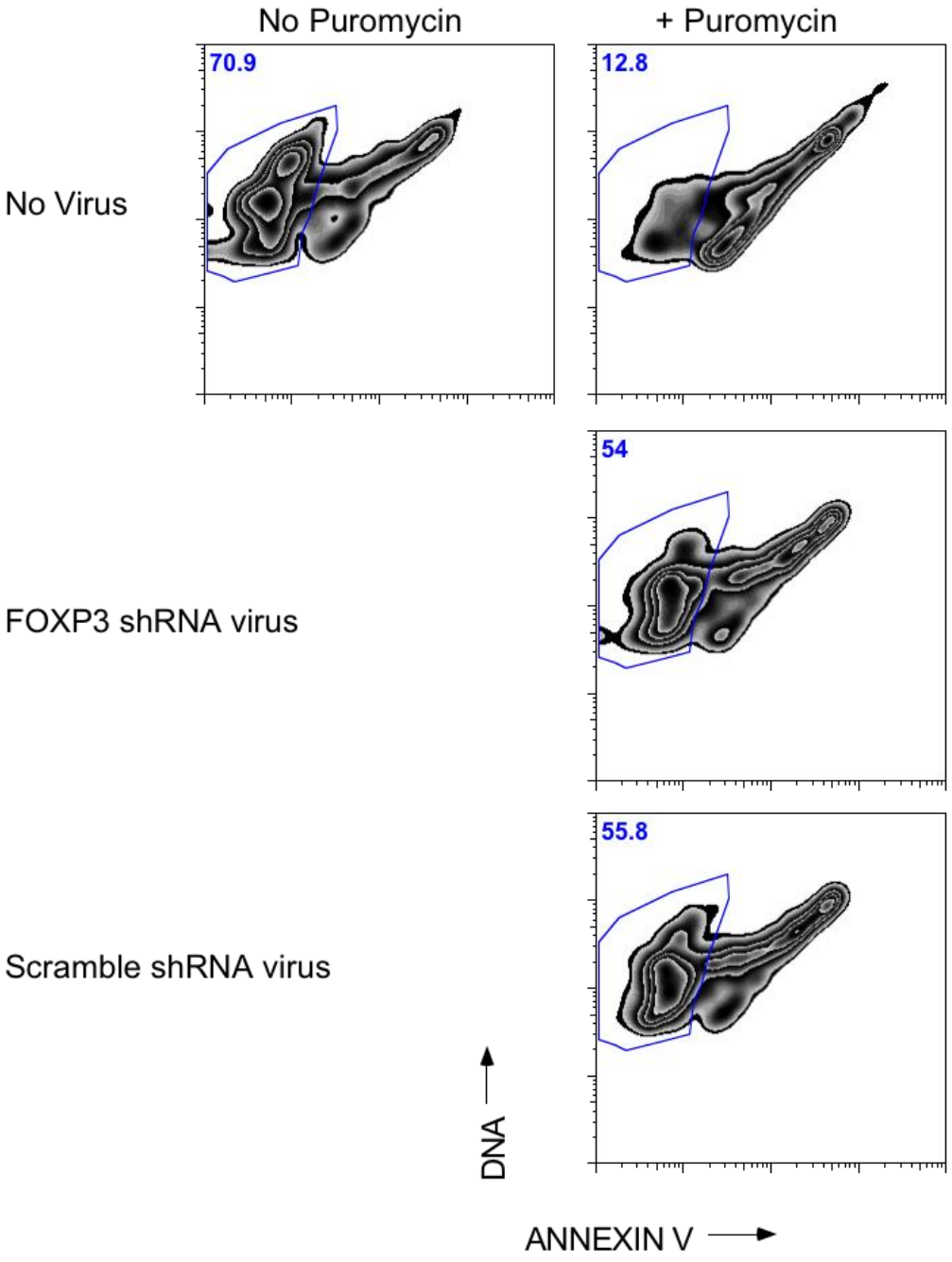


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Figure 53: FOXP3 shRNA lentivirus achieves partial protein knockdown of FOXP3

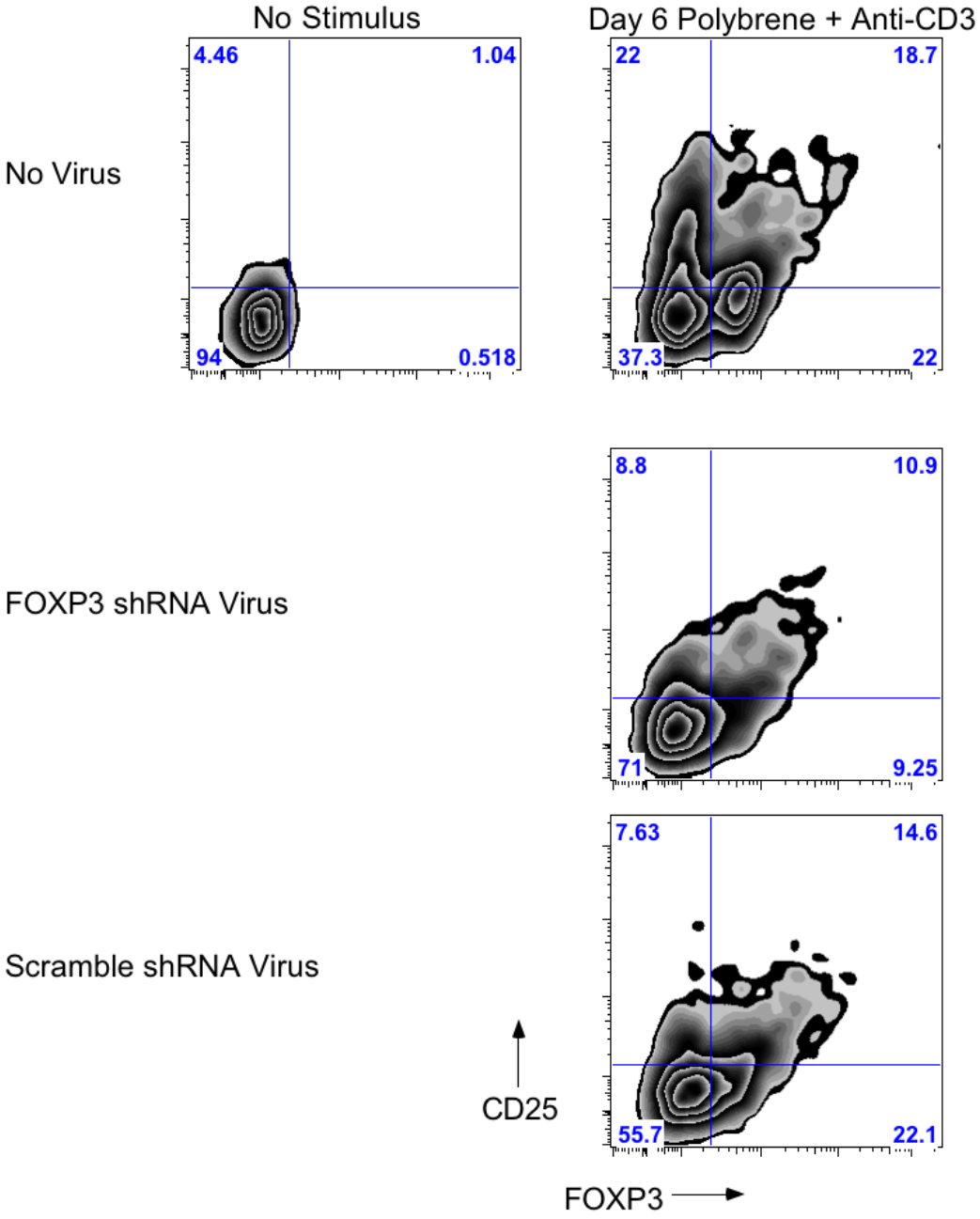
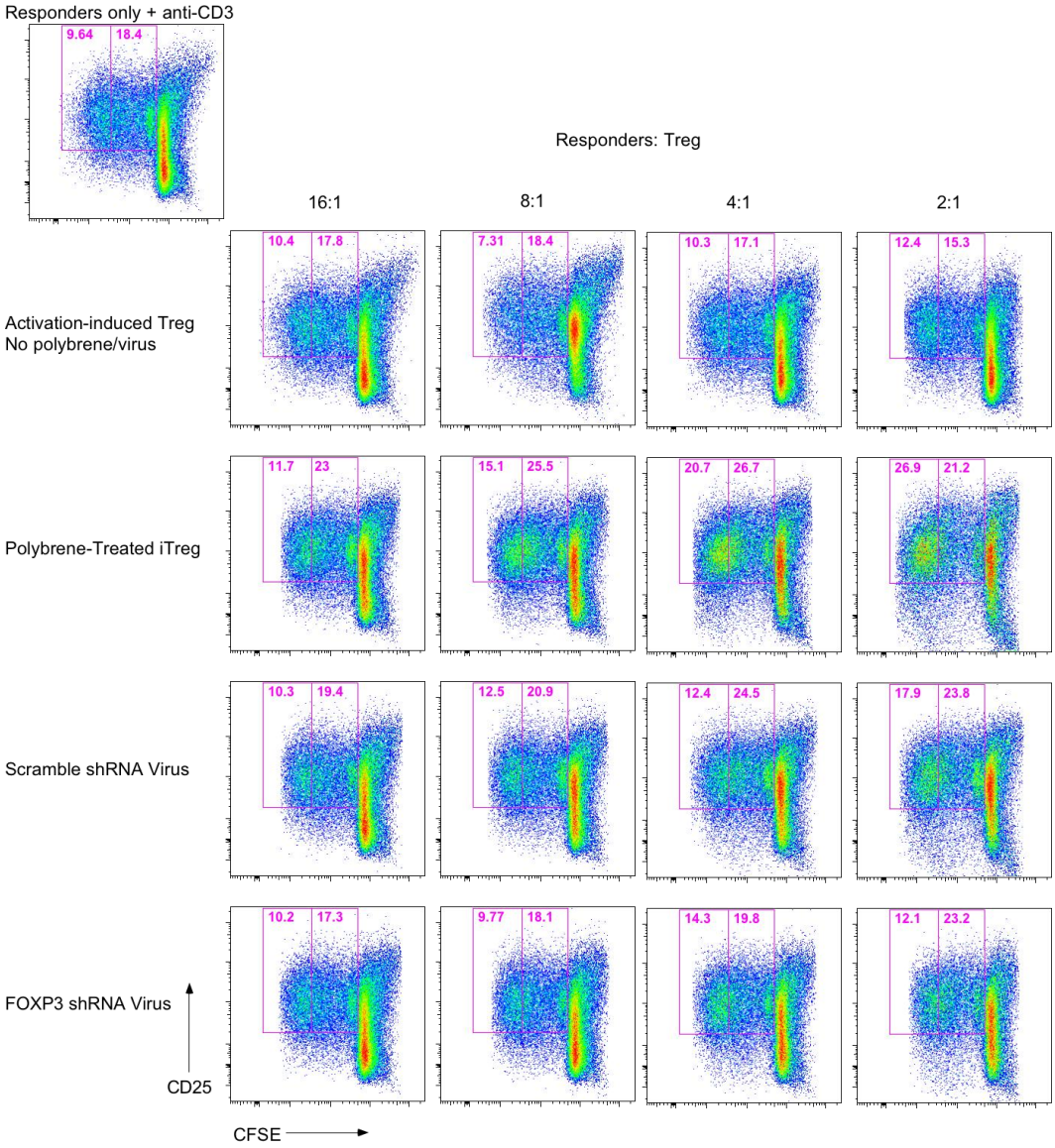


