CNS-SPECIFIC, AUTOREACTIVE CD8+ T CELLS HAVE A REGULATORY ROLE IN AUTOIMMUNE DEMYELINATION

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by

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Multiple Sclerosis (MS) is an inflammatory disorder characterized by the destruction of myelin sheaths, which encase neurons of the central nervous system. A great deal of our current knowledge about the immune pathogenesis of MS derives from work in its murine model, experimental autoimmune encephalomyelitis (EAE), which can be induced by inoculation with a specific neuroantigen or by adoptive transfer of CNS-specific activated T cells. The vast majority of studies in MS and EAE have focused on the role of CD4+ T cells in this disease, with the underlying assumption that MS, like EAE, is a CD4+ Th1-mediated autoimmune

disease. However, several reports have implicated both CD4+ and CD8+ T cells in the pathogenesis and regulation of these diseases. In this study, we show the presence of antigen-specific, autoreactive CD8+ T cells in several models of EAE. Furthermore, through series of adoptive transfer studies, we show that these cells play a regulatory role in the pathogenesis of autoimmune demyelination. Using novel in vivo killing assays, we show that these cells retain their killing capacity and that they target both activated APC and CD4+ T cells that have been loaded with the specific antigen. These cells are also shown to produce cytokines that may be involved in disease regulation. We also show that these cells modify antigen-presenting capacity of APC. In conclusion, our studies provide strong evidence that antigen-specific CD8+ T cells are involved in regulatory processes in the context of autoimmune demyelination.

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ABBREVIATIONS

μg – Micrograms

μl – Microliters

μM – Micromolar

Ab – Antibody

Ag - Antigen

APC – Allophycocyanin

APC – Antigen Presenting Cell

BSA – Bovine Serum Antigen

CD – Cluster of Differentiation

CFA – Complete Freund's Adjuvant

CFSE - 5- (and 6-) carboxyfluorescien diacetate succinimidyl ester

CNS – Central Nervous system

ConA – Concanavalin A

CTLA - Cytotoxic T-Lymphocyte Antigen

DC – Dendritic Cells

EAE – Experimental Autoimmune Encephalomyelitis

ELISA – Enzyme-Linked Immunosorbant Assay

FACS – Fluorescence Activated Cell Sorting

GA – Glatiramer Acetate

HAT – Hypoxanthine Aminopterin Thymidine

HLA – Human Leukocyte Antigen

IACUC - Institutional Animal Care and Use Committee

IFA – Incomplete Freund's Adjuvant

IFN – Interferon

IL – Interleukin

LNC – Lymph Node Cell

LPS - Lipopolysaccharide

MBP – Myelin Basic Protein

MHC – Major Histocompatibility complex

MIP – Macrophage Inflammatory Protein

MOBP – Myelin-associated Oligodendrocyte Binding Protein

MOG – Myelin Oligodendrocyte Glycoprotein

MRI – Magnetic Resonance Imaging

MS – Multiple Sclerosis

MTb – Mycobacterium tuberculosis

NK – Natural Killer

PBS – Phosphate Buffered Saline

PE – Phycoerythrin

PF – Proliferation Fraction

PFA - Paraformaldehyde

PLP – Proteolipid Protein

pMBP – MBP peptide

pMOG – MOG peptide

pOVA – OVA peptide

pPLP – PLP peptide

PT – Pertussis Toxin

OVA - Ovalbumin

RAG – Recombination Activating Protein

SCID – Severe Combined Immunodeficiency

SDS - Sodium Dodecyl Sulfate

SEB – Staphylococcus Enterotoxin B

SI – Stimulation Index

SpC – Splenocytes

TAP – Transporter associated with Antigen Processing

TCR – T-cell Receptor

TCV – T Cell Vaccine

TGF – Tumor Growth Factor

Th – T helper

TLR – Toll-like Receptor

TNF – Tumor necrosis factor

Treg – Regulatory T Cells

Ts – Supressor T Cells

wbMBP – whole bovine Myelin Basic Protein

Chapter I: Introduction

Multiple Sclerosis

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system. It affects approximately 250,000-350,000 people in the United States (1). MS manifests itself in several different forms, all of them characterized by any combination of a number of symptoms including limb weakness, pain, memory loss, optic neuritis, fatigue and cognitive dysfunction. MS is usually diagnosed in early adulthood through clinical diagnosis and confirmed through radiographic identification of CNS lesions by MRI. Histologically, these lesions have been shown to be filled with infiltrating leukocytes (2). Females are three times more likely than males to get this disease (3). There are four characteristic forms of MS, primary progressive, secondary progressive, relapsing remitting and progressive relapsing. All of these forms become more debilitating over time. The most prevalent form is relapsing remitting MS, in which patients will go through periods of latency, when no symptoms are manifested, and then enter symptomatic periods of varying lengths(4) There are several effective therapies for MS, but these only aim at the amelioration of symptoms associated with the disease (12-16). There is currently no cure.

While suggestions of people with MS have been noted as early as the Middle Ages, it wasn't until the 19th century that it became a subject of annotated study. Jean-Martin Charcot is generally credited for being the first to "frame" the disease, describing it in great detail in 1868 (5). Charcot discovered the characteristic "plaques" in the brain of an affected woman that would become that hallmark of the disease. At this time, however, all we knew about MS came from observation alone.

Despite the long, rich history of MS research, the etiology of the disease remains unknown. There is evidence, through concordant twin studies, of a genetic predisposition associated with certain HLA haplotypes as well as other non-MHC genes(6-8). For example, studies have shown that immediate family members show a 2-5% increased risk to develop MS and identical twins have a 25% increased risk to develop the disease (9). There have been many studies to deduce the genes linked to the disease. The highest concordance appears to be linked to the MHC class II alleles HLA-DR and –DQ, with HLA-DR15 having been shown to be of high concordance in Caucasians (10, 11). HLA-DQ association seems to be more prevalent in other populations with a lesser prevalence (12). HLA class I genes A3 and B7 have also been suggested to have involvement in MS (13, 14). There have also been other studies suggesting the involvement of non-MHC genes in MS as well, such as CTLA-4, IL-1 and the estrogen receptor (1)

A purely genetic explanation, however, is insufficient. It is evident that there must be some environmental factors that also play a vital role in disease induction(15). For example, studies have shown a north-south gradient of disease prevalence in the northern hemisphere and an opposite gradient in the southern hemisphere, with the prevalence decreasing as one nears the equator. Individuals that move from an area of high incidence to an area of low incidence before the age of fifteen show a decreased risk for developing MS as compared to individuals that relocate past the age of fifteen (16). Aside from a geographical standpoint, infectious agents are also thought to play a role in the pathogenesis of the disease. There have been observations made in both MS and EAE that suggest this involvement. For example, studies have shown that MS patients have increased relapses following viral infections (17). In EAE, it has been shown that mice containing transgenic, MBP-specific TCRs develop the disease in nonpathogen-free

environments, but remain healthy in specific pathogen free facilities (18). Several infectious agents have been implicated, both viral (19, 20) and bacterial (21). It has been hypothesized that MS onset, as well as other autoimmune diseases, is triggered by an immune response to self proteins that resemble proteins produced by an infecting pathogen (a phenomenon known as molecular mimicry), although studies have so far been unable to produce a direct correlation (22-25). Other studies suggest that infection leads to bystander activation of myelin-specific T cells (26-28).

Several indications point to autoimmune-mediated destruction of myelin as the major effector of disease. Mononuclear cell infiltration of the CNS is evident upon histological examination (29, 30). MS patients harbor immune responses against CNS antigens (31). It has been shown that healthy humans harbor these autoreactive T cells in their peripheries as well (32). Evidence suggests that autoreactive CD4+ T cells become activated in the periphery (32, 33) and upregulate adhesion molecules that allow them to cross the blood brain barrier (34, 35). Once inside the central nervous system, they are reactivated by resident (or possibly trafficking) APCs (36). Due to the cytokine influence of activating APCs, these cells become pro-inflammatory Th1 and Th17 cells, which activate other leukocytes, including macrophages, B cells and CD8+ T cells (Frohman, Racke review). It is thought that these cells mediate the damage to myelin, producing the symptoms seen in the disease.

As mentioned above, the evidence of immune involvement in the demyelination process has led to the development of the therapies currently in use for MS. Immunosuppressive drugs such as interferon beta therapy (37, 38) and mitoxantrone (39) have been used with limited success in MS patients. Natalizumab, another therapy, also works by suppressing the immune response through preventing the migration of white blood cells into the CNS by blocking

adhesion molecules (40). Other therapies, such as glatiramer acetate (GA, Copaxone) have been shown to modulate the immune response, causing a switch from Th1 CD4+ T cells to Th2 CD4+ T cells (35). Each of these therapies is effective in some, but not all, patients. Also important to note is that these therapies aim at decreasing the amount and severity of symptoms; they are not curative.

Experimental Autoimmune Encephalomyelitis

Much of what we know about MS comes from research done in an animal model of the disease, experimental autoimmune encephalomyelitis (EAE). EAE has its roots in adverse reactions to humans that received the rabies vaccine developed by Louis Pasteur (41). It was noted that many patients developed sudden onset paralysis or paresis. Studying this condition in monkeys, it was discovered that these patients showed perivascular inflammation and lymphoid infiltration, making it distinctly different from rabies pathology (42).

In the 1930's, Dr. Thomas Rivers was investigating this phenomenon by injecting vaccine preparations into rhesus monkeys. He noted that the monkeys developed ataxia and paresis (43). In similar experiments, rabbits injected with emulsified rabbit brains became afflicted with an ascending paralysis (44). Further experimentation utilized complete Freund's adjuvant and the disease was able to be provoked with a minimal number of injections (45). This disease, termed EAE for the first time, provided a link between brain-specific immune responses and induced encephalitis.

EAE has since been established in many different models. Different species have been used, including rats, mice, guinea pigs, rabbits, dogs and monkeys (46). One of the most studied species is the mouse. Several different strains of mice have been extensively studied, including

B10.PL, SJL/J and C57BL/6. These different models have allowed the discovery that there are many different encephalitogenic antigens that are able to induce disease when emulsified in complete Freund's adjuvant and injected.. For example, B10.PL mice are immunized with a peptide fragment of myelin basic protein (MBP Ac 1-11) (47). SJL/J is immunized with a peptide from an entirely different protein, proteolipid protein (PLP) 139-151 (48). C57BL/6 mice have been shown to develop disease when immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (49) as well as whole bovine MBP (50). These are just a few of the several mouse models of EAE that have been established.

Besides the immunizing peptide, another difference in the disease between mouse strains involves the disease course. The typical disease involves ascending paralysis, which starts with the tail becoming flaccid and involves the hind limbs as the disease progresses. In severe cases, the forelimbs can also be involved, rendering the animal in a moribund state. Some models, such as the B10.PL model, typically display an acute disease course in which the mouse will have onset of disease about ten days post immunization, be symptomatic for a variable period of time, usually 1-2 weeks, then recover with no lingering effects (46). Other models, such as the SJL/J model, show a relapsing-remitting form of the disease, similar to that which is seen in most humans with MS. These mice will go through waves of being symptomatic interspersed with periods of recovery (47). The C57BL/6 model displays a more chronic disease phenotype (48). These mice remain in a chronic symptomatic state after onset of disease.

From a pathology standpoint, EAE displays several characteristics of human MS, making it a useful tool for studying human demyelination. Demyelination with sparing of other nervous system architecture is a major feature of both diseases (51). Histopathologically, both diseases

feature perivascular infiltration of mononuclear cells, as well as central nervous system (CNS) lesions that are distributed in time and position (52).

CNS Demyelination and the T Cell

As research progressed and more models were established, attention turned to the mechanism of demyelination. A series of studies using thymectomized rats provided a great insight into the disease process. In the first study, neonatally thymectomized rats were challenged with emulsified MBP as young adults. None of the rats developed symptoms, although histology showed CNS infiltration (53). This study also showed that a cell-mediated response, measured by MIF release, was associated with the inflammation. A different study confirmed these results and took them a step further. Gonatas et. al. reconstituted the thymectomized mice with thymocytes before antigen challenge. These mice developed EAE comparable to controls and also developed anti-MBP antibodies, something not seen in the thymectomized rats (54). Both of these studies strongly indicated the need for thymus-derived cells in the induction of EAE. This was the early evidence that T cells played a critical role in autoimmune demyelination. Bernard and colleagues showed that, as was seen in rats, thymectomized mice did not develop EAE or an anti-MBP antibody response (55). These mice, however, developed EAE when they were injected with sensitized T lymphocytes. Purified T lymphocyte populations were also shown to transfer disease into naïve normal mice.

After T cells were determined to be the effector cells of EAE, work began to establish which specific T cells were responsible for the disease. Early investigations involved reconstituting T cell populations in SJL/J mice that had been thymectomized and irradiated. Mice reconstituted with Lyt-1+2- splenocytes (which would later come to be synonymous with T

helper cells), followed by immunization with spinal cord homogenate, showed signs and symptoms of EAE in 100% of immunized mice, while only 25% of mice reconstituted with Lyt-1-2+ splenocytes (which would later become synonymous with cytotoxic T cells) developed disease (56). 100% of mice injected with both Lyt-1 and Lyt-2 cells, followed by immunization with spinal cord homogenate, also developed. These experiments offered early evidence that T helper cells were responsible for onset of disease, as the small amounts of disease seen in the mice reconstitutued with Lyt-2+ splenocytes could be attributed to contaminating Lyt-1+ cells. These results also suggested that Lyt-2+ cells did not regulate the disease.

In another study, Lewis rats that had been actively immunized with MBP or received adoptively transferred lymphocytes were treated with a monoclonal anti-CD4 antibody. In both cases, mice that received the treatment showed amelioration or protection from EAE compared to mice that received control antibodies (57). These studies showed that preventing activation of helper T cells through antibody blockade could stay disease induction despite the fact that encephalitogenic cells were present in the recipient rat.

Other experiments performed around the same time indicated that Lyt-2, or CD8+, T cells did not appear to play a role in disease pathogenesis or suppression. In one set of experiments, CD8+ T cells were depleted in a long-term basis in Lewis rats that were actively immunized or injected with sensitized CD4+ T cells. These rats showed no difference in disease onset, duration or severity when compared to control. The same study showed that these rats demonstrated resistance to relapse in a manner similar to normal controls (58). A similar study was performed using SJL/J mice. These mice were depleted of Lyt-2 T cells using a single intraperitoneal injection of monoclonal antibodies, then disease was induced either with spinal cord homogenate or through transferred sensitized splenocytes. In both actively induced and

adoptively transferred disease, the Lyt-2-depleted mice failed to demonstrate any significant differences in disease course (59). Likewise, the mice showed no differences in relapsing events when compared to control mice. These studies in both rats and mice ,indicated that CD8+ T cells played no role in disease pathogenesis, disease suppression or relapse resistance.

Another critical discovery that proved to be important for understanding the disease processes was the link between the MHC variability between mouse strains and their disease susceptibility. Experiments with MBP p1-9 peptide showed that while it was able to induce disease in certain strains (B10.PL and PL/J mice, for example), other strains of mice, such as the B10.S and SJL/J mice, were resistant to disease induction with this neuroantigen. This evidence correlated with a different study that suggested MBP 1-9 peptide was H2-A^u restricted (47). Further studies went on to demonstrate correlation between the MBP peptide and a specific variable (V) gene segment (Vβ8) on the T cell receptor of cells that were encephalitogenic in these mice. Using PL/J and (PL/JxSJL) F1 mice immunized with MBP 1-9 peptide, it was shown that blockade of the Vβ8 region was shown to ameliorate and/or prevent disease (60, 61). These studies provided the basis for the hypothesis that specific MHC-neuropeptide-TCR interactions are responsible for disease. It should be noted that this observation, however, is model specific. Attempts to establish this Vβ8 correlation in other EAE models proved unsuccessful.

The results of these early studies focused the vast majority of research on disease progression and amelioration on the CD4+ T cell. As knowledge in the immunology of T cells progressed, studies were performed to determine the specific type of CD4+ T cell was responsible for disease. Studies in B10.PL mice showed that it was the "Th1" type T cells that caused disease progression in MBP-induced EAE (62, 63). These T cells are defined by their

cytokine profile (interferon-γ, IL-2, IL-18), which induces inflammatory cells in a cell-mediated type of response. These experiments corresponded well with the observations that EAE was a cell-mediated autoimmune disease. Therapeutic research also focused on Th1 T helper cells. It was shown that certain immunomodulatory therapies functioned by pushing the Th1/Th2 ratio towards the Th2 response, which appeared to repress the relapsing exacerbations seen in mice and also in patients with relapsing-remitting MS (64). These observations gave further credence to the hypothesis that T helper cells are the cause of autoimmune demyelination and other immune system cells were by and large ignored.

The more recently discovered phenomenon of epitope spreading characterizes an additional CD4+ T cell property in autoimmune demyelination. Early descriptions of this phenomenon showed that autoreactive T cells are not fixed on a given epitope, as was previously thought (63). Much to the contrary, it was found that the repertoire of autoreactive T cells evolved over time, even if the disease stimulus was a peptide and not an entire protein. Further studies showed that "determinant spreading" was not restricted to other epitopes of the immunizing protein, but that it could spread to other proteins as well (65, 66). This phenomenon correlates well with observations in MS, namely that these patients show immune responses to multiple myelin antigens (31, 67) as well as the observation of increased MBP autoreactivity following viral infections (68, 69) indicating that inflammation as a result of one peptide can lead to reactivity to other similar, and possibly self, peptides. Epitope spreading has also become an interest because of its therapeutic potential. It has been shown that induction of tolerance to a known spreading cascade can stop disease progression in the SJL/J model. This property, however, translates with difficulty to multiple sclerosis because , as has been discussed, the

inciting peptide or peptides of multiple sclerosis are unknown, and most likely vary from person to person (70, 71).

New Observations in Autoimmune Demyelination

More recent research, however, indicates that Th1 cells are not the sole pathogenic determinant of the disease, nor is the Th1-type response wholly responsible for disease induction and progression. For example, while IL-12 is one of the major effector cytokines of the Th1 response, both IL-12 and IL-12 receptor knockout mice are susceptible to EAE (72, 73). Further studies revealed that p40, the IL-12 subunit used in studies to indicate IL-12 involvement, is also found in another cytokine, IL-23. When IL-23 and IL-12 were specifically targeted using their unique subunits (p19 and p35, respectively), IL-23 proved to be the cytokine essential for EAE induction (72). It was later shown that IL-23 is critical for the propagation of CD4+ T cells that produce the cytokine IL-17 (74). These IL-17-producing CD4+ T cells (now called Th17 cells), and not Th1 cells, are critical for disease development (75).

Observations involving other Th-1 effector cytokines also indicate that they are not wholly responsible for disease induction. For example, interferon gamma (IFN-γ) has been shown to be unnecessary for disease induction (76-78). In fact, IFN-γ actually ameliorated EAE in immunized mice (79). Blocking of tumor necrosis factor-alpha (TNF-α), another inflammatory cytokine that is secreted in response to Th1 stimulation, actually worsens or precipitates CNS demyelination (80).

These observations led to a renewed interest in the possibility that other immune cells may be involved in the pathogenesis and/or regulation of the disease. There have been several studies linking myeloid cells to the disease processes of EAE and MS. Bone-marrow-derived

dendritic cells were shown to be able to transfer EAE into naïve, wild-type mice after being incubated with the neuropeptide MOG 35-55 (81). It was shown that these cells activated encephalitogenic T cells, but had to be alive in order to incite disease. Activated dendritic cells have also been found in the central nervous system of multiple sclerosis patients, whereas DC in healthy humans usually do not infiltrate the CNS (82). Studies have also shown that mice given immunomodulatory therapy develop type II monocytes, which secrete the regulatory cytokine IL-10 and direct differentiation of regulatory T helper cells (83). This evidence suggests a regulatory role for these cells.

Lymphocytes other than T cells have also been shown to be involved in EAE and MS. It has long been known that B cells can be found in the cerebrospinal fluid of MS patients (84). These have been found to undergo oligoclonal expansion, indicating reactivity to neuroantigens (85). The role of B cells has been extensively studied, and there exists ample literature arguing different possible roles for them in demyelination (reviewed by Antel, (86). For example, B cells, antibodies and plasma cells have all been found in the CNS of MS patients (87, 88). MOG-reactive antibodies have also been found in MS lesions (89). Other studies have shown that antibodies may also play a role in remyelination either by binding directly to MBP (90) or through blocking signals that inhibit growth processes (91). Natural killer cells have been implicated in disease remission (92, 93). Other studies indicate that the presence of natural killer cells correlates with increased disease incidence (94). Another subset of lymphocytes known as NK T cells has also been implicated in MS and EAE. These cells have only been studied recently, and most studies indicate that they play a regulatory role in autoimmune demyelination (95-98) either through influencing development of regulatory dendritic cells (96) or possibly through downregulation of encephalitogenic T cells (97). These studies taken together suggest

that the disease courses of multiple sclerosis and EAE are far more complex than originally thought and that further studies are necessary to determine the roles of these various cell types.