Figure 54: Polybrene-treated iTreg suppressive ability is not detectable by flow-based suppression assay



FOXP3 shRNA virus and scrambled shRNA virus were used to test the role of FOXP3 in activation-induced suppression. Polybrene was used as a co-transfection agent. The untreated activation-induced Tregs control and the polybrene-treated iTreg control failed to suppress autologous responder cells as expected. Technical difficulties prevented the furtherance of this knockdown strategy.

DISCUSSION

We devised a novel flow cytometry-based suppression assay, whereby the putative suppressor cells were excluded from the analysis of proliferation, avoiding the pitfalls of the thymidine-based assay through taking advantage of proliferation dyes and long-term cellular tracking dyes. Using this assay, CD4+ regulatory T cells were characterized to great length. We showed that the flow cytometry based suppression assay consistently detects suppressive activity in activated T cells. As the thymidine based suppression assay measures the total proliferative activity of all the cells in culture, the suppressive activity of activated cells could may be masked by their proliferation especially when the suppressors are not anergic. This phenomenon may be responsible for the failure of early Treg experiments to detect the suppressive activity of activated T cells. Many papers also do not use purified population of CD25+ T cells from activated T cell cultures. The suppressive property of activated T cells can be missed by the insensitivity of the thymidine based suppression assay and not by the flow based suppression assay.

The novel suppression assay showed that activated T cells clearly possess suppressive properties at even very low ratios. Suppression was not due to overcrowding of cells as there is no suppression by non-activated CD4+CD25- or freshly isolated CD4+CD25- T cells. Activation-induced suppression was not due to IL-2 consumption because even high doses of IL-2 did not abrogate suppression in contrast to nTreg. Suppression was clearly contact-dependent and was not affected by even high doses of soluble anti-TGFβ or anti-IL-10. FOXP3

expression by all activated human T cells was seen even when adult or cord blood CD4+CD25-CD45RA+ naïve T cells in serum free X-vivo media and plate bound anti-CD3/anti-CD28 was used.

One of the best-understood subsets of immune regulatory cells is the CD4+CD25+ Treg. FOXP3+ cells in mouse are thought to define Treg. FOXP3 represses the transcriptional activity of NFAT and NFκb among other mechanisms. The role of FOXP3 in humans is thought to be more complex. *Ex vivo* CD4+CD25-FOXP- T cells, upon activation, express FOXP3 and possess suppressive ability. Whether this holds to be true in mouse cells is currently controversial and is being investigated by collaborators in my lab. Previous notions about naturally-occurring Treg from the thymus may be re-examined by these new findings. Human Tregs, as a lineage, could be expanded from an existing pool or induced *de novo* in the periphery. Most evidence supports the former possibility in mice while human Tregs are probably derived by a combination or mostly the latter. Generation of human Tregs is important to the extent that many studies have proposed *in vitro* expansion and therapeutic adoptive transfer in diseases of hyper-immune activation (autoimmunity, allergy, asthma). If human FOXP3+ cells represent only a transient population, then harmful or sub-therapeutic results may be explained. FOXP3 status must be interpreted in light of immune activation as a product of the FOXP3 level.

These and other findings address a matter of great controversy as to whether activated T cells possess suppressive properties. While we and others have shown previously that they do indeed possess suppressive properties, many

others were not able to detect the suppressive property. We hypothesized that the differences in the results obtained by different groups is due to the peculiarities of the widely used thymidine based suppression assay. The thymidine based suppression assay measures the total proliferation of all cells in culture. Hence if the suppressive population in question is not anergic then it would not be able to detect the suppressive activity.

One reason for disparate results in measuring the suppressive property of activated cells could be the wide use of thymidine based suppression assays which fail when the suppressor population is not anergic. We directly compared the performance of the two assays in detecting suppressive property of activated T cells and natural Tregs. We found that while the performance of the two assays was comparable when the putative suppressors were anergic; performance was dramatically different when the putative suppressors were not anergic. This was especially true in the case of activated cells since by definition they are not anergic when they are activated. Hence, it would be very hard to measure their suppressive property during that phase. Our assay would also work well in the case of natural Tregs since it is difficult to obtain a pure population of anergic Tregs. We showed that our assay would be vastly superior in measuring the suppressive property compared to thymidine based suppression assays even when the population is a mixed population of natural Tregs and other contaminating cells.