CD8+ T cells

The fact that the existence of the CD8+ T cell has only been discovered in the past 30 years says nothing of its importance in the immune response. Its role in many different types of inflammatory processes has been well-established and, with the advent of certain modern technologies, the characterization of this cell has shown that it plays a vital role in the body's defenses against tumors and intracellular pathogens.

The discovery of "small lymphocytes" that actually participate in immune function has only been made within the last fifty years (reviewed by Masopust, (99). In fact, it wasn't until the early 1960's that the evidence of thymus-derived lymphocytes became known (100). Around the same time, it was shown that thymus-derived lymphocytes could specifically target and kill allogeneic targets (101). Additional roles for these lymphocytes continued to be discovered over the next twenty years. An important discovery occurred in the mid-1970s when it was discovered that these cytolytic "T cells" could be phenotypically distinguished based on the surface expression of Ly-2 and Ly-3, which later became characterized and renamed as CD8α and CD8β, respectively. Also around this time, it was discovered that these cells functioned in an MHC-restricted fashion (102, 103). This discovery, coupled with the characterization of the TCR in the early 1980's (104-109) allowed researchers to piece together the puzzle of the interactions between CD8, TCR, antigen and MHC class I (110).

Since that time, knowledge of CD8+ T cells has grown exponentially. While it is not within the direction of this work to treat all of these discoveries, it is important to highlight a few

that are pertinent to the discussion at hand. The most well known, and probably most extensively characterized, role of CD8+ T cells lies in its antiviral role. These cells are critical for destroying host cells that have become infected with intracellular pathogens. The emergence of HIV in the 1980s spurred many discoveries regarding this role (reviewed by Gulzar, (111). Another important role that has been well-studied is the "tumor surveillance" function of CD8+ T cells (reviewed by Svane, (112). In both of these roles, the CD8+ T cells use cytolytic enzymes, such as perforin and granzyme, to cause eradication of altered self cells. Another important discovery that bears mentioning is that of the ability of CD8+ T cells to develop into memory cells. Similar to the B cell, these cells allow rapid response to antigens that have already presented a challenge in the past. These cells can be maintained long term through the influence of cytokines, such as IL-7 and IL-15 (113).

Another role for CD8+ T cells was first reported by Gershon and Kondo in 1971. These scientists reported that tolerance to sheep red blood cells could be transferred from tolerized mice to thymectomized mice given activated thymocytes, and that this tolerance was mediated in an antigen-specific fashion (114). It was later discovered that the lymphocyte that effected this transferrable tolerance was the CD8+ T cell (115, 116). This observation was recognized in several other models (117, 118) and through the production of different hybridoma lines able to induce tolerance, but since a specific phenotype of this "CD8+ suppressor T cell" remained unclassifiable, this hypothesis of a specific suppressor role for CD8+ T cells fell out of favor.

Role of CD8+ T Cells in Autoimmune Demyelination

Recall that early studies in EAE characterization showed that Lyt-2, or CD8+, T cells do not play a role in the disease course. Several observations, however, hinted that these cells

responded to neuroantigens. For example, studies showed that CD8+ T cells outnumber CD4+ T cells in MS lesions of all stages (119). Further characterization of these CD8+ T cells have been shown to be oligoclonally expanded (120). Our laboratory has conducted antigen-specific proliferation assays of CD8+ T cells in the peripheral blood of both MS and healthy patients, and has shown that both populations contain CD8+ T cells that proliferate to neuroantigens in an antigen-specific fashion (31). These observations suggest that these CD8+ T cells are not only present at sites of disease exacerbation, but that they, like CD4+ T cells, are specific for neuroantigens.

Another study attempted to describe the activity of these cells by examining cellular products. It was shown that myelin-specific CD8+ T cells from MS patients have been shown to secrete both proinflammatory cytokines, such as MIP-1α and MIP-1β, and matrix metalloproteinases (121). These cells were also shown to produce the lytic granules granzyme b and perforin and express CD94/NKG2A on their surface (122). It has also been shown that these cells, like CD4+ Th1 cells, produce and secrete IFN-γ. This evidence strongly suggests that, not only are these cells specific for neuroantigens, they are being activated and responding in kind to antigenic stimuli.

A possible argument to the involvement of CD8+ T cells in the pathogenesis or regulation of EAE is that this disease is induced by priming the animals with exogenous antigen. It was accepted long ago that exogenous antigens were processed through a pathway that bound them to MHC class II for presentation to CD4+ T cells. In contrast, CD8+ T cells were presented antigen in the context of MHC class I, which had been shown to be involved in the presentation of antigen produced from within the cell. This question, however, has been resolved with the discovery of a phenomenon known as cross-presentation. This phenomenon

has been extensively studied in APCs, with a major focus on dendritic cells (123). Studies have shown that DCs have the ability to express exogenous antigens within the context of MHC class I. Mechanistically, this process is still being worked out, but there is evidence that suggests that DCs have the ability to release exogenously incorporated antigens from endosomes into the cytosol, where they can be taken up by the proteosome, degraded and passed via TAP transporters into the rough endoplasmic reticulum (124). There, it is loaded onto MHC class I and transported to the surface for presentation. Other studies have shown that DCs have decreased acidification of the phagolysosomal compartment, which allow exogenously obtained antigens to retain more antigenicity (125). This would aid in retaining peptides for presentation on both MHC class I and class II. These observations certainly allow the conclusion that CD8+ T cells can be activated in the setting of EAE, despite the fact that it is induced by exogenous antigen stimulation.

Evidence for Different Roles of CD8+ T Cells in Autoimmune Demyelination

Despite all of these observations, autoreactive CD8+ T cells have generally been ignored or thought of as uncommon in both MS and EAE. A possible reason for this could be the lack of capability to phenotypically classify responding cells. Past research relied on tritiated thymidine incorporation assays and cytokine analysis on bulk cell cultures, making it impossible to determine which cells were specifically responding. Because of the early work showing the necessity of CD4+ T cells, most of these responses were assumed to be CD4+ T cells. While there is a relative paucity of research concerning the role of CD8+ T cells, there have been some studies that suggest several roles for CD8+ T cells in MS and EAE.

There have been several different studies in which a pathogenic role for CD8+ T cells has been demonstrated. The first study used C3H mice and immunized with the p79-87 peptide of MBP. CD8+ T cells were extracted from the diseased mice and used to establish T cell clone lines. These lines were shown to retain cytotoxic capacity and were able to kill target cells with pMBP 79-87 loaded onto MHC class I molecules (126). These cell lines were then transferred either intravenously or intrathecally into naïve wild type or SCID C3H mice. These mice subsequently developed EAE, but exhibited symptoms, as well as pathology, that are very different than the typical, ascending disease course that has been characterized. These mice generally developed symptoms of damage to the brain, not the spinal cord, and this was confirmed by observing multiple lesions in the brains of diseased mice, whereas traditional EAE pathology normally demonstrates most destruction in the spinal cord. These observations indicate that CD8+ T cells specifically kill cells presenting a specific antigen, and that they are capable of causing disease in this particular model.

In another model system, using C57BL/6 mice immunized with pMOG 35-55 peptide, Sun and associates were also able to show the presence of autoreactive CD8+ T cells following in vitro stimulation with the antigen. Furthermore, when adoptively transferred into naïve or RAG knockout mice, these cells caused a progressive, chronic disease of moderate severity (127). It should be noted that in the knockout setting, disease onset was delayed significantly, with some mice developing disease as late as day 60. When examined histologically, these mice showed increased destruction of myelin as well as prolonged infiltration by neutrophils and macrophages. This study also showed that β2 microglobulin knockout mice, which lack the expression of functional MHC class I on the cell surface, were not susceptible to disease

induction by adoptive transfer of activated CD8+ T cells. This observation strongly suggests that the evidence seen in the naïve setting is produced by the CD8+ T cells.

The third study, performed by Ford et.al., was the second study in which CD8+ T cells were shown to play a pathogenic role in the C57BL/6 model. Like the previous study, this group also showed that there is a population of MOG-specific CD8+ T cells. ELISA assays performed on these cultures showed that they produce IFN-γ and TNF-α, but not IL-4, IL-10 or TGF-β (128). This group also showed that CD8+ T cells could be transferred into naïve wild type B6 mice and cause disease. They also showed that disease could be induced in SCID mice by transferring activated CD8+ T cells. They concluded from this observation that the disease could not be due to endogenous MOG-specific precursors recruited to the inflammatory site. This group also added a significant finding in the pursuit of autoreactive CD8+ T cells. Until now, the pMOG 35-55 peptide had been identified as binding to I-A^b, which is a class II MHC molecule in mice. These molecules associate with CD4+ T cells, not CD8+ T cells. This study was the first to show that MOG-reactive CD8+ T cells are restricted by the MHC class I molecule, H-2D^b. They further showed that a section of the peptide, specifically pMOG 37-46, could bind to D^b, which would engage the TCR of a CD8+ T cell. Using this peptide to stimulate CD8+ T cells in culture induced high levels of IFN-γ production. It was also shown that this peptide, when emulsified in CFA, is able to induce EAE to the same degree of incidence and severity as pMOG 35-55. This observation was confirmed by the production of a tetramer that confirmed the presence of MOG 37-46-specific CD8+ T cells in the CNS of mice.

A fourth study made use of a novel transgenic system which eliminated the need for in vitro stimulation of lymphocytes. It had been previously noted that transgenic mine that overexpress the surface marker B7.2/CD86 on microglia in the CNS spontaneously develop a

demyelinating disease. In a follow-up study, it was shown that this demyelination was not abrogated by the deletion of CD4+ T cells, which was achieved by breeding the B7.2 transgenic to a I-Aβ knockout mouse (129). This observation suggested that it was not the CD4+ T cell population that was causing the disease in these mice, contrary to observations well-noted in other models. Furthermore, this study showed that these mice showed oligoclonal expansion of CD8+ T cells in the CNS at early time points of disease. Furthermore, it was shown that the disease was abrogated in B7.2 transgenic mice that had been crossed with mice deficient in the IFN-γ receptor. They concluded from these observations that the demyelination seen in this model was due to pathogenic CD8+ T cells and that this process appeared to be IFN-γ dependent.

In 1992, Koh, et. al. used CD8 knockout mice to attempt to find a possible role for CD8+ T cells. These mice were bread on the PL/J background, which exhibits a relapsing form of the disease, and immunized with whole MBP. The knockout mice were observed to have a less severe disease course than littermate controls that were also immunized(130). Both groups of mice were similar in disease onset and incidence. Disease in the knockout mice, however, was characterized by milder acute clinical symptoms and less mortality compared to the wild type mice. This observation suggests that CD8+ T cells play a pathogenic role in the disease course, but are not necessary for disease induction. In the same study, it was observed that the CD8 knockout mice also suffered more relapses than littermate controls. This observation suggests that CD8+ T cells may be involved in disease regulation by suppressing relapses.

In a similar study, Jiang et.al. investigated the role of CD8+ T cells through the use of depleting antibodies. The model used in this study (B10.PL mouse immunized with pMBP Ac1-9) exhibits an acute disease followed by recovery and resistance to relapses as well as further

disease challenge by immunization. This group observed that when CD8+ T cells were depleted from the animals before immunization, it had no effect on the disease incidence, severity or recovery (131). This observation suggested that the CD8+ T cells in this model do not appear to play any pathogenic role in the onset or severity of disease, nor do they seem to be required for recovery (although they did reappear during the final stages of recovery and it cannot be ruled out that they played a role). This is contradictory to the observations made by Koh, et. al. in the CD8 knockout setting. The suggestion was made that this could be due to the fact that Jiang and colleagues used pMBP Ac1-9 for disease induction, whereas Koh and colleagues used the whole protein, giving the mice exposure to other epitopes that may induce CD8+ T cells with pathogenic properties. Another possible reason for this discrepancy could be the fact that Koh, et.al. used PL/J mice and Jiang et.al. used B10.PL mice. These different strains do share the same MHC background, but may have minor histocompatibility differences, which could lead to different responses to a stimulus.

If these cells were depleted during the recovery phase, it did not exacerbate or increase the rare relapse rate. However, depletion of the CD8+ T cells did abolish resistance to secondary disease by immunization. These observations led to the conclusion that depletion of CD8+ T cells lead to susceptibility to reinduction of disease, indicating that they play a regulatory role. It should also be noted that the mice that developed secondary disease following CD8+ T cell depletion showed increased disease severity when compared to mice that remained susceptible to secondary disease induction without CD8+ T cell depletion.

There have also been many other studies that indicate a regulatory role for CD8+ T cells in both EAE and MS. One of the earliest studies involved the mechanism behind the observed phenomenon that tolerance to EAE induction could be induced in Lewis rats by oral

administration of guinea pig MBP (132). Lider, et.al. showed that this tolerance could be transferred by ConA-activated bulk splenocytes as well as CD4-depleted splenocytes, but not CD3- or CD8-depleted splenocytes, indicating that CD8+ T cells are responsible for the observed adoptively transferred tolerance (133). This study also showed that CD8+ T cells are able to suppress proliferation of primed LNC, as well as antibody production.

A later study characterized a specific subset of CD8+ T cells shown to exhibit regulatory properties. Initially, this group showed that mice depleted of CD8+ T cells showed overall increased disease severity compared to controls, and that this disease was similar to that seen in CD8 knockout mice. They then showed that when CD8+ T cells were depleted from CD28 knockout mice, who are characteristically resistant to disease induction, they show increased susceptibility to disease, as did mice that were double knockouts in the CD8 and CD28 loci (134). This lead to the conclusion that CD8+CD28- T cells in this model possessed regulatory properties, which was confirmed by showing that adoptive transfer of CD8+CD28- T cells into CD8 knockout mice prevented disease by active induction. Mechanistic studies revealed that CD8+CD28- T cells suppress the proliferation of CD4+ T cells in a contact-dependent manner and that these cells also effect a downregulation of stimulatory molecules on APCs when they are cocultured.

Another study that sought to phenotypically characterize a specific sub-population of regulatory CD8+ T cells was performed by Lee and colleagues. They noted that when CD8+CD122+ T cells were depleted from a pMOG 35-55-immunized B6 mouse, these mice showed no recovery from disease symptoms, contrary to the disease course in control mice (135). They further noted that transfer of CD8+CD122+ T cells from a naïve mouse could rescue an immunized mouse at the peak of disease. Mechanistically, these cells were shown to

inhibit T cell infiltration into the CNS and increase IFN-γ production. These observations suggest that these cells are naturally occurring regulatory T cells that are akin to, but function independently of, CD4+CD25+ regulatory T helper cells.

Because of the evidences for both inflammatory and regulatory roles of CD8+ T cells in both multiple sclerosis and experimental autoimmune encephalomyelitis, we sought to further characterize their role in the murine setting. Our goals were to first, establish the presence of an antigen-specific autoreactive CD8+ T cell population in the EAE setting; second, discern the function of these cells in the disease process; and third, discover the mechanism whereby these cells accomplished their function.

Chapter II: Materials and Methods

Mice

C57BL/6 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). B10.PL mice were purchased from Taconic. B6.129 CD8-/- mice were graciously given from Dr. James Forman (UT Southwestern Medical Center). B6 TAP -/- mice were graciously given from Dr. James Forman and Dr. Michael Bennett (UT Southwestern Medical Center). All mice were housed and bred in the UT Southwestern Medical Center Animal Resource Center according to IACUC protocols.

EAE Induction

EAE was induced according to the following protocol. 6-8 week old female C57BL/6 mice were immunized subcutaneously at two injection sites with 200 µg MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK) or whole bovine MBP emulsified in CFA supplemented with 4 mg/ml M. tuberculosis (H37 RA, DIFCO, Detroit, MI), both of which express a chronic, severe disease course. SJL/J mice were immunized with PLP 139-151 (HSLGKWLGHPDKF) emulsified with CFA supplemented with M. tuberculosis (MTb), which produces a relapsing and remitting course similar to that seen in human MS. B10.PL mice were immunized with MBP Ac1-11 (ASQKRPSQRSK) emulsified with CFA supplemented with MTb. On days 0 and 2, mice were also administered 250 ng pertussis toxin (List Biological Laboratories) intraperitoneally, which can produce both chronic and acute disease courses. Disease severity was monitored daily and scored according to the following scale: 0, no disease; 1, limp tail; 2, limp tail and/or hind limb weakness; 3, moderate hind limb weakness and/or partial hind limb

paralysis; 4, hind limb paralysis; 5, hind limb paralysis and forelimb weakness, moribund state; 6, death. Other protocols that were used in attempts to optimize disease include the following variations: using IFA supplemented with MTb as well as CFA supplemented with MTb, variable concentrations of PT injections from 250 ng up to 500 ng, a double immunization protocol that involved a second booster injection of 200 μg MOG 35-55/IFA supplemented with MTb (4 mg/ml) 7 days after the first injection with 250 ng PT given on days 0 and 2 of the first immunization, variable amounts of subcutaneous injection sites (2-4), mice from different sources (NCI, Taconic, Jackson Laboratories), MOG 35-55 peptide obtained from different sources (UT Southwestern, gift from Dr. Vijay Kuchroo).

Synthetic microparticles, which have been shown to induce CD8+ T cells in virus models (136), were also used in attempt to make a CD8+ T cell-effected disease. Microparticles were prepared by mixing 10 ml of 50:50 copolymer solution containing poly (D, L-lactide-co-glycolide) in methylene chloride with distilled water that contained SDS until an emulsion was formed. This emulsion was then stirred at room temperature overnight to allow the methylene chloride to evaporate. The microparticles were then stored in at 4ºC until ready to load. To load antigen, 50 mg of microparticles were mixed with 0.5 mg pMOG 35-55 in PBS buffer at room temperature. Microparticles were then separated by centrifugation, washed and suspended in PBS buffer. The suspension was then mixed with CpG DNA, a TLR stimulator, and injected intraperitoneally into naïve, wild type 6-8 week old B6 mice.

Tritiated thymidine proliferation assays

Lymph node cells and/or splenocytes from immunized mice were harvested. CD8+ cells were magnetically separated using a negative selection protocol (Miltenyi Biotech, Germany) to

recover "untouched" CD8+ cells. Purity was assessed by flow cytometry and showed CD8+ population to be >90% pure. Bulk cells or CD8+ cells were then incubated in 96-well plates for 72 hours at a concentration of 400,000 cells/well for bulk cells and 250,000 cells/well for CD8+ cells. Irradiated splenocytes from naïve mice or immunized mice were used as APCs at a ratio of 1:2 (CD8+ cells:APCs.) After 72 hours in culture, cells were pulsed with 0.5 μCi/well of [^{3}H]methyl-thymidine for 18-20 hours. Cells were then washed, harvested on glass fibers and incorporation was detected using a Betaplate counter (Wallac, Gaithersburg, MD).

CFSE-based proliferation assays

Splenocytes were harvested from mice, suspended at 1×10^6 cells/ml in PBS and incubated at 37°C for 7 minutes with 0.25 μM 5- (and 6-) carboxyfluorescien diacetate succinimidyl ester (CFSE) followed by addition of serum and two PBS washes. Bulk cells were suspended at 1×10^6 cells/ml in culture media with antigens as noted in figures. On day 5, cells were washed with FACS buffer (PBS with 1% BSA and 0.1% Na-azide) and stained with phycoerythrin-conjugated (PE-conjugated) anti-CD8 and allophycocyanin–anti-CD4 (APC–anti-CD4) (all antibodies from Caltag/Invitrogen, Carlsbad, CA). Cells were washed and fixed in 1% paraformaldehyde (BD Biosciences). Flow cytometric data were acquired on a BD FACSort four-color flow cytometer using BD CellQuest software or BD LSR II using FACSDiva software. For analysis, FlowJo software was used to gate on lymphocytes and further on the $CD4^+/CD8^-$ or $CD8^+/CD4^-$ populations. The mean background proliferation was calculated based on the proliferating fractions in media alone. The stimulation index (SI) was calculated by dividing the percent proliferation in a sample by the percent background proliferation. Different conditions were used in attempts to optimize this assay. $2x10^6$ cells were used instead of $1x10^6$

cells under the same conditions. CFSE assays were also stopped at different time points (days 3,4,5 and 7 of incubation) to determine the best time point for results. In another optimizing attempt, bulk LNC, bulk SpC, isolated CD8+ T cells or CD8-depleted splenocytes from MOG-immunized mice were incubated in the presence of no antigen, immunizing antigen (20 μg/ml) or Con A (5 mg/ml) in culture flasks for two days. The cells were then washed and transferred to culture tubes, where they were incubated for 3 additional days in the presence of IL-2 (20 pg/ml) and no antigen. Following incubation, cells were stained with anti-CD4 and anti-CD8 antibodies, then fixed in 5% paraformaldehyde. Cells were then analyzed for proliferation by flow cytometry as described.