DISTINCTIONS BETWEEN ITREGS AND NTREGS

Addition of high dose IL-2 did not make a difference in the suppressive property of activated T cells. This might be a crucial difference between the natural T_{reg}s and activated T cells because suppression of natural T_{reg}s is known to be abrogated by addition of high dose IL-2. Natural Tregs lose expression of CD127 and therefore dependent upon IL7 for survival. Dependence upon IL7 may distinguish natural from activation induced regulatory T cells.

PRIONS IN T CELL ACTIVATION

Prion protein (PrP) or prions are responsible for the transmission of spongiform encephalopathies [320]. Unlike most other infectious agents such as viruses, PrP lacks any nucleic acid and is simply a modified form of a normal protein found in mammals. The misfolded PrP configuration (PrP^{sc}) is associated with animal prion disease, scrapie, in mammals and Creutzfeld-Jacob disease (CJD) in homosapiens. Spongiform encephalopathies are rare in both humans and their domesticated large mammals. All forms of prion disease affect approximately one per million people in the US. The bovine form of spongiform encephalopathy is popularly known as mad-cow or foot-and-mouth disease. Histopathologically, spongiform encephalopathies exhibit microscopic vacuoles within neurons and their processes. The abundant vacuoles give a sponge-like appearance. PrP^{sc} is arranged as polymers of β-pleated sheets, forming deposits. Amyloid deposits may contribute to neuronal loss, atrophy, reactive gliosis, and inflammatory infiltration.

TRANSMISSION

Unlike any other infectious agent known, PrP disease may occur sporadically, be transmitted from one host to another (by neurosurgical instruments or tissue transplants), or be inherited in an autosomal dominant manner. Lacking nucleic acids, PrP^{sc} is resistant to UV radiation, high temperature and pressure, and many chemical disinfectants that are otherwise effective on most infectious agents. Sporadic CJD most often affects individuals in their sixties. Most cases of

transmitted prion disease occurred in the UK through the ingestion of cattle with mad-cow disease. Fatal familial insomnia is an inherited form.

CLINICAL PRESENTATION

The symptoms of prion disease classically include, but are not limited to the following: rapidly progressive dementia (less than a year), ataxia, jerking movement, blindness, behavioral and psychiatric disturbances, and neuropathic pain. Prion disease is always fatal, usually within the first year after diagnosis.

The physiological function of PrP is unknown. Based upon its abundance, normal cellular protein likely plays an important physiological role. Contrarily, animals lacking PrP are viable. Most problematic toward their study, $PrP^{c-/-}$ mice are relatively normal with merely subtle abnormalities. The PrP cellular form (PrP^{c}) is found in healthy individuals and is expressed ubiquitously, but highly in the CNS and hematopoetic cells, including myeloid dendritic cells [321]. Upon activation, PrP^{c} is up-regulated on T cells [322]. Once PrP^{c} misfolds into PrP^{sc}, the presence of PrP^{sc} acts as a catalyst, converting more PrP^{c} into PrP^{sc}, primarily in nervous tissue. PrP^{c} is a highly conserved 36 kilodalton transmembrane protein, consisting of α-helices, and is anchored by its C-terminus to a glycosyl-phosphatidylinositol (GPI) in lipid rafts. On its N-terminus are oligosaccharide complex oligosaccharide chains [323]. While PrP is not truly infectious as a virus, the aberrantly folded protein particles can be spread from one afflicted individual to another. Many studies have sought to uncover the physiological functions of PrP^{c} [324]. A selected few are reviewed here.

Many proteins are candidates for interaction with PrP^c. Yeast two-hydrid screens, co-immunoprecipitation, and cross-linking studies have identified lists of putative PrP^c interactors. All of the candidate's physiological relevance to PrP^c remain uncertain. The smallest fraction of transmembrane PrP^c is cytoplasmic. Therefore many have been unlikely candidates, not being transmembrane or secreted proteins. Like most other GPI-anchored proteins residing in the lipid raft, PrP^c may play an important role either directly in signal transduction or indirectly as scaffolding for such processes [325].

PHYSIOLOGICAL ROLE OF CELLULAR PRION PROTEIN

Compelling while insufficient evidence suggests that PrP^c may possess a role in neurons. As prion disease pathology occurs primarily in the brain, it stands to reason that the major focus of study has been at the site. Immortalized neurons from mice lacking PrP^c are more susceptible to serum deprivation-induced apoptosis. In serum deprivation conditions, $PrP^{c-/-}$ cells maintain mirochondial cytochrome c and membrane potential levels as compared to wild type cells [326]. PrP neuroprotection may involve inhibition of the mitochondrial proapoptotic pathways. The moiety of serum responsible for signaling to PrP^c or the intracellular target of PrP^c is unknown. The intracellular binding partner may have been already identified as one of the putative interactors. Antibodies are thought to bind PrP^c, which leads to dimerization of PrP^c at the plasma membrane and phosphorylation of extracellular regulated kinase (ERK1/2), promoting neuronal survival. Decreased endogenous PrP^c expression in neurons has been associated with increased bax-mediated cell death [327]. Contrarily, over-

expression of PrP^{c} in some cell line increases susceptibility to apoptosis to staurosporine through the action of caspase 3 [328, 329]. Over-expression in MCF7 breast cancer cell lines increases resistance to TNFα [330]. It is unclear if over-expression of PrP may lead to a conversion to the lethal PrP^{sc} conformation. $PrP^{c-/-}$ neuronal cell lines resist the oxidative damage caused by copper [331]. PrP^{c} may act to detoxify the brain from the effects of copper. Whether PrP^{c} possesses superoxide dismutase activity or modulates the activity is still being debated [332, 333 C, 334]. PrP^{c} may play a critical role in the balance between neuroprotective and neurotoxic activity in the brain. A large, but rather inconclusive, body of evidence points to a role of PrP^{c} in regulating neuron survival, differentiation, growth, synapse formation, cell adhesion, and apoptosis. Other cell types that highly express PrP^{c} are still understudied in this regard. In consideration of the expression of PrP^{c} in the blood, bone marrow, skin [335], stomach [336], breast, and kidneys [337], alternative hypotheses of its physiologic function have been proposed. Hematopoesis, inflammation, bacterial infection, and T cell activation involvement have been suggested as the function of PrP^{c} [338]. PrP^{c} may act in the innate or adaptive immune system as a microbial pattern recognition receptor, similar but independent to TLR/NOD. The N-terminus cationic and heparin-binding domain of recombinant PrP^{c} has an antimicrobial effect toward *S. aureus* ATCC 29213, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *C. parapsilosis* ATCC 90018 isolates [339]. The peptide disrupted the membranes of the microorganisms. The N-terminus of PrP^{c} has been shown to be cleaved by oxidative stress or reactive

oxygen species [340]. Similar to kininogen and other heparin-binding peptides, cleavage or release of the N-terminus of PrP^c could play an important antibacterial role during the innate immune response during an infection [341, 342]. This hypothesis may more plausibly account for the ubiquitous expression of PrP^c in the skin, gut, lymphoid tissue, brain, blood, and kidney.

ROLE OF PRIONS IN T CELLS

Following stimulation with the mitogen concanavalin A (ConA), activated T cells up-regulated PrP^c [343]. ConA induced-lymphocyte proliferation was diminished in mouse T cells lacking PrP^c. Mice lacking PrP^c display more clinically severe EAE, suggesting that PrP^c may possess an important role in T cell immune modulation [344]. Leukocyte infiltration into the spinal cord, cerebellum, and forebrain was increased in mice after were immunized with MOG. MOG-primed T cells and macrophages/glia from PrP^c-deficient mice with EAE expressed more IFNγ and iNOS, respectively, in spinal cords compared to controls. TNFα and IL-1β were not significantly affected in spinal cords. In the $PrP^{c-/-}$ forebrain and cerebellum, transcripts for IFN-γ, TNF-α, IL-1β, iNOS, and RANTES were increased in during EAE compared to controls.