In assays using purified CD8+ T cells, "untouched" CD8+ cells were isolated using a negative selection magnetic bead protocol (Miltenyi Biotech) using MACS LS columns or an AutoMACS machine. Cells were then stained with CFSE as described. CD8+ cells were suspended at 0.5-1 x 10^6 cells/ml with irradiated splenocytes (3500 rads) used as APCs at a 1:5 ratio. Tubes were then incubated for 5 days in the presence of no antigen, immunizing antigen or Con A. Following incubation, cells were stained with anti-CD8 antibodies and fixed in paraformaldehyde. Cells were then analyzed for proliferation by flow cytometry as described. APCs were used from various sources: CD8-depleted splenocytes from naïve wild type B6 mice, CD8-depleted splenocytes from MOG-immunized mice, and CD8-depleted splenocytes from OVA-immunized mice. In all cases, the APCs were irradiated (3000-3500 rads) using a gamma-irradiator.

In assays to determine APC involvement in CFSE proliferation assays, CD8+ T cells were purified from splenocytes of mice that had been immunized with pMOG 35-55/CFA or

pOVA 323-339/CFA 25 days prior. These cells were then incubated at concentrations of 0.5-1x10^6 cells/ml with irradiated APCs, which consisted of CD8-depleted splenocytes from the mice immunized with a different antigen (CD8+ T cells from MOG-immunized mice & APCs from OVA-immunized mice and vice versa). The CD8+ T cell:APC ratio was 5:1. Cells were incubated in the presence of no antigen, pMOG 35-55 antigen, pOVA 323-339 antigen or Con A. Cells were incubated for 5 days, then stained with anti-CD8 antibodies and fixed in paraformaldehyde. Cells were analyzed by flow cytometry as described.

In assays using various cytokines, CFSE assays were set up in a similar fashion to that described above. Bulk splenocytes were harvested from immunized mice and cultured in test tubes in the presence of no antigen, pMOG 35-55 or Con A at a concentration of 1x10^6 cells/ml. To these cultures, various cytokines were added: IL-2, IL-12, IL-7 or IL-23. Cultures were incubated for 5 days at 37ºC. Cells were then stained with anti-CD4 and anti-CD8 antibodies and analyzed by flow cytometry as described.

A different CFSE protocol was also used to test for CFSE toxicity. Briefly, cells were incubated at a concentration of 10-20x10^6 cells/ml. Cells were then incubated for 10 minutes with CFSE at a concentration of 0.05 μM at 37ºC in the presence of 5% FCS. Cells were then washed twice in the presence of FCS (2% v/v) and PBS.

For analysis of other models of EAE, the CFSE protocol remained the same. Bulk LNC, bulk splenocytes and purified CD8+ T cells from the indicated models were stained with CFSE and incubated in the presence of no antigen, immunizing antigen or ConA. For the CD8+ T cell cultures, irradiated CD8-depleted splenocytes were used as APCs. Cell preparation and flow cytometric analysis were performed as described above.

Production of Autoreactive CD8+ T Cell Clone

6-8 week old female B6 mice were immunized with pMOG 35-55/CFA supplemented with MTb (4 mg/ml). 25 days post-immunization, mice were sacrificed and lymph node cells and splenocytes were isolated. CD8+ T cells were then purified from splenocytes by negative selection using magnetic beads. CD8+ T cells were then stained with CFSE and incubated in 24-well plates at concentrations from $1x10^6$ to $5x10^6$ cells/ml in the presence of pMOG 35-55 (20 μg/ml) and IL-2 (10 pg/ml), as well as irradiated CD8-depleted splenocytes from OVA 323-339/CFA-immunized mice. After five days of culture, cells were pooled and CFSE-dilute cells were sorted by flow cytometry into 96-well plates. Cells were then cultured in the presence of CD8-depleted splenocytes from OVA/CFA-immunized mice, pMOG 35-55 and conditioned media obtained from cultures containing lymphocytes and Con A (5 mg/ml).

In another protocol, bulk LNC and SpC were obtained from MOG-immunized mice (25 days post-immunization). Cells were then cultured in flasks at concentrations of $5x10^6$ cells/ml in the presence of pMOG 35-55 (20 μg/ml), pMOG 35-55 + IL-2 (10 pg/ml), or pMOG 35-55 + IL-12 (10 pg/ml). Following three days of culture, CD8+ T cells were purified by positive selection using magnetic beads. These cells were incubated in 96-well plates at $1x10^3$ cells/well in the presence of irradiated APCs and pMOG 35-55 ± IL-2 or IL-12.

Production of CD8+ T cell hybridoma

A mouse tumor cell line (obtained from Dr. Kenneth Rocke, Univ. Massachusetts) that constitutively expressed CD8 was grown at 37ºC until cells were in log phase of proliferation. CD8+ T cells were purified from SpC of MOG-immunized mice 25 days post-immunization.

Cells were then fused according to established protocol. Following fusion, cells were incubated in HAT media to select for fused cells. Surviving cells were analyzed by flow cytometry for expression of CD3 and CD8. Cells that were shown to express both surface markers were expanded and tested for constitutive expression of CD3 and CD8.

Adoptive Transfer Experiments

For adoptive transfer of EAE, C57BL/6 mice were immunized with MOG 35-55 as outlined above. Splenocytes were harvested either 10 or 25 days post-immunization. These cells were then incubated for 72 hours in culture media consisting of Dulbecco's Modified Eagle's Medium supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, HEPES buffer, Non-essential amino acids, sodium pyruvate and 2-mercaptoethanol. MOG 35-55 (20 μg/ml) and mouse IL-2 (10 pg/ml) were also added to the culture media. Live cells were then separated using a Ficoll gradient. CD4+ and/or CD8+ cells were then positively selected using magnetic beads (Miltenyi Biotech, Germany). 5-15 x 10^6 CD4+, CD8+ or bulk splenocytes were injected intravenously into naïve, wild-type B6 female mice and monitored for disease according to the previously outlined scale. In some experiments, 250 ng pertussis toxin was also administered intraperitoneally on days 0 and 2. Other variations of the protocol include the addition of IL-12 (1-5 ng/ml) to cultures during 72 hour incubation.

Protection adoptive transfer experiments

Female C57BL/6 mice were immunized with MOG 35-55 or OVA 323-339 as described. 25 days post-immunization, the mice were sacrificed and the splenocytes were harvested. These cells were incubated in culture media containing MOG 35-55 or OVA 323-339 (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours at 37°C at a concentration of 7.5 x 10^6 cells/ml. Live cells were

then separated on a Ficoll gradient. CD8+ cells were purified by positive selection using magnetic beads. 5-10x10^6 CD8+ cells from the MOG-immunized mice and the OVA-immunized mice were then injected into naïve, wild-type B6 mice via tail vein. These mice were then immunized the next day with MOG 35-55 according to our protocol. Mice received pertussis toxin on days 0 and 2 post-MOG immunization. Mice were then randomized and monitored for disease.

Therapeutic adoptive transfer experiments

CD8+ cells from MOG-immunized and OVA-immunized mice were isolated and prepared in the same manner as described for protection adoptive transfer experiments. These cells were then injected via tail vein into wild-type mice that had been previously immunized with MOG 35-55/CFA and had been normalized according to disease score at the time of adoptive transfer. These mice were then randomized and monitored daily for disease.

Co-adoptive transfer experiments

Bulk splenocytes were taken from mice that had been immunized with MOG 35-55/CFA or OVA 323-339/CFA 25 days prior. Bulk splenocytes and lymph node cells were pooled from mice immunized with MOG 35-55/CFA 15 days prior. Bulk cultures were incubated for 72 hours in the presence of IL-2 (10 pg/ml) and the immunizing antigen (20 μg/ml). CD8+ T cells were isolated from the 25-day-immunized splenocytes and CD4+ T cells were isolated from the 15-day-immunized bulk cells, all by positive magnetic selection. CD8+ T cells from either the OVA- or MOG-immunized mice were then mixed with CD4+ T cells from the 15-day MOG-immunized mice at a ratio of 2:1. These mixes were then injected into naïve, wild type B6 mice and monitored for disease.

CD8+ T cell kinetics studies

6-8 week old female C57BL/6 mice were immunized with pMOG 35-55/CFA supplemented with MTb (4 mg/ml). Mice were sacrificed on days 10, 15, 20, 25, 30 and 35 post-immunization. LNC and SpC were harvested from immunized mice. CD8+ T cells were isolated from LNC and SpC by negative selection using magnetic beads. Bulk cells and CD8+ T cells were then stained with CFSE. Cultures were set up in the presence of no antigen, pMOG 35-55 (concentrations indicated) or Con A (5 mg/ml). For CD8+ T cell cultures, irradiated CD8-depleted APCs from OVA-immunized mice were used at a T cell:APC ration of 1:5. After five days of culture, cells were stained with anti-CD4 and/or anti-CD8 antibodies and fixed in paraformaldehyde. Cells were analyzed for proliferation as described above.

Cytokine detection

Cytokine detection was performed using a flow-cytometric cytokine bead array (CBA) kit (Becton Dickenson, Franklin Lakes, NJ). Briefly, supernatants were taken directly from tubes containing cells for the CFSE-based proliferation assay described above. These supernatants were centrifuged to remove any cells and stored at -20ºC. At the time of the assay, supernatants were thawed and analyzed according to the manufacturer's protocol for detection of mouse Th1/Th2 cytokines on a BD LSR II instrument using FACSDiva software. Data was analyzed using BD CBA software.

ELISA

ELISA assays were performed in 96-well plates that were purchased precoated or coated overnight in our laboratory. For cytokine analysis of CD4+ T cells, supernatants were taken

from the following cultures at 48, 72 and 96 hours: purified CD4+ T cells from a MOG-immunized mouse + CD4-depleted APCs from a MOG-immunized mouse; CD4+ T cells from a MOG-immunized mouse that had been "protected" by injection of MOG-specific CD8+ T cells + CD4-depleted APCs from a MOG-immunized mouse; CD4+ T cells from a MOG-immunized mouse that had been injected with "control" CD8+ T cells from an OVA-immunized mouse. Samples were analyzed in duplicate for the presence of IFN-γ or IL-4 on premade 96-well plates according to the manufacturer's protocol. Plates were analyzed by optical density for presence of cytokine according to protocol. Samples were measured against standard concentrations and concentration was calculated using Microsoft Excel software.

ELISA assays to measure cytokine production by APCs were measured in a similar fashion. CD4-depledted APCs were obtained from splenocytes of mice from the following groups: MOG-immunized, MOG-immunized mice that had been "protected" with MOG-specific CD8+ T cells sixteen days prior, MOG-immunized mice that had been injected with "control" CD8+ T cells from an OVA-immunized mouse sixteen days prior. All of the groups were incubated in 48-well plates at a concentration of $1x10^6$ cells/ml. Cultures were set up under the following conditions: no antigen, IFN-γ (10 units/ml), IFN-γ (100 units/ml), or lipopolysaccharide (1 mg/ml). Supernatants were collected at 48, 72 and 96 hours and frozen down. For the ELISA, 96-well plates were prepared as follows. Plates were coated with capture antibody in coating bicarbonate buffer overnight at 4ºC. Plates were then washed with PBS/0.05% Tween wash buffer. Plates were then blocked with 1%BSA/PBS buffer for 1 hour at room temperature, followed by another washing step. Supernatants were thawed and placed on coated 96-well plates and incubated at room temperature for two hours. Plates are then washed five times and detection antibody is added. Plates are then incubated at room temperature for

another hour. Plates are washed again and streptavidin-peroxidase enzyme reagent is added to the wells and incubated at room temperature for 30 minutes. Plates are then washed seven times and substrate solution containing TMB/TBABH is added. Plates develop for 10 minutes at room temperature in the dark after which 1M sulfuric acid is added to stop the reaction. Optical density was measured at 450 nm and samples were compared to standard controls to determine cytokine concentrations of TNF-α, IL-10 or IL-12. Standard error of measure and t-test analysis was then performed using Microsoft Excel software.

Epitope spreading experiments

LNC and SpC were harvested from diseased mice in all of the EAE models described above. CD8+ T cells were isolated by negative selection using magnetic beads. Bulk cells and CD8+ T cells were stained with CFSE according to the described protocol. Proliferation assays were then performed by incubating bulk cells and purified CD8+ T cells in the presence of no antigen, specific neuroantigens (indicated in the figure), or Con A. Irradiated splenocytes from naïve or OVA323-339/CFA-immunized mice of the same strain as the CD8+ T cells were used as APCs.

In vitro suppression assays

CD4+ T cells were purified from pooled LNC and SpC of CD8 knockout mice that had been immunized with pMOG 35-55/CFA supplemented with MTb 10 days prior. These cells were stained with CFSE and used as the responder cells in this assay. CD8+ T cells from wild type C57BL/6 mice that had been immunized with MOG 35-55 or OVA 323-339 emulsified in CFA (+ MTb 4 mg/ml) were used as suppressor cells. Tubes were set up such that each tube contained $1x10^6$ responder cells and increasing numbers of CD8+ T suppressor cells. Tubes

were then incubated in the presence of no antigen, pMOG 35-55 (20 μg/ml), pOVA 323-339 (20 μg/ml) or Con A (1 mg/ml). Tubes were incubated for 5 days, after which they were stained with anti-CD4 and anti-CD8, then fixed in paraformaldehyde. Suppression was then measured flow cytometrically by calculating change in percent proliferation as compared to the control tubes containing the immunizing antigen in the absence of any suppressor cells.

CD8+ T cell influence on APCs

6-8 week old C57BL/6 female mice were immunized with pMOG 35-55/CFA (+ MTb) or pOVA 323-339/CFA (+ MTb) according to our active disease protocol. 20 days post-immunization, splenocytes were harvested and incubated for 72 hours in the presence of pMOG 35-55 or pOVA 323-339 (20 μg/ml) and IL-2 (10 pg/ml) at a concentration of $7.5x10^6$ cells/ml. Following culture, dead cells were removed by Ficoll gradient and CD8+ T cells were purified by positive selection using magnetic beads. These cells were then injected ($6x10^6$ cells/mouse) into naïve wild type mice. The next day, the mice were immunized according to our active disease protocol along with naïve mice to serve as disease controls. 15 days post-immunization, the mice were sacrificed. CD4+ T cells were purified from LNC and SpC of the mice and stained with CFSE. CD4-depleted subsets were stained with CMTPX and irradiated for use as APCs. Cultures were then set up such that CD4+ T cells from each subset were incubated with irradiated CD4-depleted APCs from each subset. Cells were incubated in the presence of pMOG 35-55 antigen for five days. Following culture, cells were stained with anti-CD4 antibody and fixed in paraformaldehyde. Proliferation responses in each of the groups were then measured by flow cytometry.

In vivo killing assays

Splenocytes from naïve B6 mice, TAP knockout mice or K^bD^b knockout mice were harvested for use as target cells. These cells were stained with 0.25μM CFSE (CFSE Hi) or 0.05 μM CFSE (CFSE Lo) and incubated overnight in the presence of MOG 35-55 or OVA 323-339 (20 μg/ml) and Con A (10 μg/ml). CFSE Hi and CFSE Lo cells were then mixed in a 1:1 ratio. Approximately 20 x 10^6 cells were then injected via tail vein into naïve, CFA-immunized, or MOG-immunized wild type or CD8 knockout mice at 25 days post-immunization. 48 hours after injection, the mice were sacrificed and splenocytes were harvested and purified. These cells were then fixed in 1% paraformaldehyde and collected using a BD LSR II flow cytometer. Data was then analyzed using FlowJo software. Killing was measured as a difference in ratios between the MOG-immunized mice and either naïve or CFA-immunized mice as controls.

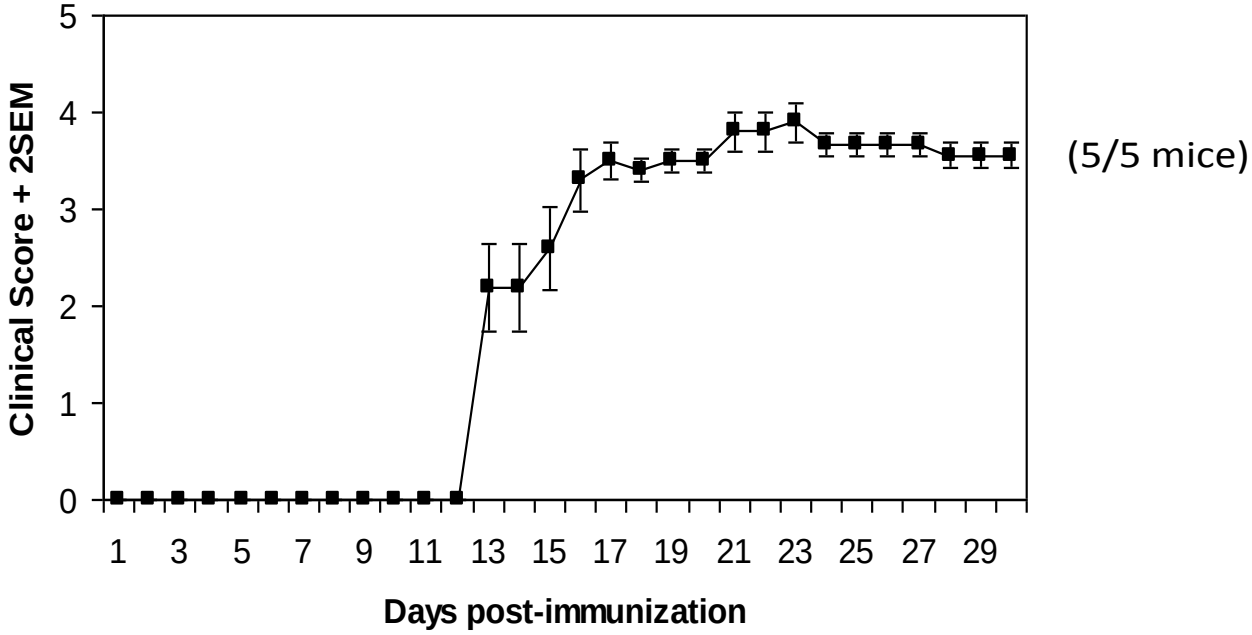
For assays containing two dyes, target cells were isolated as described above. Cells were then split into four populations. Two populations were stained with CMTPX (Invitrogen, Eugene, OR) according to the manufacturer's protocol. Simultaneously, all four populations were stained with CFSE, two receiving CFSE Hi concentration and two receiving CFSE Lo concentration. Cells were then incubated overnight in culture media containing either MOG 35-55 or OVA 323-339 (20 μg/ml) and Con A (10 μg/ml). All groups were then mixed in a 1:1:1:1 ratio and injected via tail vein into MOG-immunized or control mice. 2 days later, mice were sacrificed and splenocytes were harvested. Analysis was then performed as previously described.

For specific cell populations, CD4+ cells were purified via positive selection using magnetic beads. CD4+ and CD4-depleted subsets were then stained separately according to the procedure described above. Analysis showed CD4+ population to be >90% pure and CD4-depleted population to contain <10% CD4+ cells.

Chapter II Figure Legends

Figure 1: Active Disease Induction in C57BL/6 Mice. Wild-type female B6 mice were immunized on day 0 with MOG 35-55/CFA supplemented with M. tuberculosis toxin (4 mg/ml). The representative figure shows the disease course for this model.

Figure 2: Active disease induction in other EAE models. A. Wild-type female SJL/J mice were immunized on day 0 with PLP 139-151/CFA supplemented with M. tuberculosis toxin (4 mg/ml). The representative figure shows the typical disease course for this model. B. Wild-type SJL/J mice were immunized on day 0 with either MOBP 15-36/CFA or MOBP 37-60/CFA. MOBP 37-60-immunized mice showed decreased disease from that which has been described in other studies.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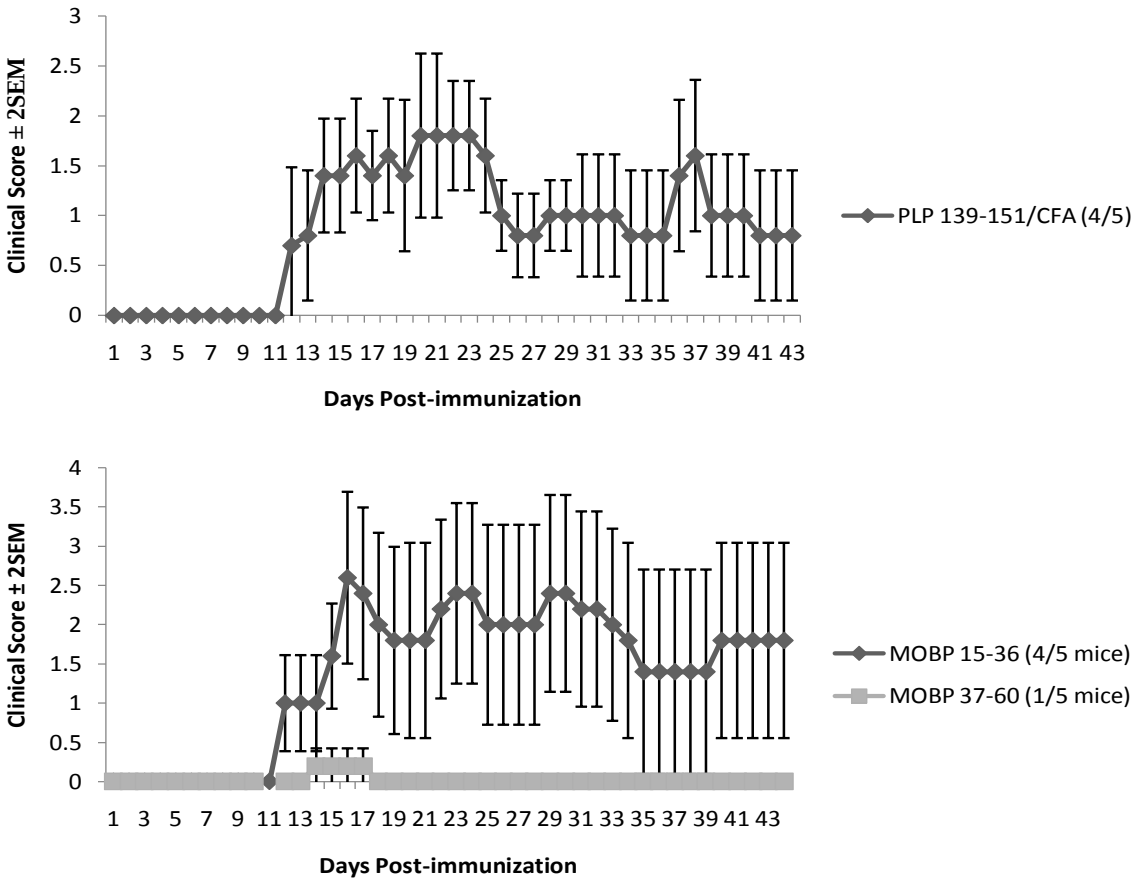
0
1
2
3
4
5
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
Days post-immunization
(5/5 mice)


Clinical Score ± 2SEM
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
Days Post-immunization
PLP 139-151/CFA (4/5)
Clinical Score ± 2SEM
4
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43
Days Post-immunization
MOBP 15-36 (4/5 mice)
MOBP 37-60 (1/5 mice)

Chapter III: Searching for Autoreactive CD8+ T Cells

Much of the knowledge that we have obtained regarding the immunology of demyelinating diseases comes from the studies of the murine models. The function and mechanisms of CD4+ T cells have been well-studied both in vitro, through standard proliferation assays and cytokine profiling, and in vivo, through adoptive transfer experiments using wild-type as well as genetically-altered mice. Studies have also shown the ability of these cells to participate in epitope spreading. However, because of early studies, it has long been assumed that CD4+ T cells are responsible for most, if not all, of the T cell responses that were seen experimentally. Now, we have the technological ability to view different cell populations, both as part of a bulk culture and individually through effective separation techniques. This allows us to answer questions as to the true responding populations.

As we established disease in our colony, we also sought to discover if, as is seen in multiple sclerosis, there is an antigen-specific, autoreactive CD8+ T cell response. We first wanted to reproduce proliferation assays that have been previously published. These prior studies used thymidine incorporation assays to measure proliferation. We therefore harvested both lymph node cells (LNC) and splenocytes (SpC) from diseased C57BL/6 (B6) female mice at days 10, 20 and 30 post-immunization. These cells were then incubated in 96-well culture plates with no antigen, immunizing antigen and a superantigen (SEB) or mitogen (Concanavalin A). 72 hours after the cultures were set up, cells were pulsed with tritiated thymidine for 20 hours, followed by harvesting and measurement of radioactive uptake. We discovered that both bulk SpC and bulk LNC showed increased proliferation in the presence of the immunizing

antigen, suggesting the presence of an autoreactive cell (Figure 3). However, because these assays were performed on bulk populations, it was impossible to determine which of the cells might be proliferating. In order to determine the identity of the cell, we needed a different test.

CFSE (5' and 6' carboxyfluorescein diacetate succinimidyl ester) is an intracellular dye that covalently binds to cytoplasmic proteins found within the cell when cleaved by intracellular esterases (136, 137). As cells divide, they distribute the CFSE among the daughter cells, with each cell receiving approximately half of the CFSE that the original cell contained. This sequential halving will continue to occur as the cell continues to divide. Because the dye is fluorescent, it can be tracked using a flow cytometer. The dilution of the dye can then be used to measure proliferation of a specific cell (137, 138). In the past, this dye has been used for cell tracking as well. Studies from our lab have utilized the dye to discover autoreactive CD8+ T cells in the MS setting (31, 139, 140). We therefore already had a working protocol for cell staining.

In our initial experiments, B6 mice were immunized with MOG 35-55 peptide emulsified in IFA supplemented with 4 mg/ml MTb. 0/5 mice developed disease. We sacrificed the mice at day 30 and harvested the LNC and SpC. We then stained each population individually with CFSE and aliquoted $1x10^6$ cells into tubes. Tubes were incubated with either no antigen, varying levels of pMOG 35-55 (from 80μg/ml to 0.1 μg/ml), or a positive control (Con A or SEB) for seven days. Cells were then stained with anti-CD4 and anti-CD8 antibodies. and analyzed by flow cytometry for proliferation (Figure 4). We observed that the CFSE-stained cells were detectable by flow cytometry, as had been seen by us and others, but we were unable to see any proliferation in either the CD4+ or CD8+ populations. However, we were able to conclude that the CFSE assay protocol that was developed for staining human cells is sufficient for staining

mouse cells as well. There were several different parameters that could explain why we saw no proliferation. Since the mice did not develop disease, it is possible that the cells never developed a response in vivo, and would therefore not be expected to respond upon in vitro stimulation. It is also possible that the mice were sacrificed too late after injection to see activated cells, although this was highly unlikely since usual disease in this model is a chronic disease and would likely have autoreactive CD4+ T cells throughout the disease course. Another possibility was that the antigen concentrations used for in vitro activation were not optimal to see disease proliferation. It is also possible that seven days was too long of an incubation period, allowing the proliferating cells to divide to the point where CFSE detection would be minimal or possibly dying through activation-induced cell death. We concluded that we needed to optimize this assay in order to answer our question.

We conducted many different experiments for the purposes of optimization. We varied the protein content and protocols of our culture media, which proved to be insignificant. We also varied and optimized the concentration of stimulating protein in the culture. These assays showed that optimal proliferation was seen between the concentrations of 1 μg/ml and 40 μg/ml. Using these findings, we were able to detect an antigen-specific response in both LNC and SpC of MOG-immunized mice (Figure 5). In our previous studies, we had determined significant proliferative responses could be calculated by the following criteria: 1) The change in percent of cells in the proliferating fraction (ΔPF = % proliferation seen in stimulated population - % proliferation in "no antigen" control) must be at least 1%, and 2) The stimulation index (SI = % proliferation in stimulated population / % population in no antigen population) must be ≥ 2. These calculations also allow for variability between experiments as the background levels in the no antigen control vary from experiment to experiment. We do not, as of yet, have a tested

explanation for this variability. It could be due to responsiveness to fetal calf serum in the culture media, or perhaps contamination during the experimental set-up. Using SI as a means to determine significant proliferation allows us flexibility to determine significance from experiment to experiment. As can be seen in the figure, there is an expected CD4+ T cell response when cells from a MOG-immunized mouse are incubated in the presence of pMOG 35-55 ($SI \geq 3$). Because of the flow cytometric ability to characterize specific subsets, we were also able to show that there is an autoreactive, MOG-specific response in the CD8+ T cell subset as well ($SI \geq 8$).

Further optimization experiments were also conducted in the CFSE assay system. By this time, our protocol for active disease immunization was well-established, so that we were able to use mice that were showing signs of disease. All of the mice in this experiment showed grade 2 clinical scores at the time of the experiment, which was 25 days post-immunization. The mice were sacrificed and LNC and SpC were extracted. Each group was independently stained with CFSE and incubated with no antigen, pMOG 35-55 (40 μg/ml or 1 μg/ml) or a positive control stimulus (SEB or ConA). Cell concentrations were varied with one group containing $1x10^6$ cells in 1 ml and another group containing $2x10^6$ cells in 1 ml. The tubes were then incubated for 3,4,5 and 7 days to find which time point yielded the optimum window to measure proliferation. At each time point, cells were stained with anti-CD4 and anti-CD8 antibodies and fixed. At the end of the experiment, cells were analyzed by flow cytometry for proliferation. From this data, we concluded that the optimal time for recovery of autoreactive CD8+ T cells was day 5 of incubation (Figure 6), as days 3 and 4 showed lower levels of proliferation and day 7 showed higher levels of background proliferation in our no antigen control populations. An optimum

cell concentration of $1x10^6$ cells/ml was also shown, as $2x10^6$ cells/ml also produced higher background levels in the no antigen control tubes (data not shown).

We also sought to optimize the assay by changing the culture conditions for the incubation period. We noticed that in another publication, CD8+ T cells were able to transfer disease after a specific incubation protocol, so we decided to find out if proliferation would be detectable in a CFSE assay. In this variation, bulk LNC, bulk SpC, CD8+ cells (obtained by negative selection) and CD8-depleted cells were incubated in tubes in the presence of no antigen, pMOG 35-55, or Con A, and also IL-2 (10 pg/ml) for five days. In a parallel experiment, bulk LNC, bulk SpC, CD8+ T cells and CD8-depleted cells were incubated in tissue culture flasks for two days in the presence of no antigen, pMOG 35-55, or Con A. After two days, cells were washed and transferred to tubes. They were incubated for three more days in the presence of IL-2 alone (20 pg/ml). All cells were then stained for surface markers and fixed in paraformaldehyde. Cells were then analyzed for proliferation by flow cytometer. We saw no additional benefit to using this incubation protocol we did not see improved proliferation in either T cell subset (data not shown).

We also tested the various immunizing protocols of the mice that were used to elicit disease. Among these protocols were mice immunized with 200 or 400 μg pMOG 35-55 (both groups showed signs of clinical disease) and mice that had been double immunized (100% disease incidence). Both of these protocols also showed antigen-specific CD8+ T cell responses (data not shown). However, we found that CD4+ and CD8+ T cell proliferation, as detected by flow cytometry, was fairly consistent among the various protocols. From this data we concluded that the active disease protocol that we established for disease induction in our laboratory was sufficient for our studies concerning detection of autoimmune responses.

Our next goal was to confirm that the antigen-specific CD8+ T cell response that we were seeing was a real response. We questioned whether or not this response may be the product of bystander stimulation since there were antigen-specific T helper cells in the same culture, and these cells could be expressing stimulatory cytokines leading to a non-specific response. In order to test this question, we isolated CD8+ T cells from the LNC and SpC of immunized mice. We used a magnetic technique known as "negative selection" that allowed us to isolate CD8+ T cells that had not been bound by antibody for the purpose of purification (141, 142). Doing this ensured that the cells would not be inadvertently activated by binding to the CD8 coreceptors. We first performed a tritiated thymidine assay to measure proliferation in this purified population. For this assay, CD8+ T cells were purified as described and incubated in triplicate with varying concentrations of pMOG 35-55 peptide along with irradiated SpC from a naïve mouse that served as APCs. The assay was performed as described above. The results of this assay showed a dose-dependent response in the cultures (Figure 7a). This assay suggested that we were seeing a true autoreactive response in the CD8+ T cell subset. However, since this was a thymidine proliferation assay, we could not conclude with certainty that the proliferation we were seeing was due to CD8+ T cells, and not other cells in the culture (i.e. contaminating CD4+ T cells, APCs that survived the irradiation process). In order to be certain, we then performed a CFSE assay on these purified CD8+ T cells by incubating them with irradiated, CD8-depleted SpC from a naïve mouse and with one of the following conditions: no antigen, pMOG 35-55 or Con A. Following a 5 day incubation period, cells were stained for CD4 or CD8 and analyzed by flow cytometer (Figure 7b). As seen in the figure, CD8+ T cells are capable of responding to pMOG 35-55 in the absence of activating CD4+ T cells. This confirms that the response that we observe in the bulk cultures of this model is a true CD8+ T cell response.

The results that we had seen in our CD8+ T cell proliferation assays had shown that the cells had been able to proliferate. These results, however, were not consistent and results seemed sub-optimal. We hypothesized that perhaps the CD8+ T cells needed to be stimulated by activated APCs instead of naïve APCs. To test this hypothesis, we set up a CFSE assay with cells from mice that had been immunized 14 days prior and showed clinical disease scores of 4+. For the assay cultures, we used bulk SpC, bulk LNC and CD8+ T cells obtained by negative selection from the SpC population. In this experiment, we used irradiated cells from the CD8-depleted subset of cells. All other conditions remained the same (Figure 8). As seen in the figure, these cells showed a much higher proliferative response (SI $\geq$ 50). We concluded that activated APCs were superior stimulators of our autoreactive, antigen-specific population and used these APCs as part of our protocol for subsequent CFSE assays.

The results from these experiments, however, posed a different question for us: are these cells increasing in proliferation because they are incubated with activated APCs, or is the proliferation due to bystander activation caused by cytokines from CD4+ T cells in the APC population that are reacting to the pMOG 35-55 peptide in the culture? To answer this question, we decided to perform tritiated thymidine incorporation assays using bulk cells from mice that were immunized with either pMOG 35-55 or pOVA 323-339 (Figure 9a). These cells were then stimulated with either pMOG 35-55, pOVA 323-339 or wbMBP (used as a nonspecific control antigen). The results of this experiment showed that the cells only proliferated when stimulated with APC exposed to the immunizing peptide. This lead to the conclusion that, if activated APCs from an OVA-immunized mouse were used with CD8+ T cells from a MOG-immunized mouse, they would not be stimulated by the antigen, and thus act only as APCs, not as responding cells. We could therefore be confident that they would not produce cytokines that

would stimulate the CD8+ T cells in a bystander-type manner. We confirmed the results from the thymidine assays using our CFSE protocol with CD8+ T cells from mice that had been immunized with MOG 35-55 (Figure 9b). SpC from immunized mice were harvested and CD8+ T cells were harvested and separated by negative selection. CD8+ T cells were incubated with irradiated CD8-depleted populations from OVA-immunized mice in the presence of MOG 35-55 or OVA 323-339. As seen in the figure, CD8+ T cells only responded to the pMOG 35-55 antigen. We could therefore conclude that, if used as APCs, cells from OVA-immunized mice would not be stimulated by pMOG 35-55 antigen and confirm that the CD8+ T cell response was MOG-specific..

We next decided to test the effects of different cytokines on the proliferative capacity of the CD8+ T cells. The goal for this assay was to see if any cytokines known to be involved in either disease induction or CD8+ T cell maintenance could increase proliferation and/or cell viability, which may be useful in establishing CD8+ T cell lines (another pursuit of this project). We followed our established CFSE protocol, but added the following cytokines to the cultures: IL-2, IL-12, IL-23 and IL-7. IL-2 (10 pg/ml) was tested because it has been shown to be involved in general T cell stimulation (143). IL-12 (10 ng/ml) was tested because studies showed IL-12 was important for the establishment of EAE (144). IL-23 (3 ng/ml) was then later shown to be essential for EAE, so we tested this cytokine also (72). IL-7 (10 ng/ml) has been reported to be important for the maintenance of memory CD8+ T cells (145). The cultures were incubated for five days. CD8+ T cells were incubated with either naïve irradiated APCs or irradiated APCs from the CD8-depleted population of the immunized mouse for comparison (Figure 10). As can be seen, incubation with different cytokines did allow significant proliferation in several conditions. IL-2 was able to stimulate at both pMOG 35-55

concentrations; IL-7 did not promote proliferation to significant levels; IL-12 promoted significant proliferation in both populations when naïve APCs were used, but not when activated APCs were used; IL-23 was able to promote proliferation when either type of APCs were used, but only at low levels of [pMOG 35-55]. These positive results were expected, as each of these cytokines has been shown to promote T cell growth. We were surprised that the IL-7 results were negative in most cases, because we have surmised that the CD8+ T cell response we have seen is a memory response. However, we did not conduct titration experiments to optimize concentrations; we used published concentrations that had been used in previous studies. We can therefore presume that these results could be due to incorrect culture kinetics. Although many of these tests yielded positive results, they did not show significant proliferative differences over CFSE assays in which no additional cytokines were used. Therefore, we concluded the addition of exogenous cytokines was unnecessary for optimal proliferation. We also concluded that since no additional proliferation was seen, CFSE proliferation assays would be more indicative of biological processes without the addition of exogenous cytokine stimulation. For these reasons, we also did not pursue any further titration experiments to optimize cytokine concentrations.

After establishing the presence of an antigen-specific CD8+ T cell response in the MOG 35-55/B6 model, we wanted to test if this was perhaps a model-specific phenomenon. We immunized SJL/J mice with pPLP 139-151(200 μg/ml) in CFA containing MTb. 10 days post-immunization, the mice were sacrificed and LNC and SpC were harvested. In a similar experiment, B6 mice were immunized with whole bovine MBP, which has been shown in another publication to produce a CD8+ T cell response (50). 12 days post-immunization, LNC and SpC were harvested. In yet another model system, LNC and SpC were harvested from

B10.PL mice immunized with pMBP Ac1-11. In all cases, CD8+ T cells were purified from bulk cells using magnetic sorting to obtain "untouched" CD8+ T cells (purity >90% by flow cytometry). CFSE assays were then performed on bulk cells and CD8+ T cells in each of the different models. As seen in figure 11, each of the different models of EAE contained antigen-specific CD8+ T cell responses against the immunizing antigen. These results indicated that the autoreactive CD8+ response that we see in the MOG/B6 model is not unique to MOG-immunization (B6 mice immunized with wbMBP respond to wbMBP in culture), nor are they a model-specific phenomenon (two other models with different strains of mice showed antigen-specific CD8+ T cell responses).

Chapter III Figure Legends

Figure 3: Bulk Cells from MOG-immunized Mice Respond in a Dose-dependent Manner to MOG 35-55 Antigen Stimulation. Female wild type B6 mice were immunized with 200 μg MOG 35-55/CFA subcutaneously and given 250 ng pertussis toxin intraperitoneally on days 0 and 2 post-immunization. On day 25 post-immunization, the mice were sacrificed and lymph node cells and splenocytes were harvested and pooled. Pooled cells were then cultured in 96-well plates at a concentration of 400,000 cells/well with either no antigen, varying levels of MOG35-55, or ConA. On day 3 of culture, cells were pulsed with tritiated thymidine for 18-20 hours. Cells were then harvested, washed and analyzed for uptake on a β plate scintillation counter. This figure is representative of 10 independent studies.