T cell activation and TCR signaling may regulated by PrP^c [345]. Using RNA interference (RNAi) in mice, PrP^c expression was decreased by 70 percent in peripheral leukocytes, leaving CNS expression unaffected. PrP^c RNAi mice suffered clinically severe EAE compared to control mice. Sub-optimal $PLP_{p139-151}$ immunization during EAE induction was required in order to reduce mortality to that of control mice. Antigen-specific T cell proliferation, activation, and survival

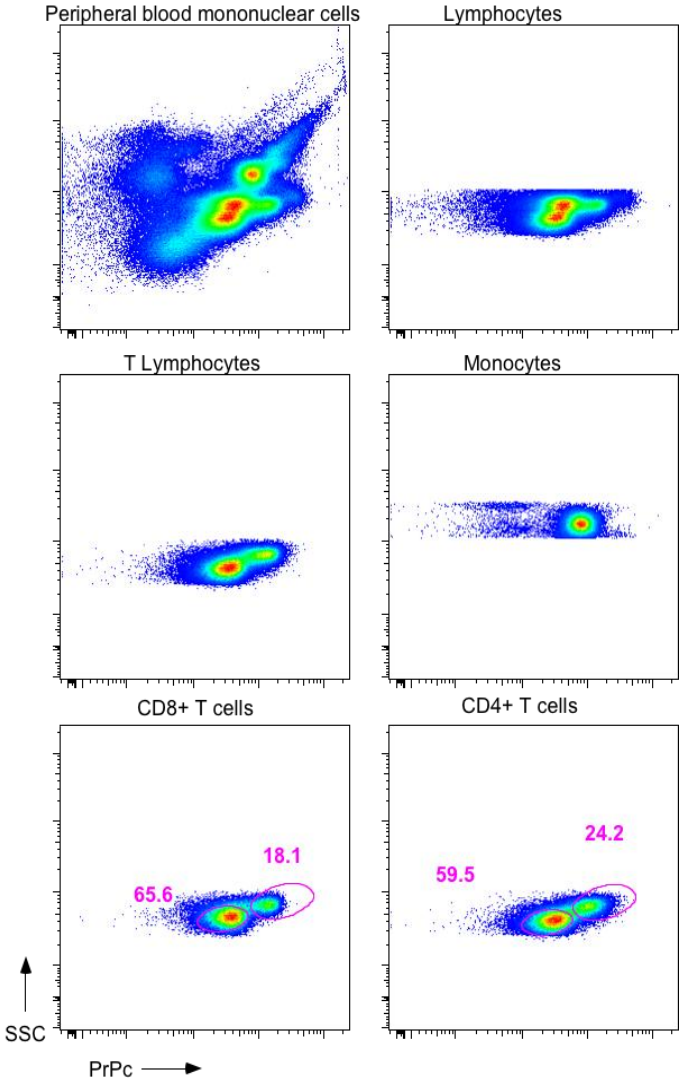
were increased through PrP^c RNAi. Transgenic TCR mice with T cells specific to MBP_{1-11} acquired spontaneous EAE when treated with PrP^c RNAi, but not when treated with scrambled RNAi. PrP^c RNAi in combination with anti-CD3/anti-CD28 treatment enhanced TCR signaling by up-regulating zeta-chain-associated protein-70 (ZAP70) phosphorylation and nuclear factor of activated T cells/activator (NFAT) protein 1 transcriptional activity. PrP^c RNAi mice had increased differentiation of myelin-specific T cells towards Th1 and Th17 cells, while APC were not affected by PrP^c RNAi. MBP_{1-11} TCR transgenic mice developed spontaneous EAE when treated with PrP^c RNAi. Mice that overexpress PrP^c had reduced clinical severity of EAE. Other studies have suggested that PrP^c participates in T lymphocytes activation [322, 346, 347]. Likewise, using human cell, I showed that anti-CD3 stimulated CD4+ T cells up-regulated PrP^c and that PrP^c expression was correlated with CD25 and FOXP3 expression. This suggests that PrP^c may possess a role as a negative regulatory of proximal TCR signaling in human CD4+ Tregs while limiting Treg survival. The precise roles of PrP^c and especially PrP^{sc} in T cell activation remain poorly understood and under-appreciated.

How T cell activation may play a role in the abnormal PrP^{sc} configuration is poorly understood. Severe combined immunodeficiency (SCID) mice challenged with PrP^{sc} are resistant to intraperitoneal and subcutaneous inoculation while sensitive at a low level disease after intracebrebral injection [348]. Immunocompetent or bone-marrow reconstituted SCID mice were highly susceptible to prion disease, suggesting that normal lymphoid structure is required

for transmission and infectivity of PrP^{sc}. Mice that lack PrP^{c} expression in all tissues except T cells and over-express PrP^{c} on T cells are resistant to scrapie [349]. Cell-mediated immune responses appear unaffected in scrapie mice [350]. Scrapie mice lack a detectable PrP^{sc}-specific immune response. PrP^{sc} may accumulate in splenic follicular dendritic cells (FDC) where it is carried to lymph nodes and transmitted to the CNS [351, 352]. How carrier FDCs interact with T cells and nervous cells in order to spread PrP^{sc} remains unclear.

PRPC IS EXPRESSED UBIQUITOUSLY BY HEMATOPOETIC CELLS AND HIGHEST IN A SUBSET OF ACTIVATED/REGULATORY T LYMPHOCYTES

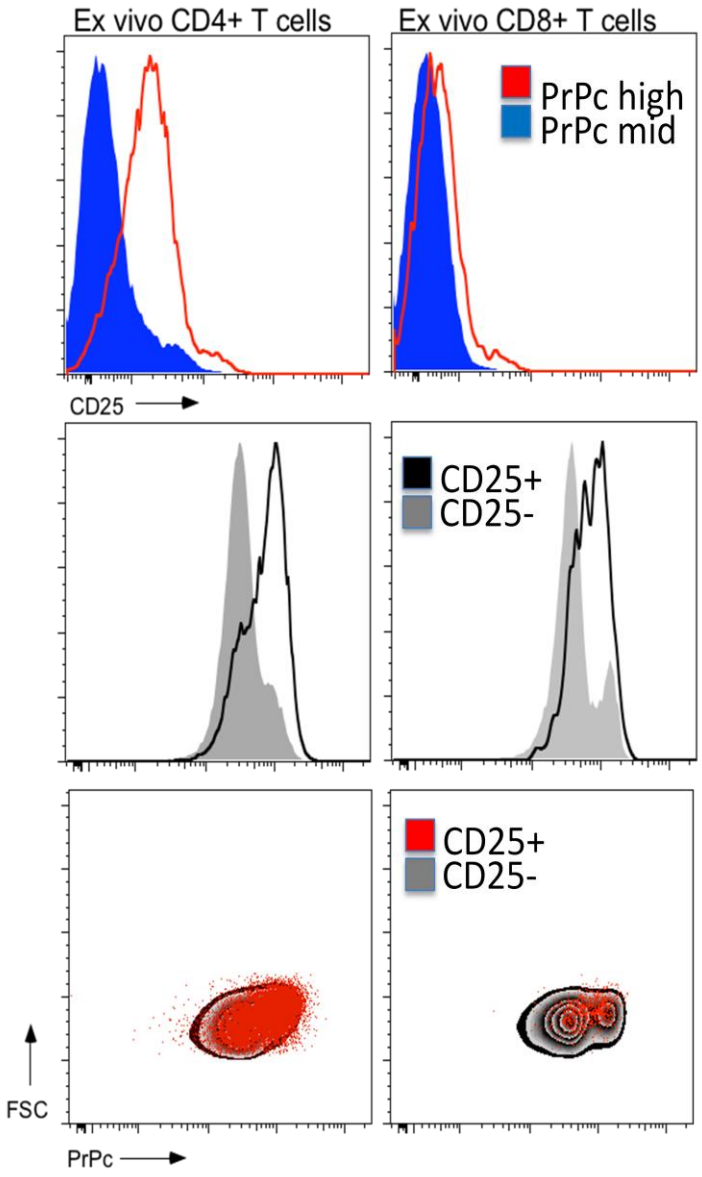
Figure 55: PrPc is expressed ubiquitously by hematopoetic cells and highest in a subset of activated/regulatory T lymphocytes



Ex vivo healthy donor PBMCs were surface stained and acquired for FACS analysis.