Figure 4: Lymphocytes from MOG/IFA-immunized Mice Do Not Show Antigen-Specific Proliferation. 8-week-old female B6 mice were immunized with 200 μg MOG35-55/IFA supplemented with MTb (4 mg/ml) and give intraperitoneal PT on days 0 and 2 (250 ng/injection). 30 days post-immunization, bulk splenocytes and LNC were pooled and stained with CFSE. They were then incubated for 7 days at a concentration of $x10^6$ cells/ml with the indicated antigen. Following the incubation, cells were washed and stained with anti-CD4 and anti-CD8 antibodies. They were then analyzed by flow cytometry for proliferation. Lymphocytes were gated using forward and side scatter analysis. The CD8high population in the dot plots represents the CD8+ cell population in the culture. The numbers represent the

percentage of proliferating cells as compared to the total cell number in the specific subpopulation (i.e. CFSE dilute CD8+ cells/Total CD8+ cells). This figure is a representative of 3 independent experiments.

Figure 5: Both CD4+ and CD8+ T Cells from MOG/CFA-immunized Mice Respond to MOG 35-55 in a Specific, Dose-dependent Manner. 8-week-old female B6 mice were subcutaneously immunized with 200 μg MOG 35-55/CFA and intraperitoneally injected with 250 ng PT on days 0 and 2 post-immunization. 20 days post-immunization, bulk splenocytes and LNC were pooled and stained with CFSE. These cells were then incubated for 5 days at a concentration of $1x10^6$ cells/ml with the indicated antigen. Following the incubation, cells were washed and stained with anti-CD4 and anti-CD8 antibodies, then fixed in paraformaldehyde. They were then analyzed for proliferation by flow cytometry. Lymphocytes were gated using forward and side scatter analysis. The CD8high population in the dot plots represents the CD8+ cell population in the culture. The CD8low population represents CD4+ cells in the same culture that have been gated according to a CD4hi population during analysis. The numbers represent the percentage of proliferating cells as compared to the total cell number in the specific subpopulation (i.e. CFSE dilute CD8+ cells/Total CD8+ cells). This figure is a representative of 40 independent experiments, with SI for CD4+ T cells and CD8+ T cells ranging from 2-20.

Figure 6: CFSE Proliferation Analysis is Optimal at Day Five of *In Vitro* Stimulation. Bulk SpC were harvested at day 20 post-immunization from B6 mice immunized with 200 μg MOG 35-55/CFA + MTb (4 mg/ml) and given PT i.p. on days 0 and 2 post-immunization. Cells were

stained with CFSE and incubated with the indicated antigen for 3 (A), 4 (B), 5 (C) or 7 (D) days in test tubes at a concentration of $1x10^6$ cells/ml. Cells were then stained with anti-CD4 and anti-CD8 antibodies and fixed in paraformaldehyde. Proliferation was analyzed by flow cytometer. Numbers indicate percentage of proliferating cells as described in figure 5.

Figure 7: Purified CD8+ T Cells Show Antigen-Specific Autoreactivity. A. . 8-week-old female B6 mice were immunized with 200 μg MOG 35-55/CFA + MTb (4 mg/ml). Mice also received 250 ng PT i.p. on days 0 and 2. "Untouched" CD8+ T cells were isolated from bulk splenocyte populations at day 25 post-immunization using a magnetic negative-selection protocol. These cells were incubated with irradiated, CD8-depleted antigen presenting cells for 3 days at a concentration of 400,000 cells/200 μl/well. They were then pulsed with tritiated thymidine for 18-20 hours. Following the pulse, cells were washed, harvested and counted as described earlier. This figure is a representative of 10 independent experiments. B. Untouched CD8+ T cells were purified from bulk splenocytes as described in part A. They were then stained with CFSE and incubated for 5 days in the presence of the indicated antigen. Irradiated, CD8-depleted SpC from a naïve wild type mouse were used as APCs. CD8 + T cells were incubated at a concentration of $1x10^6$ cells/ml and the CD8+ T cell:APC ratio was 1:5. Following the incubation period, cells were washed, stained with anti-CD8 antibodies, fixed in paraformaldehyde and analyzed by flow cytometry. The numbers represent the percentage of proliferating cells as measured by CFSE dilution. This figure is a representative of 10 independent experiments, all of which showed SI >2.

Figure 8: Activated APCs Promote Better Proliferative Responses in CD8+ T Cells from MOG-immunized Mice. Untouched CD8+ T cells were obtained from immunized mice according to the protocol described in figure 7b. These cells were stained with CFSE and incubated at a concentration of $1x10^6$ cells/ml in the presence of the indicated antigen. Irradiated CD8-depleted splenocytes obtained from the negative magnetic selection of the SpCs from the immunized mice were used as APCs at a 1:5 T cell:APC ratio. Following 5 day incubation, cells were stained with anti-CD8 antibodies and fixed in paraformaldehyde. Proliferation was analyzed by flow cytometry as previously described. Numbers indicate percentage of cells in the proliferating fraction.

Figure 9: CD8+ T Cells from MOG-immunized Mice Respond in an Antigen-Specific Manner. A. 8-week-old female B6 mice were immunized with 200 µg of either MOG35-55/CFA or OVA323-338/CFA and given 250 ng pertussis toxin i.p. on days 0 and 2. On day 25 post-immunization, splenocytes were harvested from the mice and incubated in 96 well plates at a concentration of 400,000 cells/200 µl/well with no antigen, MOG35-55 (20 µg/ml), OVA323-338 (20 µg/ml) or wbMBP (20 µg/ml). On day 3 of incubation, cells were pulsed for 18-20 hours with tritiated thymidine. Cells were then washed, harvested and measured for thymidine incorporation. This figure is representative of 3 independent experiments. B. Untouched CD8+ T cells were purified from bulk splenocytes of a MOG/CFA immunized mouse 25 days post-immunization as described in figure 7b. These cells were stained with CFSE and incubated ($1x10^6$ cells/ml) in the presence of irradiated APCs (1:5 = T cell:APC ratio) from a naïve mouse as well as no antigen, MOG35-55, OVA323-338 or ConA. Cells were then incubated for five days, washed and stained with anti-CD8 antibodies. They were then fixed in paraformaldehyde

and analyzed for proliferation by flow cytometry. This figure is representative of two different experiments.

Figure 10: Exogenous Cytokines Do Not Influence Antigen-Specific CD8+ T Cell Response. CD8+ T cells were harvested and purified from MOG-immunized mice as described in figure 7b. These cells were then stained with CFSE and incubated for five days ($1x10^6$ cells/ml) in the presence of the indicated antigen and irradiated APCs obtained from either naïve mice or CD8-depleted SpC from MOG-immunized mice. Cells were additionally incubated with IL-2 (10 pg/ml), IL-12 (10 ng/ml), IL-23 (3 ng/ml) or IL-7 (10 ng/ml). Following incubation, cells were washed, stained with anti-CD8 and anti-CD4 antibodies, fixed in paraformaldehyde and analyzed for proliferative responses by flow cytometry. * indicates significant proliferation ($\Delta PF \geq 1$ and $SI \geq 2$).

Figure 11: Antigen-Specific CD8+ T Cell Responses Can Be Found in Multiple Models of EAE. The following groups of mice were immunized: B6 mice (200 μg MOG 35-55), B6 mice (200 μg wbMBP), SJL/J mice (200 μg PLP 139-151) and B10.PL mice (200 μg Ac 1-11). All peptides were emulsified in CFA supplemented with MTb (4 mg/ml). All mice received 250 ng PT i.p. on days 0 and 2. 20 days post-immunization mice were sacrificed and splenocytes were harvested. CD8+ T cells were purified by negative selection using magnetic beads (purity > 90% by flow cytometry). Bulk splenoctyes and purified CD8+ T cells were stained with CFSE. Cells were then cultured with no antigen, respective immunizing antigen (40 μg/ml), or Con A (5 μg/ml) for five days at a concentration of $1x10^6$ cells/ml. Irradiated splenocytes from the CD8-

depleted subsets following sorting from the respective mice were used as APCs at a T cell:APC ratio of 1:5. Following incubation, cells were washed, stained with anti-CD8 antibodies, fixed in paraformaldehyde and analyzed for proliferation by flow cytometry. The figure shows graphic representations of proliferation in the bulk CD4+ population (A), bulk CD8+ population (B) and purified CD8+ population (C) in each of the models. This figure is a representative of at least two assays per model system.

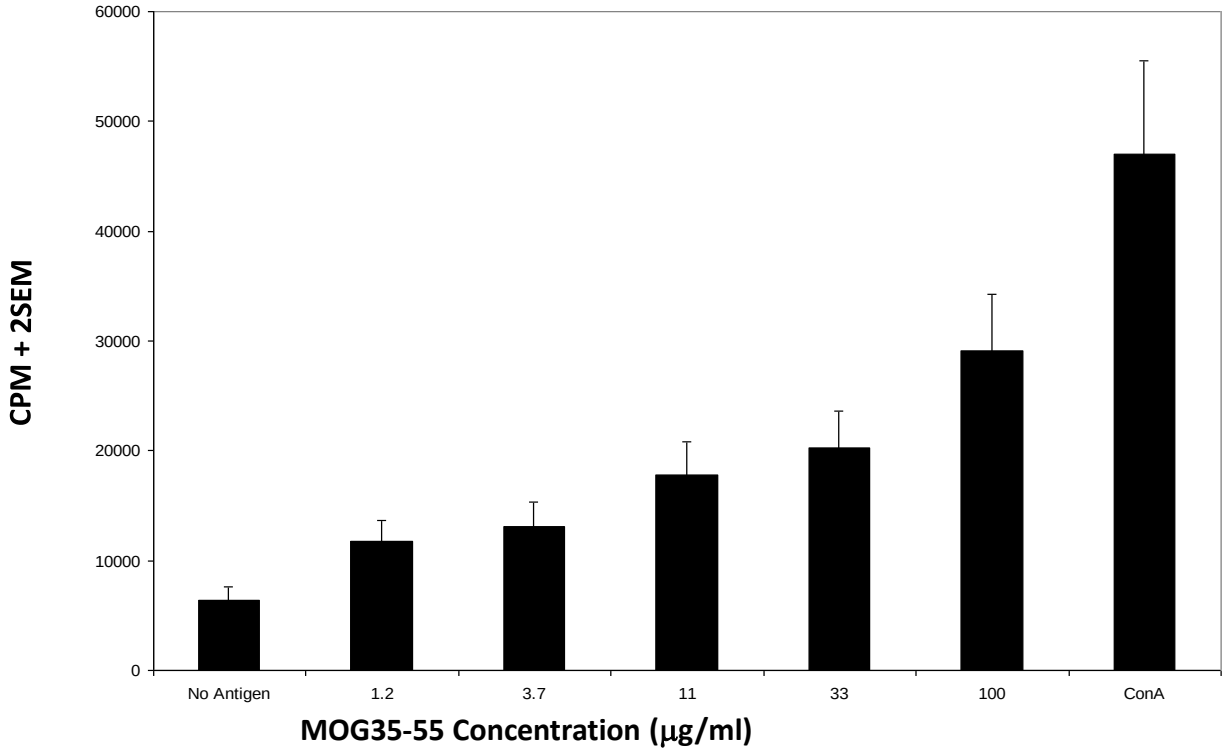


Figure 3: Bulk Cells from MOG-immunized Mice Respond in a Dose-dependent Manner to MOG 35-55 Antigen Stimualtion.

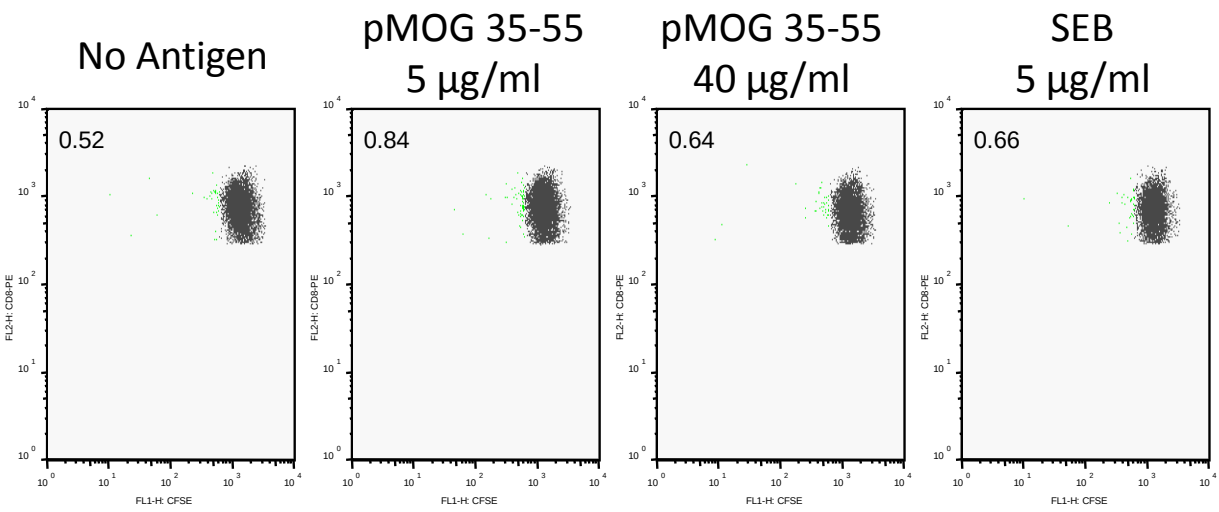


Figure 4: Lymphocytes from MOG/IFA-immunized Mice Do Not Show Antigen-Specific Proliferation.

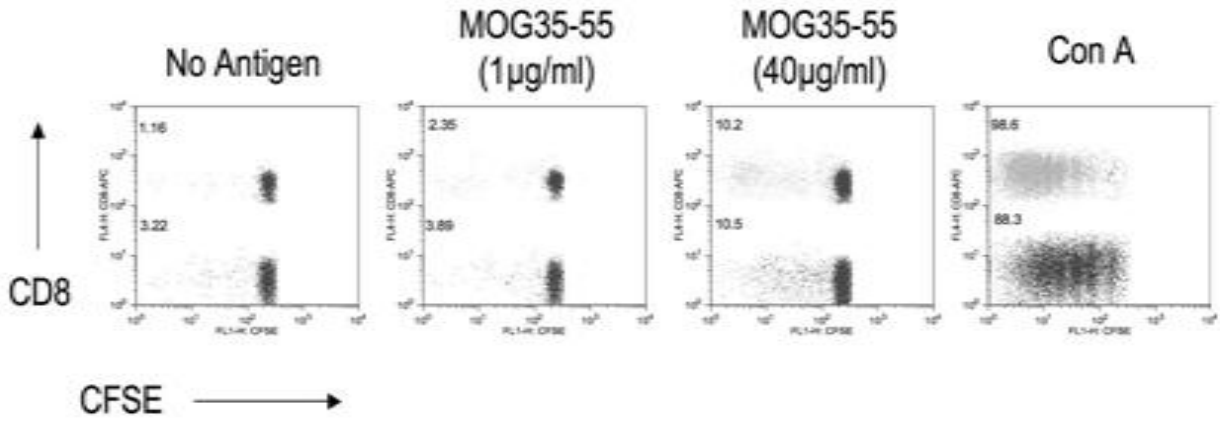


Figure 5: Both CD4+ and CD8+ T Cells from MOG/CFA-immunized Mice Respond to MOG 35-55 in a Specific, Dose-dependent Manner.

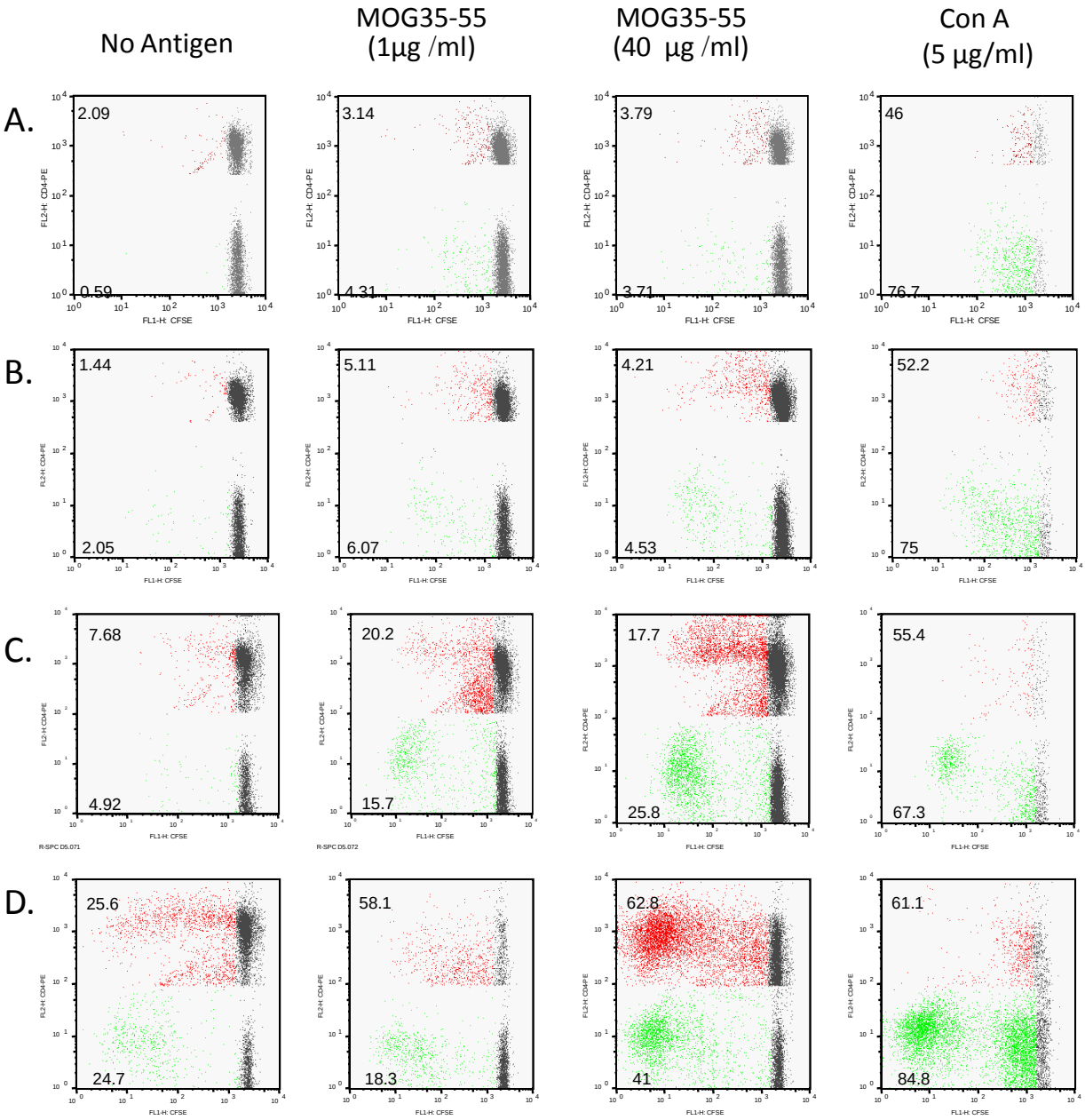


Figure 6: CFSE Proliferation Analysis is Optimal at Day Five of *In Vitro* Stimulation.

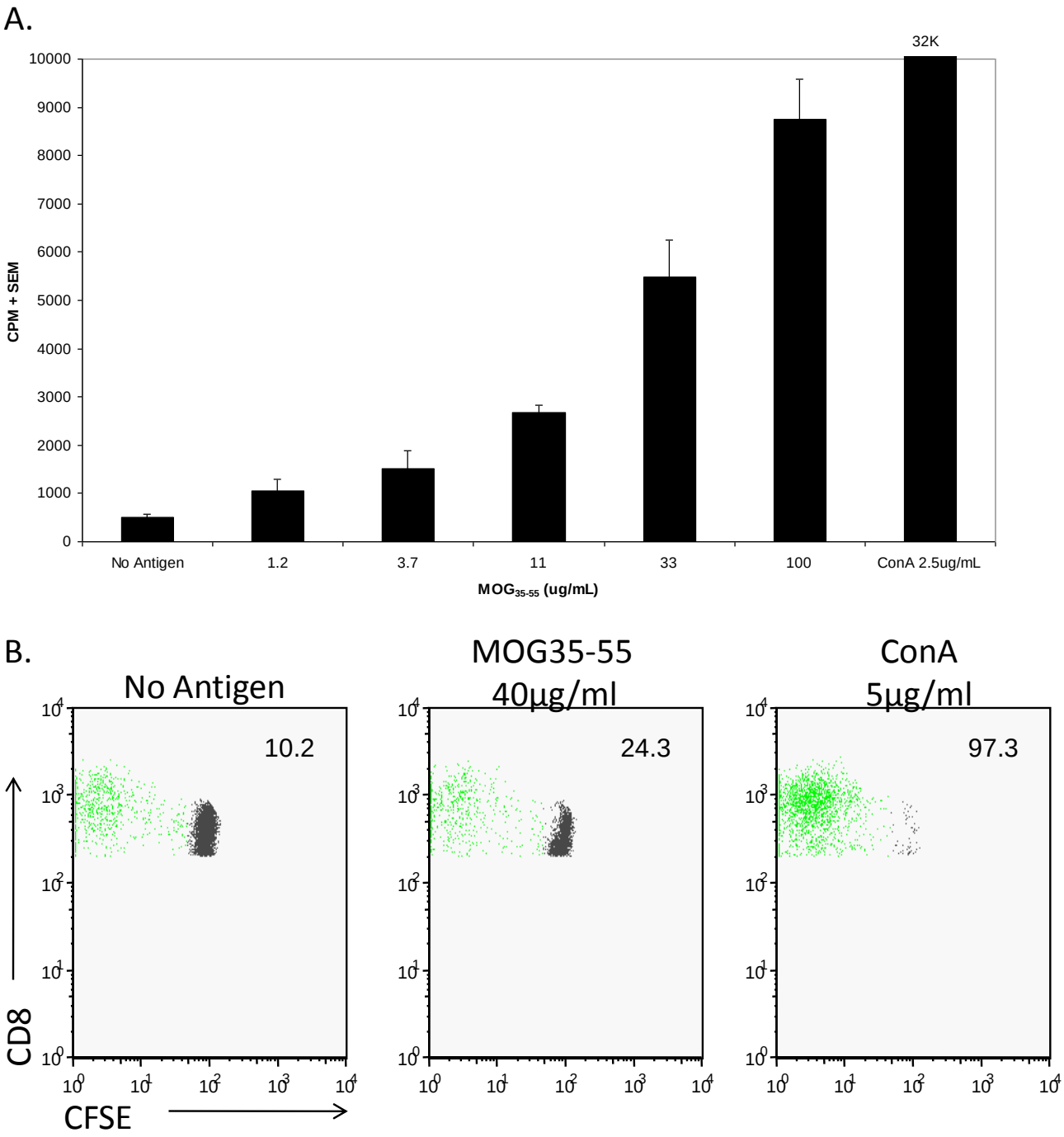


Figure 7: Purified CD8+ T Cells Show Antigen-Specific Autoreactivity.

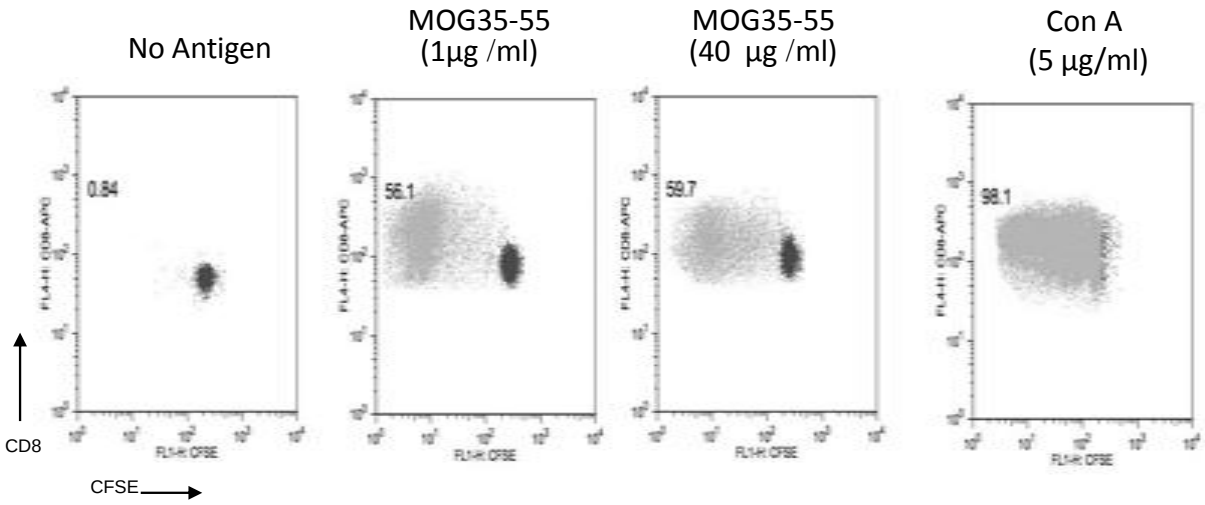


Figure 8: Activated APCs Promote Better Proliferative Responses in CD8+ T Cells from MOG-immunized Mice.

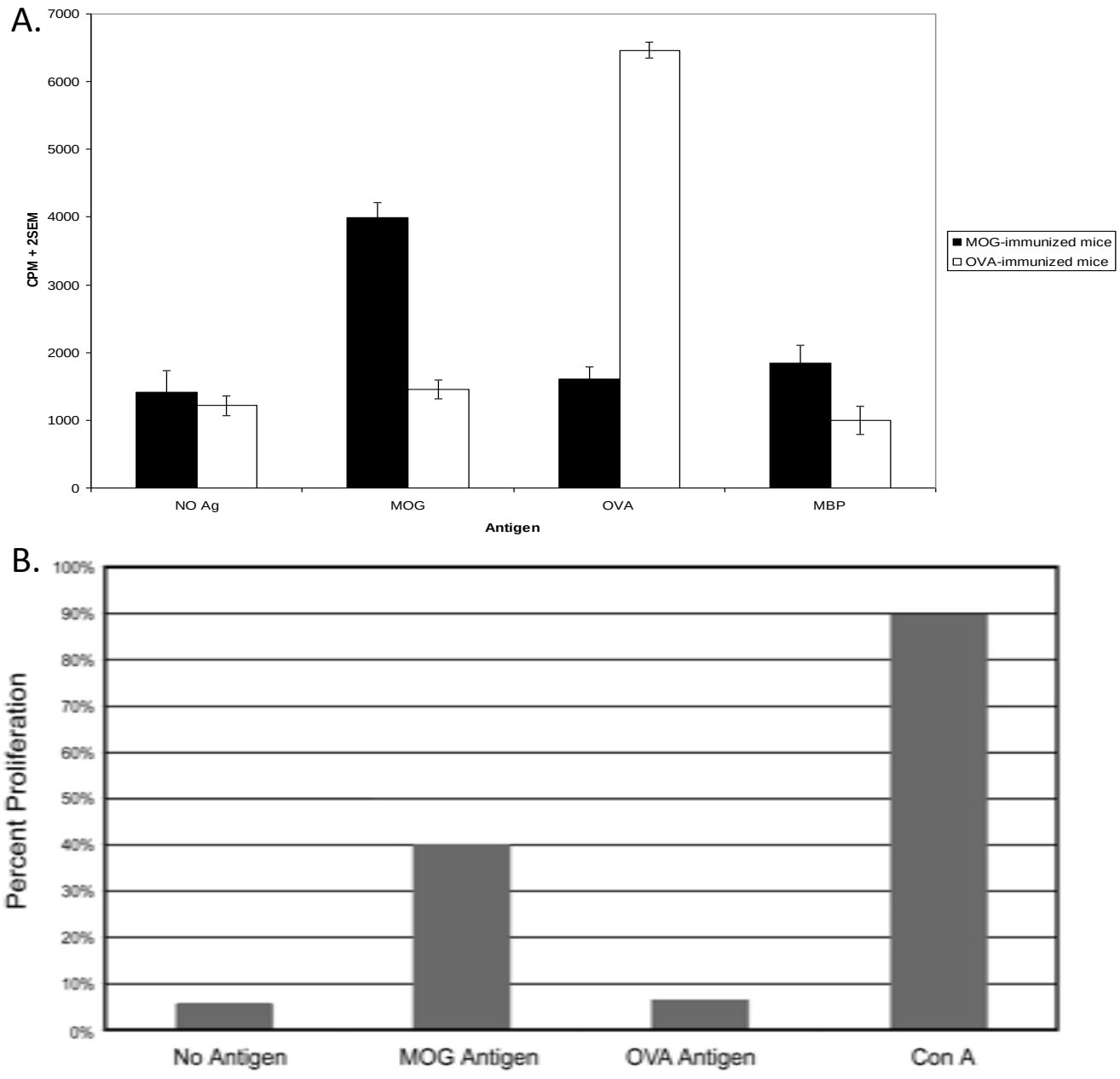


Figure 9: CD8+ T Cells from MOG-immunized Mice Respond in an Antigen-Specific Manner.

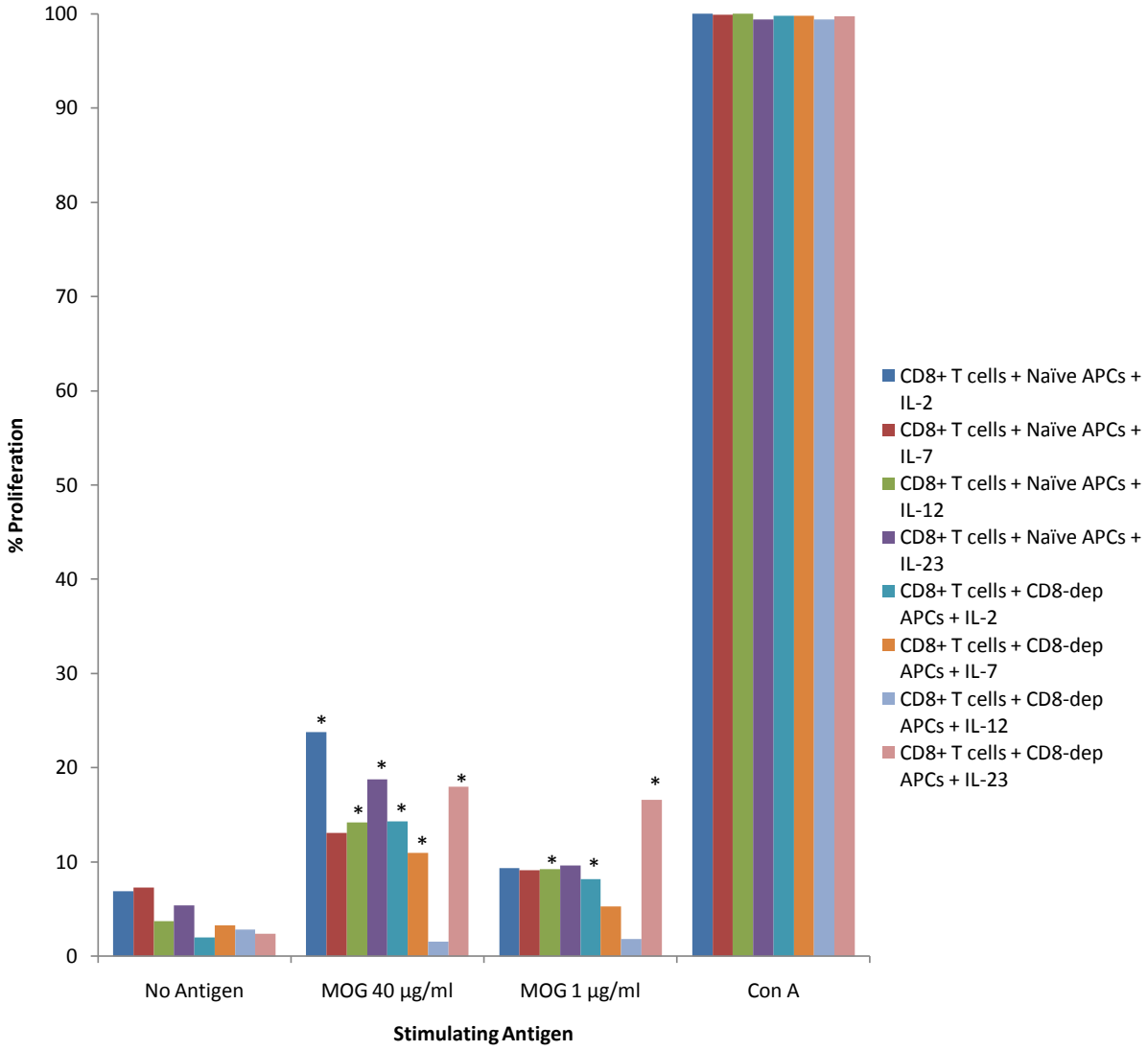


Figure 10: Exogenous Cytokines Do Not Influence Antigen-Specific CD8+ T Cell Response.

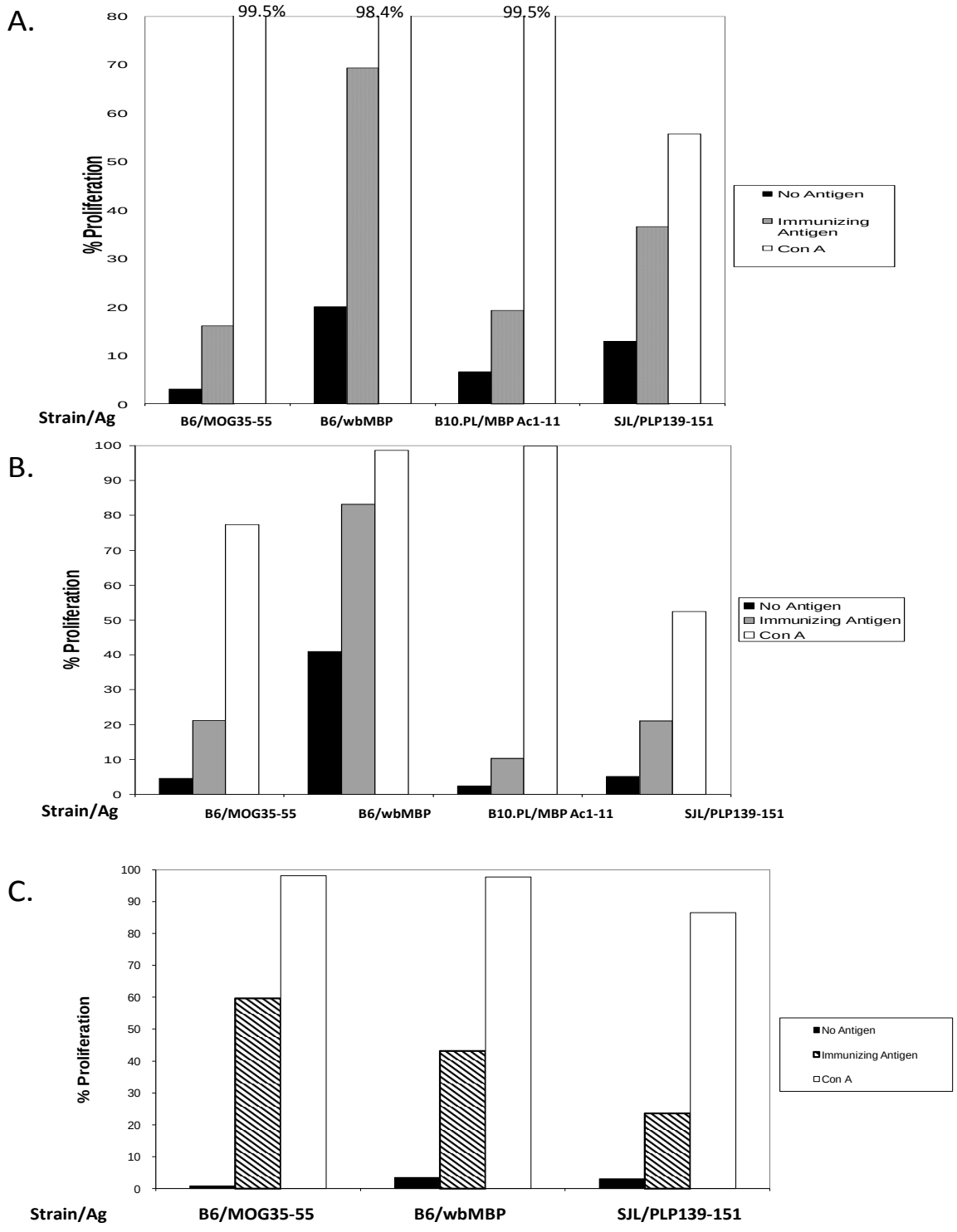


Figure 11: Antigen-Specific CD8+ T Cell Responses Can Be Found in Multiple Models of EAE.

Chapter IV: Producing an Autoreactive CD8+ T Cell Clone

After having concluded that an antigen-specific, autoreactive CD8+ T cell response is reproducibly identifiable in our B6 model of EAE, we wanted to produce an immortalized CD8+ T cell line from an autoreactive primary bulk population. The intent of this line would be to maintain a reservoir of antigen-specific, autoreactive CD8+ T cells that could be drawn from for future mechanistic studies and perhaps the creation of a transgenic model for further characterization and also to characterize the TCRs of the autoreactive CD8+ T cells involved in the MOG-specific response.

We first attempted to derive a cell line from primary CD8+ T cells. LNC and SpC were harvested from MOG 35-55-immunized mice and pooled. Unfortunately, despite numerous attempts to maintain these cultures, the cells never maintained growth past 5 days. Additional attempts were made using exogenous cytokines (IL-2 and IL-12) to stimulate and maintain growth, but these attempts failed as well. There are ongoing studies in the lab to elucidate the affects of different cytokines on cell growth with the intent that these discoveries can help to establish a stable CD8+ T cell line.

Since our attempts to establish a TCR-specific T cell line had thus far proven fruitless, we decided to try to employ a different strategy that would give us an immortal clone from which we could analyze the T cell receptor and create a T cell transgenic mouse model. This strategy involved creating a CD8+ hybridoma cell line that expressed an autoreactive, MOG-specific TCR. We obtained a hybridoma cell line that had been published to constitutively express CD8 on the cell surface (a kind gift from Dr. Kenneth Rock, U. Mass.) (146) We isolated CD8+ T cells from SpC of MOG-immunized mice. We then fused the CD8+ T cells to the hybridoma

cell line according to a published protocol (147). Unfortunately, we discovered that the cell line had lost resistance to the selection media, and therefore could not be selected according to the protocol. We therefore sought to characterize the cells that had fused by detection of CD3 expression using flow cytometry. This technique seemed to be successful, as there were only 5 out of 96 wells that appeared to have significant CD3+ populations. However, repeat flow cytometric analyses on passaged cells showed that, over time, the cells lost their CD3+ expression. Further attempts to hybridize the cells ended in the same result.

Efforts in the laboratory are ongoing for the establishment of an autoreactive, CD8+ T cell line, but this pursuit has proven far more difficult than expected. Despite efforts to improve culture media as well as fusion protocols, all attempts thus far have been unable to produce a stable T cell clone or hybridoma cell line. Further experimentation is required to elucidate the reasons for difficulties we have encountered. Perhaps a different hybridoma cell line sensitive to HAT selection could be used. Unfortunately, we were unable to find another line that constitutively expressed CD8. We would therefore have to create our own CD8-expressing fusion partner, which would extend the length of time originally planned for this project. For the purposes of this study, we decided to press forward with our analysis of CD8+ T cell function using only primary CD8+ lymphocytes as our source of cells.

Chapter V: Mechanism of CD8+ T Cell Regulation – Adoptive Transfer Experiments

Adoptive transfer of autoreactive cells allows us to characterize the function of different cell types during disease. Normally, EAE is established by active immunization, which generally involves the emulsification of the encephalitogenic peptide in complete Freund's adjuvant, which contains the inflammatory *Mycobacterium tuberculosis* toxin. It has been proposed that the role of adjuvants in EAE is to manipulate the environment so that the immunogenicity of the immunizing peptide is increased and so that it is recognized and processed by APCs in an inflammatory context (148). It is also hypothesized that adjuvants such as PT incite a general state of inflammation in the mouse, and this may also aid in the development of disease. As can be inferred, this process creates the disease artificially and therefore only allows speculation as to the similarities into the etiology of MS. Adoptive transfer studies allow cellular participation in disease to be studied without the necessity of adjuvants or inflammatory mediators. It has long been shown that activated CD4+ T cells from a diseased mouse can cause disease in a naïve mouse without the necessity of emulsified antigen. It was then inferred that these cells were at least one of the causative agents of EAE in rodents. Indeed, these studies were integral in the emphasis that research took on the CD4+ T cells as the major cell involved in the EAE (and MS) pathogenic process

After identifying and confirming the presence of an antigen-specific, autoreactive CD8+ T cell population in immunized mice, we next sought to determine their function. There have been several publications that suggest pathogenic and/or regulatory roles for CD8+ T cells in autoimmune demyelination. Two of these studies were done in the B6-MOG model that we had established in our laboratory, and both of them showed that purified CD8+ T cells were able to adoptively transfer disease, and thus play a pathogenic role (127, 128).