EX VIVO CD25+ T CELLS EXPRESS HIGHER LEVELS OF CELLULAR PRION PROTEIN

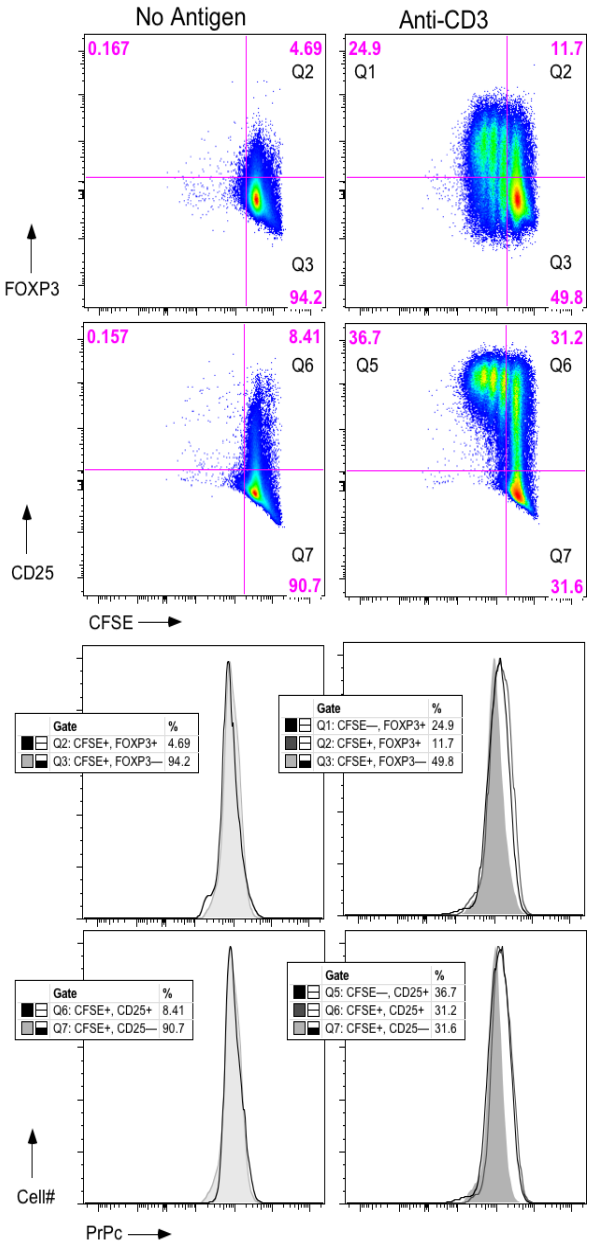
Figure 56: Ex vivo CD25+ T cells express higher levels of cellular prion protein.



Ex vivo healthy donor PBMCs were surface stained and acquired for FACS analysis.

AFTER ACTIVATION, FOXP3+ CD4+CD25+ T CELLS EXPRESSED HIGHER LEVEL OF PRION PROTEIN

Figure 57: After activation, Foxp3+ CD4+CD25+ T cells expressed higher level of Prion Protein



Healthy donor PBMCs were stimulated with anti-CD3 for 7 days and surface stained and acquired for FACS analysis.

THE ROLE OF T CELLS IN TRANSPLANTATION

IMMUNOLOGY OF ALLOGRAFT REJECTION/TOLERANCE

The contributions of direct and indirect recognition to allograft survival or acute and chronic rejection remain unclear. Direct antigen presentation involves donor APC presenting donor MHC peptides. Indirect antigen presentation involves recipient APC presenting donor MHC peptides. The indirect pathway may prolong allograft survival. The direct pathway is thought to mediate acute rejection and the indirect perhaps modulates chronic rejection [353]. However, in heart transplant, indirect alloantigen presentation to CD8+ T cells results in a bystander, non-pathogenic phenotype [354]. Thus, CD8+ T cells which recognized donor peptide in the context of host MHC were not participatory during acute graft rejection. Indirect antigen presentation, but not direct presentation, results in persistent T cell trafficking to lymph nodes, but not the spleen [355]. This suggests that indirect antigen presentation and the lymph node microenvironment are necessary for alloantigen-specific tolerization by CD8+ Tregs, potentially in contrast to the theory that CD8+ Tregs arise naturally and are competent to suppress centrally from the thymus. The context of priming by antigen presenting cells plays a critical role in the alloantigen-specific T cell response [356, 357]. Suppressor CD8+CD11b+ T cells can be generated *in vitro* through culturing naïve CD8+ T cells with alloantigen-primed CD4+ T cells [358]. Presumably, indirect alloantigen presentation *in vivo* after bone marrow transplant results in similar suppressor CD8+ cells, which down regulate immunoglobulin production [359].

STEM CELL TRANSPLANT

Hematopoietic stem cell transplant is used to treat hematologic malignancies. Allogeneic transplant exhibits an enhanced graft-versus-tumor effect, but this is accompanied by significant morbidity and mortality due to graft-versus-host disease (GvHD). In GvHD, donor T cells attack recipient epithelia the intestine, skin, and liver. Autologous transplant displays diminished graft-versus-tumor and –host phenomena, but relapses occur more frequently. Treatment of recipients with an anti-CD25 monoclonal antibody enhances allogeneic stem cell engraftment and expands alloantigen-specific regulatory T cells [360]. Blockade of the CD8 molecules, as well, inhibits down-regulation of alloantigen-specific immune responses [361]. Several other studies have shown a positive relationship between increased Treg frequency and reduced incidence of GvHD [362-364]. IL-10 induction of Tregs or production by regulatory T cells is thought to prolong graft acceptance [365-367].

UMBILICAL CORD BLOOD

A major source of multipotent hematopoietic stem cells (HSC) is umbilical cord blood (UCB). Cord blood HSC, in contrast to embryonic stem cells, are termed adult or fetal stem cells. HSC may give rise to more HSC and all formed elements in the blood. HSC are required to engraft in hematopoietic tissue transplants into patients who have undergone purposeful ablation of their hematopoietic organ, reducing the burden of occult malignant cells and resident T cells. HSC purification from UCB is accomplished through positive selection for CD34 and Thy-1 and negative selection for other lineages (CD10, CD14, CD15,

CD16, CD19, and CD20) [368]. Isolation of HSC is required to purge grafts of unwanted populations from clinically functional and transplantable HSC population. Immune reconstitution following a HSC transplant presents a risk for opportunistic infection. CD4+ T lymphocyte recovery takes between 3 and 6 months to reach >100 CD4+ T cells/μL [369]. Relative to autologous HSC, allogeneic HSC without T cells eliminates the clinical issue of GVHD and the basic requirement for immunosuppression. The benefit of GVT effect is also eliminated without T cells. Allogeneic immune reconstitution is likely to be delayed and engraftment failure may occur. With T cells depletion, the incidence of allogeneic HSC transplant engraftment failure increases. Bone marrow-derived non-HSC populations play a critical role in enhancing engraftment. The engraftment facilitating capacity of the non-HSC population was concentrated in the CD8+ compartment [370, 371]. Both CD8+ T lymphocytes and CD8+ dendritic cells were shown to facilitate HSC engraphment. Taking advantage of CD8+ cell dose, GVT may be restored without GVHD [372, 373].

UCB HSC transplant (UCB-HSCT) was first performed in 1990 on a 6-year-old boy with Fanconi anemia [374]. UCB HSC is now performed for a variety of clinically severe hematologic, immunologic, and neoplastic disorders. UCB banks, both private and public, are increasing in capacity. A HIV positive patient with newly diagnosed with acute myeloid leukemia (AML) showed long-term control of both HIV and AML after a stem-cell transplant with CD34+ peripheral HSC from a CCR5 deficient donor [375]. Compared to peripheral HSC transplant, UCB-HSCT allows more HLA disparity with less GVHD [376]. The

mechanism of increased UCB-HSCT may involve diminished CD8+ T cell cytotoxicity [377]. The diminished CD8+ cytotoxicity may potentially involve a more naïve T lymphocyte repertoire, expressing CD45RA, and more dominant immune regulatory cells, expressing FOXP3, that may both control GVHD [378]. The high proportion of CD45RA+ T cells is owed to the fact that the fetus *in utero* is exposed to low levels of environmental pathogens and vaccines compared to adults. UCB CD45RA+ T cells produce less IFNγ than adult CD4+ T cells after stimulation with alloantigen. UCB CD4+ T cells may produce less IFNγ as a result of impaired master regulator transcription factors, such as T-BET, NFAT, AP-1, and phosphorylated STAT4 [379]. Defective antigen-presenting cells in UCB, expressing less costimulatory molecules and TNFα, may impair T cell differentiation into Th1 cells [380]. Immature dendritic cells (DC) express less costimulatory molecules and promote antigen-specific tolerance [381]. DCs may drive tolerance through the generation of FOXP+CD25+CD4+ T regulatory cells [382]. Immune regulatory cells may play a dominant role over IFNγ production though, as IFNγ plays a protective effect against GVHD [383]. The fetus *in utero* is exposed to higher macrophage colony stimulating factor (M-CSF) [384] and low inflammatory signals, which may help to promote Th2 differentiation and immature dendritic cells [385] capable of producing more IL10 and less IL12 [386]. Immune regulatory cell populations in UCB may potentially have a role in transplantation tolerance.

The tolerogenic nature of T cells is important in UCB HSC transplant. GVHD, rejection, and reconstitution delay are all immunologic parameters and may

potentially be managed taking into consideration the regulatory phenotype and function of particular subsets of T cells. A master regulator transcription factor, FOXP3, is the most specific marker for regulatory T cells (Tregs) [387]. FOXP3+ cells co-express CD25 on the surface [388]. Subsets of Tregs also express CTLA4, GITR, OX40, CD62L, and CCR7 [389-393].

THE ROLE OF CD8+ TREGS IN TRANSPLANT

Regulatory CD8+ T cells are selectively resistant to general immunosuppressant agents, such as Cyclosporin A, allowing them to dominantly down-regulate lymphocyte proliferation [394]. Kidney transplant recipients treated with alemtuzumab have shown a homeostatic replenishment of regulatory CD8+ T cells [395]. Alloreactive CD8+CD103+ regulatory CD8+ T cells are expanded by rapamycin [49, 396]. Sphingosine-1-phosphate receptor modulation plays an important role in mediating CD8+ T cell responses in skin graft rejection [397]. Orthoclone OKT3 is a monoclonal antibody used to prevent rejection in heart, liver, pancreas, and kidney transplant [398-402], and OKT3 is also used to treat T1DM. OKT3 has been demonstrated to selectively expand CD8+ T cell populations relative to CD4+ T cells and induce regulatory CD8+ T cells *in vivo* [299]. Experimentally, pre-transplant cell-based therapy can be accomplished either by infusion with whole cells or MHC-derived synthetic peptides. Donor-specific transfusion, along with anti-CD2, -CD3, and –CD40L monoclonal antibodies, has successfully tolerized heart recipients as long as T cells home to the lymph node [403]. Alloantigen-specific CD8+CD103+ regulatory T cells are generated *in vitro* using mixed lymphocyte reactions containing mDCs plus

TFGbeta [404]. Regulatory CD8+ T cells are protective in liver [39, 43, 50], heart [38], and kidney [42, 297, 405] transplants. CD8+ T cells are required for cardiac allograft survival mediated by ICOS-B7 blockade [53]. Anti-ICOS monoclonal antibodies induce regulatory CD8+PD1+ T cells, which transfer protection to CD8-deficient allograft recipients. Regulatory CD8+ T cells have been explored as potential agents in cell-based therapies for GvHD [406, 407] following allogeneic bone marrow transplant.

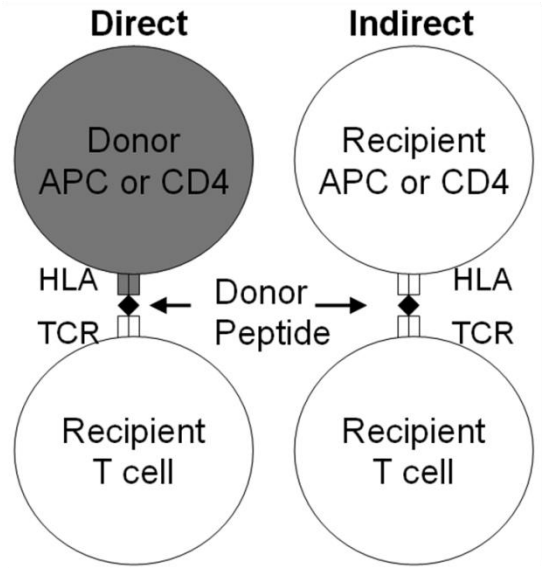
ALLOANTIGEN-SPECIFIC CD8+ TREGS

In various experimental models, both CD4+ and CD8+ Tregs exhibit alloantigen-specific suppressive ability [408]. Oral allo-antigen exposure induces γδ CD8+ T cells to produce IL-10 and tolerize recipients toward kidney allograft [297]. Pan-stimulated CD8+CD122+ T cells via anti-CD3/28 suppress allogeneic-T cell IFNγ production in a CD28-, CD80-, and CD86-dependent manner, but independently of CTLA-4, PD-1, or ICOS [300]. Alloantigen-specific CD8+PD-1+regulatory T cells are induced by anti-ICOS mAb to produce IL-4 and IFNγ suppress alloantigen-specific CD4+ T cell in cardiac allograft transplant [53]. Donor-specific transfusion, which can be used to tolerize recipients toward allogeneic liver transplantion, induces CD8+CD28- T cells capable of adoptively transferring reduced acute rejection rates to secondary recipients [296]. While less than 5% of CD8+ express CD103, which binds E-cadherin, CD8+CD103+ T cells potently suppress mixed lymphocyte reactions [49]. Alloantigen-specific CD8+ Treg generated *ex vivo* from CD8+CD25(-) cells can be induced from CD40-activated B cells [57].

IMMUNOLOGY OF ALLOGRAFT REJECTION/TOLERANCE

The contributions of direct and indirect recognition to allograft survival or acute and chronic rejection are unclear. Direct antigen presentation involves donor APC presenting donor MHC peptides. Indirect antigen presentation involves recipient APC presenting donor MHC peptides. Whether the indirect pathway initiates or enhances rejection is not understood. The direct pathway is thought to mediate acute rejection and the indirect perhaps mediates chronic rejection [353]. However, in heart transplant, indirect alloantigen presentation to CD8+ T cells results in a bystander, non-pathogenic phenotype [354]. Thus, CD8+ T cells which recognized donor peptide in the context of host MHC were not participatory during acute graft rejection. Indirect antigen presentation, but not direct presentation, results in persistent T cell trafficking to lymph nodes, but not the spleen [355]. These results suggest that indirect antigen presentation and the lymph node microenvironment are necessary for alloantigen-specific tolerization. The context of priming by antigen presenting cells plays a critical role in the alloantigen-specific T cell response [356, 357]. Suppressor CD8+CD11b+ T cells can be generated *in vitro* through culturing naïve CD8+ T cells with alloantigen-primed CD4+ T cells [37]. Presumably, indirect alloantigen presentation *in vivo* after bone marrow transplant results in similar suppressor CD8+ cells, which down regulate immunoglobulin production [359].

Figure 58: Alloantigen-presentation pathways



CD8+ TREG SUPPRESS AUTOLOGOUS AND ALLOGENEIC ALLOREACTIVE CD4+CD25(-) T CELLS IN THE BOTH DIRECT AND INDIRECT ANTIGEN PRESENTATION PATHWAYS

Suppression of alloreactive CD4+ T cell stimulation was carried out in culture with varying CD4 to suppressor ratios including the following: 1:0.125, 1:0.25, 1:0.5, and 1:1. CD8 suppressors autologous to CD4 responders or the APC were used, as well as a third party donor. Donor origin is annotated in Figure 10 and Tables 1 and 2 as A, B, and C, respectively. Similar to the previously described flow-based suppression assay, on day 7, cells are stained with florescent antibodies and fixed with 1% paraformaldehyde. Suppression was determined by comparison to CD4 response in the absence of suppressors.