For this reason, we attempted to duplicate the results of these adoptive transfer experiments to determine if these cells were pathogenic in our hands. Female B6 mice were injected with pMOG 35-55 emulsified in IFA supplemented with MTb. The mice were also given PT on days 0 and 2. 20 days post-immunization, the mice still showed no signs of clinical disease, but our observations from proliferation assays using these mice suggested that there may be autoreactive CD8+ T cells. We hypothesized that these cells may be able to transfer disease despite the fact that the immunized mice did not develop disease. To this end, the mice were sacrificed and the LNC and SpC were harvested. CD8+ T cells were separated from the remaining cells by negative selection. 72 hour cultures were then set up in the following conditions: CD8+ T cells + irradiated bulk APCs from naïve mice + pMOG 35-55 (20 μg/ml), CD8- cells + pMOG 35-55, or bulk cells (pooled LNC and SpC). After the 72 hour cultures, dead cells were removed from the populations by Ficoll gradient. The live cells were counted and injected intravenously into naïve B6 mice ($10x10^6$ cells/mouse) and monitored for disease.

Unfortunately, the mice never showed any signs of disease. This, being our first attempt, could have been for many reasons. Since the mice never developed disease, it is possible that none of the cultured cells were encephalitogenic. It is also possible that the culture conditions established were not conducive to activation or expansion. Another possibility is that the cells were not injected in sufficient numbers or with the proper technique. No members of the lab were experienced with intravenous injection at the time. We decided to perform repeat experiments to optimize a protocol for our mouse colony, as we had done with our active immunization protocol.

In another attempt to establish disease by adoptive transfer, we cultured bulk SpC and LNC from B6 mice that had been immunized with pMOG 35-55 or whole bovine MBP

emulsified in IFA supplemented with MTb 12 days prior. In both of these groups, no mice developed disease as a result of the active immunizations. For this experiment, we decided to culture the cells in bulk cultures, as opposed to purifying the CD8+ T cells pre-culture. We hypothesized that this would maintain the cells in a more biological setting and perhaps augment their activation and expansion. Therefore, these cells were cultured for 72 hours in the presence of immunizing antigen (20 μg/ml) and IL-2 (10 u/ml). Following the cultures, dead cells were removed by Ficoll and CD8+ T cells were positively selected (purity > 90% by flow cytometry). CD8+ T cells or CD8-depleted cells were then injected into naïve B6 mice. The mice were also injected intraperitoneally with IL-2 (10 units). This technique was according to a different published protocol (). Again, the mice failed to develop disease. We determined that the problem could again have been a lack of encephalitogenic T cells, since even the groups that contained CD4+ T cells failed to induce disease.

Once we established a working active immunization protocol in the mouse colony (see prior results section), we decided to attempt adoptive transfer disease again. Using findings in our previous attempts, we harvested the bulk splenocytes from diseased mice (or control mice immunized with OVA 323-339/CFA) and cultured them for 72 hours in the presence of immunizing antigen and IL-2. Following culture, both CD4+ and CD8+ T cells were purified from MOG-immunized mice and naïve mice were injected with either bulk activated cells, activated CD4+ T cells or activated CD8+ T cells ($5x10^6$ cells/mouse). We then monitored the mice for disease. For the first time, we were able to observe adoptively transferred disease (Figure 12). As expected, we observed typical disease curves for mice that had been injected with either bulk cells or CD4+ T cells. To our initial disappointment, however, the mice that were injected with the CD8+ T cells failed to develop disease. Since this was our first success

with the CD4+ and bulk cells, we repeated the experiment and got the same results. Therefore, over a series of experiments, we varied the protocol by injecting greater numbers of cells (up to $10x10^6$ cells/mouse) or by adding inflammatory cytokines (IL-2 and IL-12) to the culture conditions, according to other published protocols for adoptive transfer of disease (). In all of these successive experiments, we continued to observe the same result: bulk cells and purified CD4+ T cells were able to elicit disease, but CD8+ T cells were not.

Since we could not establish disease by adoptive transfer of CD8+ T cells despite several different variations on established protocols, we concluded that these cells were not sufficiently pathogenic to initiate disease. This led us to consider other possible roles for CD8+ T cells in the disease process. One hypothesis is that these cells play an accessory role in the pathogenesis of the disease, but require CD4+ T cell help in order to function. Another hypothesis is that these cells are not pathogenic in nature, but rather play a regulatory role in the disease. A third hypothesis is that, although these cells are autoreactive, they may not play any role at all. To test this hypothesis, we decided to adoptively transfer activated CD8+ T cells into actively immunized mice. We hypothesized that if the CD8+ T cells play any role in disease course, be it regulatory or pathogenic, they would alter disease course, and this would be observable in comparison to control mice.

For the experiment, we immunized B6 mice with either MOG 35-55 or OVA 323-339 emulsified in CFA supplemented with MTb (4 mg/ml). These mice were also given PT injections on days 0 and 2 post-immunization. 20 days after immunization, the mice were sacrificed and SpC were harvested. The cells were then incubated for 72 hours in the presence of immunizing antigen (20 μg/ml) and IL-2 (10 pg/ml). Following incubation, dead cells were removed by Ficoll gradient. CD8+ T cells were then purified by positive selection over a

magnetic column. Once purified, the CD8+ T cells were then injected intravenously into naïve B6 female mice ($6x10^6$ cells/mouse). The following day, the mice were then immunized with MOG 35-55/CFA according to our active disease protocol. The mice were then monitored for disease. We observed that the mice that received CD8+ T cells from the MOG-immunized mice had less severe disease with delayed onset and faster recovery compared to mice that received CD8+ T cells from OVA-immunized mice (Figure 13a). In a parallel experiment, we tested if ex vivo cells were able to exert the same influence on disease course as the in vitro-cultured cells. We therefore separated some of the mice that had been immunized. CD8+ T cells were purified from bulk splenocyte populations immediately following harvesting (purity > 90% by flow cytometry). These cells were then injected into naïve wild type B6 mice ($6.5x10^6$ cells/mouse). The next day, the mice were immunized according to our active immunization protocol and monitored for disease. As was seen with the cultured cells, these mice also developed delayed disease with decreased severity and quicker recovery when compared to control mice (Figure 13b). These results indicate that, contradictory to previously published reports, the CD8+ T cells do not play a pathogenic role in the disease pathogenesis of EAE in this model. In fact, this experiment suggests that these cells actually play a regulatory role in EAE. The results also suggest that, although ex vivo cells are able to ameliorate disease, cells that have been activated in vitro are superior in regulation. This observation lends confidence to the idea that it is the MOG-specific CD8+ T cell subset that is effecting this protection, and not just bulk CD8+ T cells from an immunized mice, due to the fact that in vitro culture should increase activation status and expansion of cells responding to the stimulating antigen, pMOG 35-55 in this case.

We also wanted to discover if these cells could ameliorate active disease. To this end, we prepared CD8+ T cells as described above. For further controls, we used CD8+ T cells from

naïve mice and also mice that received no CD8+ T cells. For this experiment, instead of injecting them into naïve mice pre-immunization, we injected the CD8+ T cells into mice that had been immunized 12-15 days prior. We anticipated that these mice would be at peak disease at the same time that the CD8+ T cells would be ready to transfer. Unfortunately, the mice did not become sick as quickly as anticipated. Since we could not allow the cells to continue to incubate, we injected them into the recipient mice even though they were not at peak disease. We randomized and normalized average disease scores among the 5 mice in each group and monitored them for disease. As the results of this experiment showed, the average disease scores showed no significant differences between all groups. However, because several of the mice had not developed disease at the time of injection, we cannot conclude if the CD8+ T cells played a role in disease prevention or not. A repeat of the experiment was necessary, but for the repeat we decided we would not culture the donor cells until the recipient mice all showed initial signs of disease. Waiting for all mice to get sick may result in some of the mice being past peak disease, but all of the mice would be sick and could be normalized between groups, so we decided that this would be the best strategy to answer our question. The results of this test showed that mice injected with CD8+ T cells from a MOG-immunized mouse began to see decrease in severity of symptoms within 48 hours of injection (Figure 14). Mice that received CD8+ T cells from OVA-immunized mice, however, showed no signs of recovery. The results of this experiment show that antigen-specific CD8+ T cells from MOG-immunized mice can also exert a therapeutic effect in addition to the protective effect seen earlier.

In another set of adoptive transfer experiments, we sought to discover if MOG-specific CD8+ T cells could prevent disease caused by adoptive transfer of MOG-specific CD4+ T cells. In order to test this question, we harvested and purified CD8+ T cells from mice that had been

immunized with MOG 35-55 or OVA 323-339/CFA 20 days prior, following 72 hour in vitro activation as has been outlined in other adoptive transfer experiments. CD4+ T cells were obtained from MOG-immunized mice 10 days after immunization and activated in a similar manner. Naïve B6 mice were then injected with either CD4+ T cells alone, CD4+ T cells with CD8+ T cells from OVA-immunized mice or CD4+ T cells with CD8+ T cells from MOG-immunized mice. We discovered that the mice that received the CD8+ T cells from OVA-immunized mice showed disease scores similar to that seen in mice that received CD4+ T cells alone. The mice that received CD8+ T cells from MOG-immunized mice along with CD4+ T cells, however, never developed signs of disease (Figure 15). We concluded from this study that MOG-specific CD8+ T cells can exert a regulatory effect on encephalitogenic CD4+ T cells and prevent disease in the adoptive transfer setting. This testing now confirmed in three different disease settings the ability of antigen-specific CD8+ T cells to regulate autoimmune demyelination.

Chapter V Figure Legends

Figure 12: Adoptive Transfer of MOG-stimulated CD8+ T Cells Does Not Cause Disease. Wild type female B6 mice were immunized with MOG35-55/CFA or OVA 323-339/CFA and received pertussis toxin on days 0 and 2. 25 days post-immunization, the mice were sacrificed and splenocytes were harvested. These cells were incubated for 72 hours in the presence of MOG35-55 and IL-2 (10 pg/ml). Following incubation, dead cells were removed by Ficoll gradient. CD4+ and CD8+ cells from the MOG-immunized mice were selected using magnetic sorting (purity > 90%). Next, bulk splenocytes, CD4+ cells or CD8+ cells were injected intravenously into naïve wild type B6 mice ($5\text{-}10x10^6$ cells/mouse) and monitored for disease. This figure is a representative of 5 independent experiments. In some experiments, cells were also cultured in the presence of IL-12 (10 ng/ml), but this did not affect the results of the transfer.

Figure 13: MOG-stimulated CD8+ T Cells Protect from Active Disease Induction, But OVA-stimulated CD8+ T Cells Do Not. Wild type B6 mice were immunized with MOG35-55/CFA or OVA323-339/CFA and given pertussis toxin on days 0 and 2. 25 days post-immunization, splenocytes were harvested from the mice. At this point, cells were either A. incubated for 72 hours in the presence of MOG35-55 and IL-2 (in vitro) or B. taken directly from the mouse (ex vivo). CD8+ T cells were purified by magnetic sorting (purity > 90%) and injected intravenously into naïve wild type B6 mice ($6.5x10^6$ cells/mouse). 1 day after adoptive transfer, the mice received MOG35-55/CFA subcutaneously. The mice were also given pertussis toxin and days 0 and 2 according to our usual disease protocol. These mice were then monitored

for disease. This figure is representative of 5 independent experiments for the in vitro culture and 2 for ex vivo adoptive transfer.

Figure 14: MOG-specific CD8+ T Cells Ameliorate Active Disease. CD8+ T cells were purified from the splenocytes of MOG-immunized or OVA-immunized mice 25 days post-immunization and following 72 hour in vitro culture with the immunizing antigen and IL-2. These cells were then adoptively transferred intravenously into wild type B6 mice that had been immunized with MOG35-55/CFA 13 days prior according to our protocol. Mice were selected at various stages of disease course so that the mice receiving cells from the MOG-immunized mice and those receiving cells from OVA-immunized mice were normalized for average disease score. The mice were then randomized and monitored for disease. This figure is representative of 2 independent experiments.

Figure 15: MOG-Specific CD8+ T Cells can Prevent Adoptive Transfer of Disease. Wild type B6 mice were immunized with pMOG 35-55/CFA or pOVA 323 -339/CFA. Splenocytes and LNC were harvested on day 10 or day 20 of disease. CD4+ cells were purified from the mice at day 10 of disease (all MOG-immunized) and CD8+ T cells were purified from the mice at day 20 of disease (either MOG-immunized or OVA-immunized) following 72 hour stimulation. The CD4+ T cells were either mixed with CD8+ T cells from the MOG-immunized mice, CD8+ T cells from the OVA-immunized mice or injected alone into naïve wild type mice. Mice were then monitored for disease

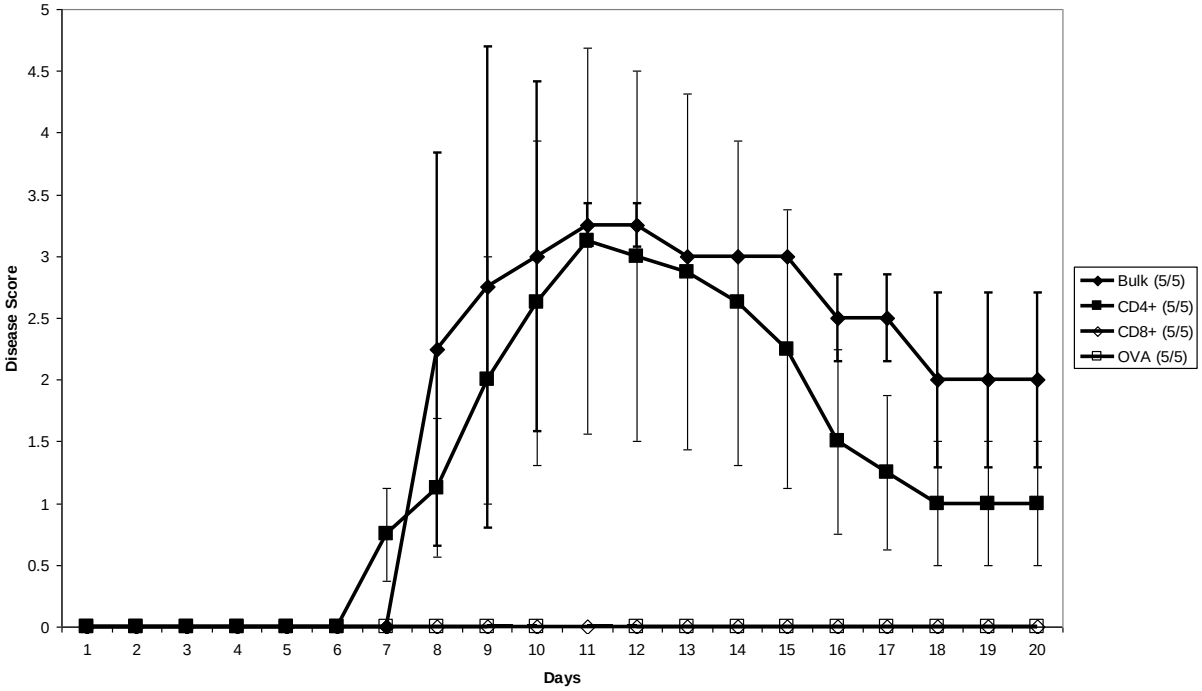


Figure 12: Adoptive Transfer of MOG-stimulated CD8+ T Cells Does Not Cause Disease.

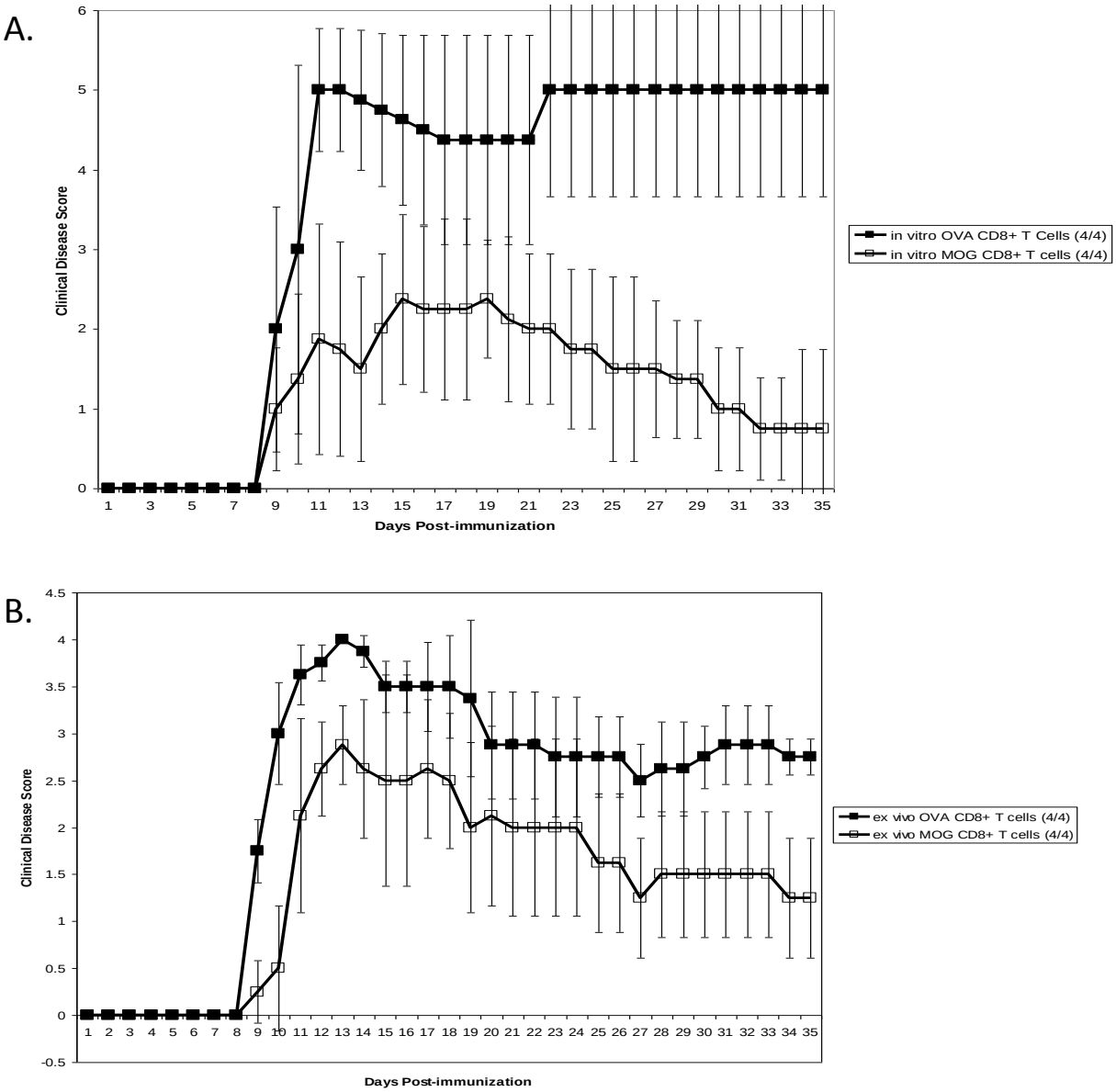


Figure 13: MOG-stimulated CD8+ T Cells Protect from Active Disease Induction, But OVA-stimulated CD8+ T Cells Do Not.