Figure 59: Alloantigen-specific CD8+ Treg suppress autologous and allogeneic alloreactive CD4+CD25(-) T cells in the both direct and indirect antigen presentation pathways.

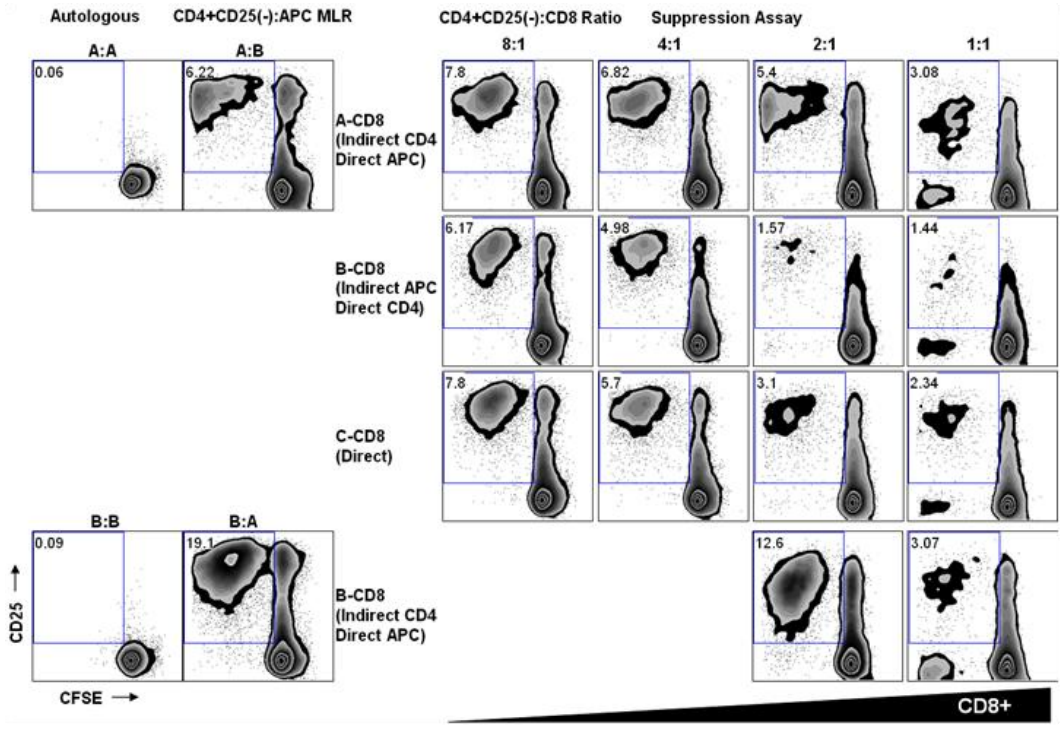
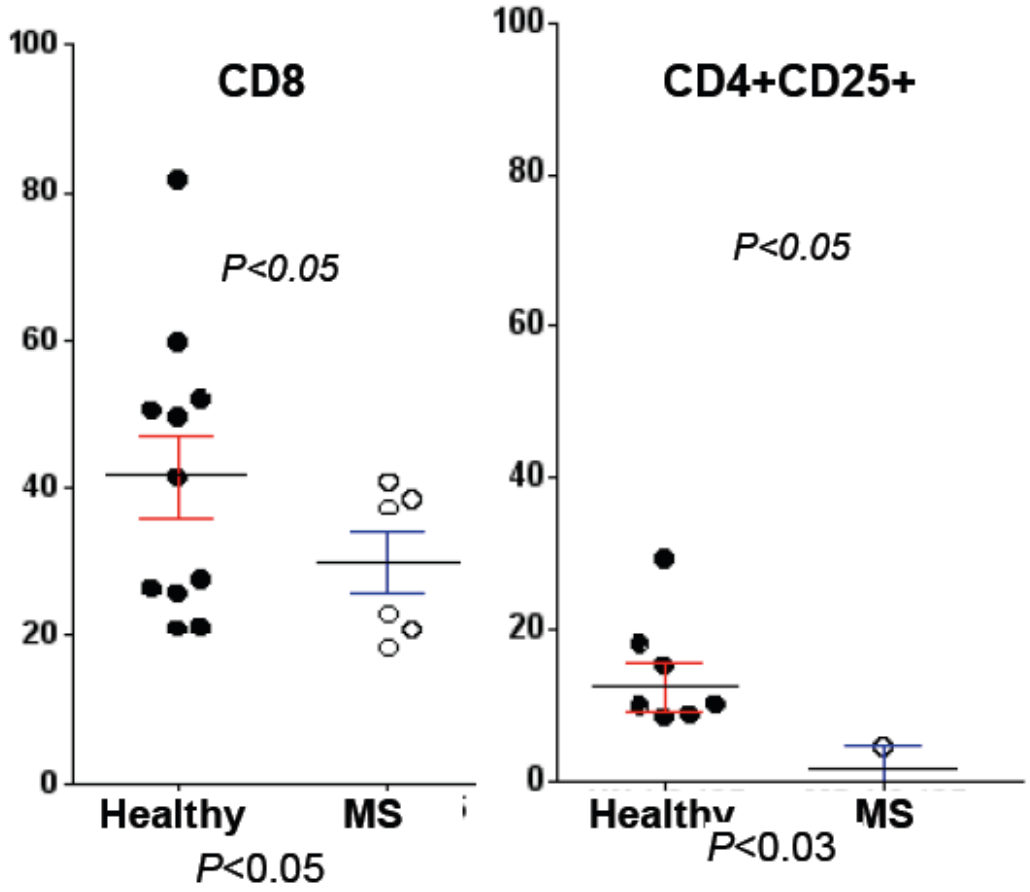


Table 2: Figure 59 Legend

Autologous No Stim CD4+CD25(-):Irrad. APC	Ex Vivo MLR CD4+CD25(-) → Irrad. APC	MLR + Ex Vivo Suppressors	Expected Result on CD4	Expected Result on APC
A:A	A → B	**A-CD8 (Recipient)**	**Indirect Suppression**	**Direct Suppression**
		B CD8 (Donor)	**Direct Suppression**	**Indirect Suppression**
		C CD8 (Third Party)	**Direct Suppression**	**Direct Suppression**
		A CD8+CD28+	Indirect Suppression	Direct Suppression
		B CD8+CD28+	Direct Suppression	Indirect Suppression
		C CD8+CD28+	Direct Suppression	Direct Suppression
		A CD8+CD28(-)	Indirect Suppression	Direct Suppression
		B CD8+CD28(-)	Direct Suppression	Indirect Suppression
		C CD8+CD28(-)	Direct Suppression	Direct Suppression

Ex vivo PBMC from 3 adults (notated A, B and C) were sorted into bulk CD8+, CD4+CD25(-), and APC. CD4+CD25(-) were cultured with allogeneic APC, and CD8+ T cell suppressive ability was compared among the adult donors. Different donor origin is annotated as A, B, and C.

Figure 60: MS patients have deficient CD4 and CD8 Treg function



Purified bulk CD8+ T cells (left panel), CD4+CD25high T cells (right panel) or CD4+CD25neg T cells (not shown) were labeled with CMTPX and used in flow cytometry-based suppression assays (similar to those shown in **Figure 15**). Autologous, CFSE-labeled CD4+CD25neg T cells were used as responders (stimulated with autologous T celldepleted APC and anti-CD3). Increasing ratios of Tregs were added (data shown here are from a Treg:responder ratio of 0.125:1 in each case). Percent suppression was calculated based on the proliferation of responders alone (without Tregs) and is represented on the Y-axis.

APPENDIX II: PERSONAL RECORD

CHRONOLOGY OF FRUITFUL COLLABORATIONS

While the published narrative of my and my collaborators contribution to biology may seem like an ordered stream, the who and how it all happened was really less well-organized, more serendipitous, mostly having to do with me being in the right place at the right time around so many other smart, diligent people. After I was accepted to the MD/PhD program at UT Southwestern Medical Center as an undergraduate, I requested an appointment with Nitin Karandikar (then an associate professor of immunology, pathology, and neurology) in March of 2005. I had read a paper entitled, "Intrathecal synthesis of oligoclonal IgM against myelin lipids predicts an aggressive disease course in MS," regarding antibodies in the CSF of MS patients [409]. It was not a particularly high impact article, but I knew something about lipids and was interested in immunology. Nitin discussed the paper out of consideration for me even though he is more interested in T cells than B cells, and he was kind to hand me a copy of one of his recent publications about myelin-specific CD8+ T cells in MS [202]. I read several of Nitin' publications and met with Nitin again during my first year of medical school to discuss doing my second rotation in his laboratory.

During my rotation in Nitin's laboratory in the summer of 2006, I was trained and supervised daily by Sterling Ortega (then the lab manager/senior technician), who later became a graduate student in our lab. Sterling taught me to run flow cytometry and supervised me acquire a 9 color experiment of CNS-specific CD4+ and CD8+ T cell responses using the BD LSRII. Sterling taught me digital

compensation and proper techniques in flow cytometry. In May, I helped Vinodh Pillai (then a senior graduate student) and Sterling do quantitative real time PCR on CD4+CD25+ cells for FOXP3, IL2, IL4, IL10, IFNγ, TGFβ and β actin as a control. In July, Sterling and Vinodh taught me to intracellularly stain cells for FOXP3. I also helped Vinodh to edit his paper manuscript, "Transient regulatory T-cells: a state attained by all activated human T-cells." After the summer, I returned to second year medical school. Vinodh and Nitin were kind to acknowledge me in their paper when it was published later that winter [91]. Nitin, Vinodh, and Sterling were excellent mentors, and I hoped that they would accept me into the laboratory.

When I returned to Nitin's laboratory in June of 2007 for my "third rotation," Nitin carved out a more-or-less independent project for me that would follow in the footsteps of a previous post-doc, Deepani [104]. In our discussions, Nitin asked me to design an experiment so that we could measure and observe the suppressive ability of CNS-specific CD8+ T cells. This proved to be one of the most pivotal conversations we ever had. After reading several papers using suppression assays, on July 2, I conducted my first 6 day flow-based suppression assay after having tinkered with CMPTX staining of the CD8+ suppressor cells as a strategy for gating them out in the analysis. On July 10, the 7 day flow-based suppression assay was standardized, after having determined the appropriate CFSE-stained responder to CMTXP-stained suppressor ratios and attempting to adapt acquisition to the HTS on the LSR. By the end of July, the suppression assay was validated with CD4+CD25+ cells as a positive control for suppressive

ability and CD4+CD25- cells as a negative control. Vinodh (then a post-doc) and I began using PKH26 staining of APCs in the suppression assay. Unlike CMPTX and CFSE that make protein adducts, PKH is a lipophilic proliferation dye that non-colvalently partititions into lipid membranes. The PKH fluorescence was off the scale, and there were problems with toxicity. By August, the simpler CMTPX and CFSE only assay was ideal in my mind, and I began functional studies with healthy human cells in August. After working out all the kinks, I held to this established protocol for the flow-based suppression assay for all the years of my graduate school. My first summer back in the laboratory felt like a success. I was grateful that Vinodh remained in our lab to cross-pollinate my ideas. Thankfully, Nitin agreed to take me into the lab as his newest graduate in the summer of 2007. Now that we had a working suppression assay as a tool, we put it to use with real samples. In the fall of 2007, I sorted healthy human CD8+ T cells into subsets using CD28 and tested their suppressive ability. By October, I had characterized 11 healthy human donors and began my first experiment with an untreated MS patient with my suppression assay. In November, I generated my first antigen-specific T cell lines after 5 weeks of culture with the help of Larry Anderson (then an assistant professor in internal medicine). I also examined cellular prion protein expression in healthy human PBMC with the collaboration of Olaf Stuve (then an associate professor in neurology).

By the spring semester of 2008, I was assigned to train an undergraduate, Liz Gunter, in collaboration with the UT Dallas Green fellowship program. Promising results led to the first new MS patients recruited from the MS clinic in

St. Paul, in coloration with Elliot Frohman, professor of neurology. By February, I was able to grow T cell lines for 10 weeks in culture and conducted suppression assays with transwells. So far, it was clear that healthy and untreated MS patients possessed neuroantigen-specific suppressive ability by CD8+ T cells. Foreign-antigen specific T cell responses were undeterred. In May, we began using cord blood as a source cells, in collaboration with Parkland OBGYN, and induced FOXP3 expression in naïve T cells. Vinodh and I conducted several functional assays together, characterizing FOXP3 in activation-induced CD4+ Tregs. We presented posters of our work at FOCIS in Boston that summer. By the end of the fall semester in 2008, I had shown that CD8+ T cell could suppress a mixed lymphocyte reaction and had tested if supernatants from CD8+ T cells could inhibit CD4+ T cell responses. By December, I had collected 5 more cord blood donors for functional assays. Vinodh and I spent several weeks writing grants together, and I submitted my first grant to the NIH in December for the F30 individual predoctoral MD/PhD fellowship. Nitin helped to hone my scientific writing and presentation skills.

In the spring semester of 2009, I was co-assigned to train a rotating graduate student, Elizabeth Dimitrova. With the help of Larry, I attempted to use a IFNγ ELISPOT assay as a readout of suppression and explored the mechanism of CD8+ T cell suppression with anti-TNFa/HLA class I antibodies. Several sets of T cell lines were grown and used in dual antigen thymidine-based suppression assays. These experiments helped to answer important questions about the

specificity and likely HLA restriction of the suppressive neuroantigen-specific CD8+ T cells.

Having had considerable success, Nitin and I decided to move into riskier and bolder strategies with the help of Todd Eagar (then an assistant professor in neurology). In the summer of 2009, Vinodh and I attempted knockdown strategies with lentivirus vectors in primary T cell lines. Vinodh wanted to knockdown FOXP3, and I aimed for perforin and granzyme B. Vinodh ultimately settled on purchasing lentivirus particles from Santacruz while I toiled to culture plasmid-transfected HEK293T cells with fugene and lipofectamine, culture bacteria, conduct Maxipreps, concentrate virus, infect T cells with polybrene, and select them with puromycin. It was an excellent learning experience, but not a great source of published data. After Vinodh left the lab to start his residency, I tried my best to show that FOXP3 was required or was not sufficient for functional suppressive ability. Ultimately, I failed. My grant scored mediocre, and I prepared to resubmit a revision in August. Nitin encouraged me to get back up and stick to my guns.

In the fall semester of 2009, I was assigned to train another rotating graduate student, Chris Peña. I resumed conducting suppression assays with several untreated MS patients and my first acute exacerbation of MS patient in October. Nitin and I experimented with human tonsil immune cells, in collaboration with the Children's Hospital, and I continued mechanistic experiments with a panel of antibodies toward molecules thought to have some effect in my suppression assays. I wrote another grant proposal with Nitin's help in December. That fall

semester, the suppression assays from several MS patients began to suggest that MS patients during an acute exacerbation were fundamentally different from quiescent MS patients in my CNS-specific CD8 suppression assay. We ramped up patient recruitment, in collaboration with Ben Greenberg (then associate professor of neurology). Jason Mendoza (then a post-doctoral fellow) was also critical in the recruitment of MS patients in the fall 2009 and spring 2010 semesters. Nitin and I began work on the manuscript entitled, "Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis" that spring 2010 semester. I gave a talk and presented posters at FOCIS in Boston with work done in collaboration with Jason and Vinodh, (then a pathology intern at MGH in Boston) that June. Nitin and I spent several months revising and re-drafting my paper for publication in several scientific journals, and it ultimately was published on January 11, 2011 in the Journal of Autoimmunity [2]. The continued effort has focused on the question of the mechanism of CNS-specific suppression. My experience in graduate school continues to be diverse and rich with collaboration due to many gifted scientists. The richness was not only in the questions answered but also in the relationships forged. The scientists of this institution were world-class in terms of their unfettered curiosity and ambitious contributions to human health and biology. Many other important people were critical to the work, as I have included only a few in consideration of brevity. I look forward to continuing to work in such a collaborative environment where training and mentorship are so customary and highly valued.

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