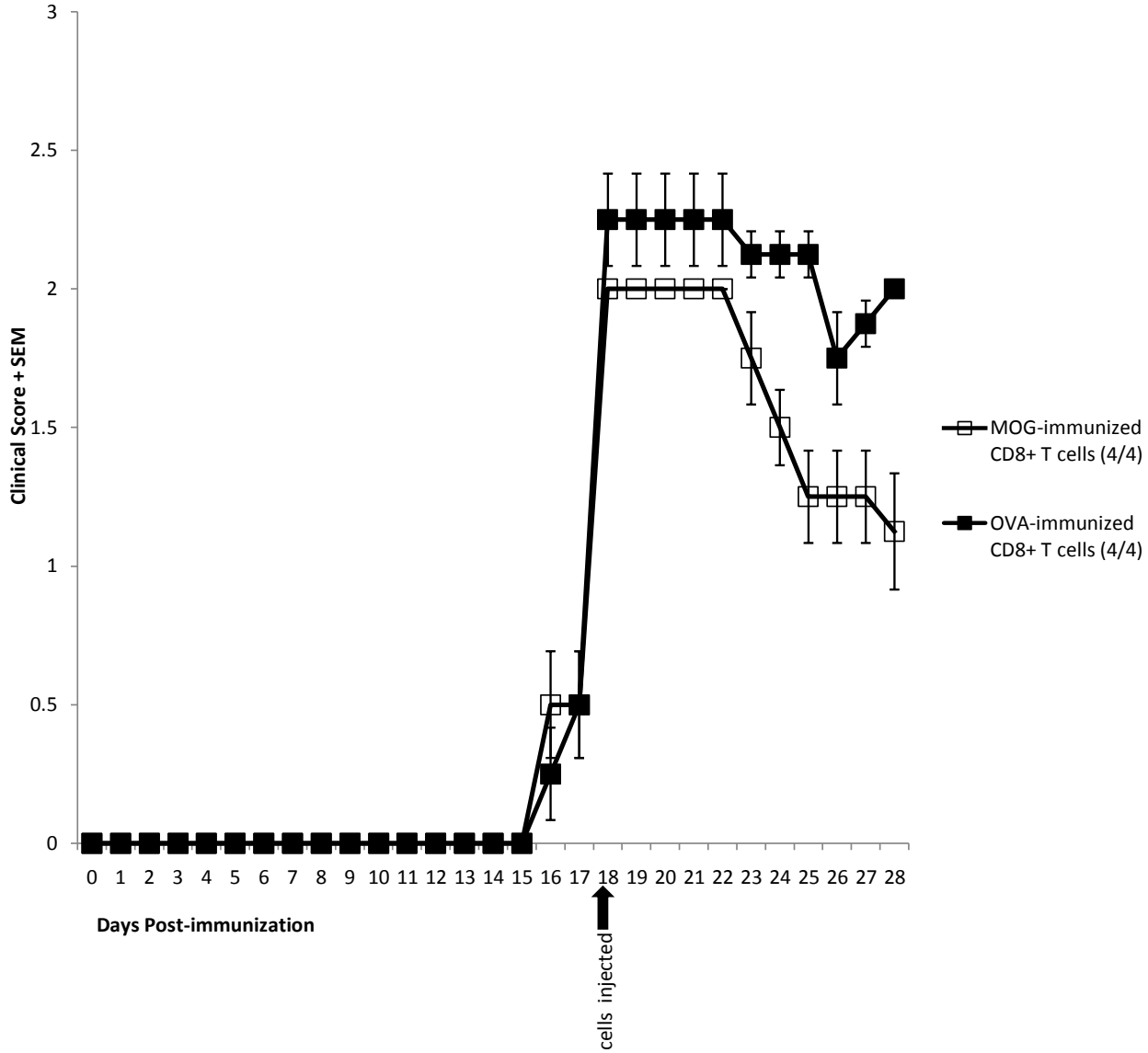


Figure 14: MOG-specific CD8+ T Cells Ameliorate Active Disease.

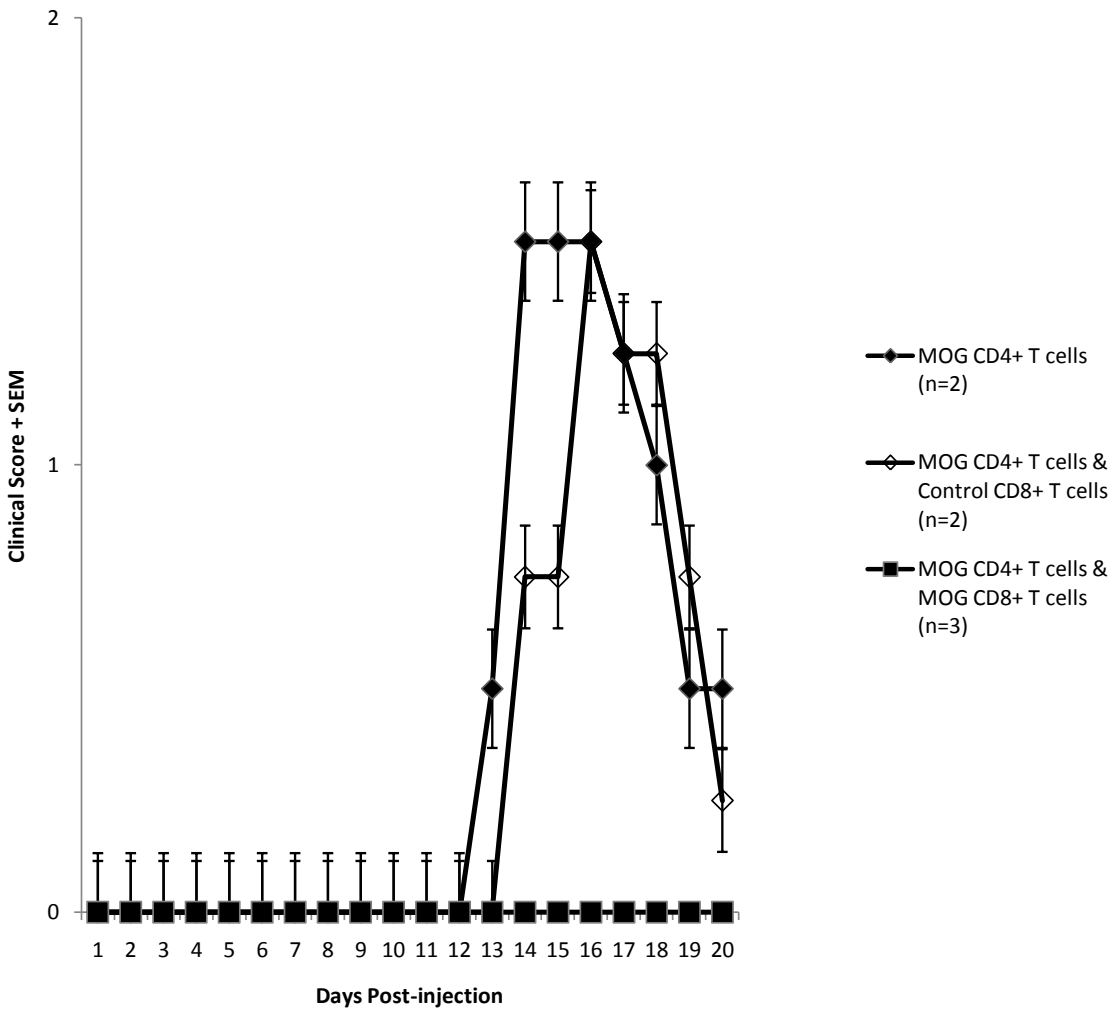


Figure 15: MOG-Specific CD8+ T Cells can Prevent Adoptive Transfer of Disease.

Chapter VI: Mechanism of CD8+ T Cell Regulation – Properties of Autoreactive Cells

Now that we had established a regulatory role for CD8+ T cells in the EAE setting, we next focused on possible mechanisms by which these cells may be regulating the disease. We first sought to characterize the kinetics of CD8+ T cell proliferation during the onset and progression of disease course. We also sought to discover if there were any cytokines produced by these cells and, if so, which ones were present. In another set of experiments, we attempted to deduce if these cells participate in the phenomenon of epitope spreading. Another property we explored was that of the ability of these CD8+ T cells to suppress activated CD4+ T cells.

Kinetics of CD8+ T Cell Response in EAE

Normal disease kinetics for EAE in actively immunized, wild-type B6 mice in our laboratory typically show disease beginning in between days 10-13. Over the next 5-7 days the disease climbs steadily to reach a peak at around day 15-20. Following the peak, the clinical scores would variably drop (usually around 1 clinical score) after which disease would level and be maintained in a chronic state. Occasionally, we would observe that the mice would exhibit a relapsing-remitting disease course, with short periods of recovery followed by exacerbations of disease. This course, however, seemed to be random and we could not repeatably reproduce it (data not shown). From studies of others, we can assume that CD4+ T cells are involved in the early disease kinetics of EAE. However, the presence of autoreactive CD8+ T cells has only been recently shown, and therefore the participation at different stages in the disease course is important to describe in order to gain understanding into their role. If these cells were present only early on in the disease course, this would suggest that they are involved to some degree in disease initiation, be it regulatory or pathogenic. If the cells were present during later stages of

the disease course, then it would suggest that they are involved in the maintenance and/or regulation of the disease in its chronic phase.

In order to deduce the kinetics of the CD8+ T cell response during the course of disease, we first immunized B6 mice according to our protocol. At days 15, 25 and 35 (day 15 is usually around the peak of disease) and we sacrificed mice and harvested the SpC and LNC for a CFSE assay. We then isolated CD8+ T cells from the splenocyte population using magnetic separation for negative selection. We stained the CD8+ T cells and incubated them with irradiated APCs from the CD8-depleted subset (T cell: APC ratio 1:5). We observed that the presence of CD8+ T cells could be observed at each of these time points to varying degrees (Figure 16). We consistently observed that the greatest proliferation occurred around day 20-25, as is depicted in the figure. Attempts to see proliferation as early as day 10 of disease were seen in bulk populations, predominantly in the lymph node cells (data not shown), but these results were not consistent from experiment to experiment. As disease progressed, we found autoreactive CD8+ T cells to be more consistently found in the spleen. Since mice normally get sick around day 10-13, this evidence suggested to us that autoreactive CD8+ T cells are present during most, if not all, of the active disease course. However, they are not consistently detectable during the initiation phase of the disease and appear to be most prominent during the early chronic phases of the disease, although they can be reproducibly detected later in the chronic disease phase.

Cytokines

Another question we sought to answer in order to characterize these cells involved the production of cytokines. Cytokines are an important part of T cell function. CD4+ and CD8+ T cells have been shown to produce cytokines. CD4+ Th cells have traditionally been divided into

Th1 and Th2 characterizations based on profile of cytokine production. Because of the inflammatory nature of the Th1 cells, which are known to be stimulated by IL-12, they were assumed to be involved in the pathogenesis of the disease. This hypothesis was backed up by studies that showed the presence of IL-12 and its necessity for disease induction. Recently, this evidence was challenged by the fact that IL-12 and IL-12R knockout mice both develop disease. Investigations pursuant to these findings led to the characterization of CD4+ T cells with different cytokine profiles, called Th17 cells. Studies have now shown evidence for roles of both Th1 and Th17 cells in EAE. CD8+ T cells and the cytokines that they produce have been relatively understudied. A few studies have described CD8+ T cells that play an apparent regulatory role in the disease, and they have been shown to effect this role through the production of IFN-γ. This role for IFN-γ is an unexpected one, given its association with inflammatory processes. The cells that have been described, however, are thought of as antigen-nonspecific for the most part.

In order to find out which, if any, cytokines are produced by these cells, we first sought to utilize a flow cytometry-based cytokine bead array. In this assay, multiple cytokines commonly associated with Th1 or Th2 T cells are measured. We froze down supernatants from CFSE assays containing CD8+ T cells from MOG-immunized mice, APCs from CD8-depleted subsets and different antigens (no antigen, MOG 35-55, Con A). These samples were thawed and incubated with fluorescent antibodies specific for IL-2, IL-4, IL-5, IFN-γ and TNF-α. Repeated tests using this experimental procedure yielded no detectable cytokines from the culture supernatants taken from cells incubated with MOG 35-55 antigen. We did find some levels of IL-2, IFN-γ and TNF-α in the supernatants from the cells incubated with Con A. This is not unexpected, since Con A is known to be a mitogenic activator of T cells. There are several

reasons to explain the lack of detectable cytokine in the MOG 35-55-cultured supernatants. One reason could be that these cells are not producing any cytokines. However, there is published literature to suggest that this is not the case (149). Perhaps the level of cytokine produced by these cells was below the limit of detection. This would require optimization of our culture protocols. This type of experimentation was not feasible due to the expense of this assay system. Another possibility is that the cells were not responding to the MOG 35-55 antigen. However, the CFSE assays performed on the same cells from which the supernatants were taken demonstrated a proliferative response. We therefore decided to use standard ELISA assays to test the supernatants for cytokine presence.

For our ELISA assays, we used supernatants that were frozen down from CFSE proliferation assays at 48, 72 and 96 hours of culture. Supernatants were from tubes that cultured cells at both $1x10^6$ and $2x10^6$ cells/ml. These cells were being cultured with no antigen, pMOG 35-55 (the immunizing antigen, 20 μg/ml), pOVA 323-339 (specificity control, 20 μg/ml) or Con A (2 μg/ml). Irradiated CD8-depleted SpCs from OVA-immunized mice were used as APCs (T cell:APC ratio = 1:5). The results of the ELISA assays show that the autoreactive CD8+ T cells produce IFN-γ (Figure 17a) and that this result is not due to activity from the irradiated APCs, since no IFN-γ production was observed in the cultures incubated with the OVA 323-339 peptide. We also observed that these cells to produce IL-4 in response to MOG 35-55 stimulation, but not OVA 323-339 stimulation, which again indicates that the cytokine is being produced by antigen-specific CD8+ T cells.

Epitope Spreading

We also attempted to find out if CD8+ T cells participate in a phenomenon known as epitope spreading. This phenomenon is known to occur in the autoreactive CD4+ T cell population (as has been described in other sections of this work), but there is no evidence as to CD8+ T cell populations. Briefly, during the course of disease, it has been shown in SJL/J mice that as disease progresses, the specificity of the predominant responding CD4+ T cell changes from one epitope to another, even one antigen to another (70, 71).. As mentioned earlier, we noticed that disease in the B6/MOG model in our hands occasionally produced relapsing-remitting disease as well. We therefore hypothesized that perhaps there is also epitope spreading in this model. It could also be hypothesized that epitope spreading occurs even in the absence of a relapsing-remitting disease course and is part of a chronic course as well. In either case, epitope spreading among CD8+ T cells has not been shown.

In order to study this phenomenon in CD8+ T cells, we established EAE in four different models of disease (SJL:PLP 139-151, SJL:MOBP 15-36, SJL:MOBP 37-60, B6:MOG 35-55) using standard protocols described in the Materials and Methods section of this work. At the time of sacrifice, all of the mice showed disease of varying clinical scores (2-4). LNC and SpC from each model were harvested, and CD8+ T cells were separated from SpC by negative selection. APCs were taken from naïve mice of the same strain as the immunized mice. A CFSE assay was then performed on bulk cells, as well as CD8+ T cells, from each model using six different antigens that have been shown to have specific encephalitogenic responses. In all cases, the cells in both the bulk cultures and the CD8+ T cells showed mild proliferation to the immunizing antigen, but no epitope spreading was observed in the CD8+ T cell population.

These assays were also repeated using APCs from OVA-immunized mice, to find out if activated APCs are required to promote proliferation. As the results show, none of the CD8+ cells, either in bulk cultures or in purified cultures, proliferated to any antigens other than the immunizing antigen (Figure 18). This suggests that these cells are not involved in epitope spreading, but it must be stated that these results are not conclusive. There are many reasons as to why we may not have detected a spreading phenomenon. First, we did not take samples at multiple points along the disease course. Second, the peptides and proteins that we used to survey autoreactivity have all only been shown to bind to MHC class II. While we know that pMOG 35-55 can stimulate both CD8+ and CD4+ T cells, we cannot say with certainty that other peptides have the same ability. It could very well be that MOG-reactive CD8+ T cells do epitope spread to some unknown peptide fraction of neuroproteins. This study, however, would require extensive testing and is not within the scope of this study. Therefore, we did not draw any definitive conclusions as to the involvement of CD8+ T cells in the epitope spreading phenomenon.

In Vitro Suppression Assays

Because of the regulatory function that we observed in our adoptive transfer models of disease, we wanted to measure the ability of the CD8+ T cells to counteract the disease mechanisms of the encephalitogenic CD4+ T cells. In one of our models, we transferred encephalitogenic CD4+ T cells along with autoreactive CD8+ T cells. We found that the CD8+ T cells were able to suppress the ability of CD4+ T cells to cause disease. This evidence lead us to hypothesize that autoreactive CD8+ T cells possess the capability to suppress the function of encephalitogenic CD4+ T cells.

In order to further characterize these cells, we also set up flow cytometry-based suppression assays to measure their ability to suppress MOG-specific CD4+ T cells. For this assay, we used CD4+ T cells from LNC and SpC of CD8 knockout mice immunized 10 days prior with MOG 35-55/CFA for responder cells. For suppressor cells, CD8+ T cells were purified from wild type B6 mice that had been immunized 20 days prior with either MOG 35-55 or OVA 323-339/CFA. Suppression was then measured by incubating $1x10^6$ responder cells with increasing amounts of suppressor cells in the presence of no antigen, pMOG 35-55, pOVA 323-339 (control peptide to evaluate specificity) or Con A for a positive control. The results of this assay showed that the CD8+ T cells from the MOG-immunized mice effectively suppressed the proliferation of CD4+ proliferation to pMOG 35-55 (Figure 19a). Furthermore, this suppression seemed to be antigen-specific, as the CD4+ T cells did not proliferate to the control antigen (an observation we had seen from previous proliferation assays). However, the results of this assay also suggested that CD8+ T cells from OVA-immunized mice could comparably suppress CD4+ T cell activation (Figure 19b). This result was also observed in repeat experiments. This phenomenon of apparent non-specific, global suppressability by CD8+ T cells is not in agreement with our results from other assays (proliferation assays and in vivo killing assays), which show a conclusive MOG-specific response. We therefore believe that the result that we are seeing is due to an inherent flaw in the assay set-up or possible contamination. Another possible explanation is the presence of regulatory CD4+ T cells in the responder population that, upon activation, may be causing suppression of neighboring CD4+ T cells. These hypotheses would both require further testing to determine the mechanism behind the suppression that we observed in these experiments. For the purposes of this work, however, the

results of this assay are inconclusive, and therefore don't contribute to any conclusions about the role of antigen-specific regulation by CD8+ T cells.

Chapter VI Figure Legends

Figure 16: Autoreactive CD8+ T cells Are Most Prevalent During Maintenance Phase of Disease. 8-week-old female B6 mice were immunized for active disease as described in the Materials and Methods sections. At the time points indicated, mice were sacrificed and LNC and SpC were harvested and pooled. CD8+ T cells were purified from bulk populations by magnetic sorting (purity > 90%), stained with CFSE and incubated in the presence of no antigen, pMOG 35-55 (40 μg/ml) or Con A (2 μg/ml) at a concentration of $1x10^6$ cells/ml. Irradiated CD8-depleted bulk cells from the same mouse were used as APCs (APC:T cell ratio = 5:1). Following a 5 day incubation period, cells were stained with anti-CD8 antibodies and fixed in paraformaldehyde. Proliferation was then measured by flow cytometry. The data shown is representative of 3 independent experiments.

Figure 17: MOG-stimulated CD8+ T Cells Produce IFN-γ and IL-4. CD8+ T cells were purified by negative selection from splenocytes of mice that were immunized according to our active disease protocol (purity > 90%). These cells were cultured with irradiated, CD8-depleted splenocytes from an OVA-immunized mouse (5:1 APC:T cell ratio) and the antigen indicated. Supernatants were collected at 48, 72 and 96 hours of culture and frozen. For ELISAs, supernatants from 72 hours of culture were thawed and incubated on precoated plates. Plates were then read and samples were compared to optical density standard curves from manufacturer for the presence of IFN-γ (A) or IL-4 (B).

Figure 18: MOG-specific CD8+ T Cell Response Does Not Spread to Different Epitopes. SpC from MOG-immunized mice were harvested 25 days post-immunization and CD8+ T cells were purified by negative selection using magnetic beads (purity > 90%). Both groups of cells (bulk SpC and CD8+ cells) were stained with CFSE and incubated with the antigens indicated at $1x10^6$ cells/ml. Irradiated, CD8-depleted SpC from MOG-immunized mice were used as APCs (T cell:APC ratio = 1:5). Following five days of incubation, cells were stained with anti-CD4 and anti-CD8 fluorescent antibodies, fixed in paraformaldehyde and analyzed for proliferation by flow cytometry. * indicates significant proliferation as established in our previous flow cytometry assays.

Figure 19: CD8+ T cells Suppress in a Non-Antigen-Specific Manner. SpC and LNC were harvested from CD8 knockout mice that had been immunized with MOG 35-55/CFA 15 days prior and used as responder cells. CD8+ T cells were purified from SpC of mice that had been immunized with MOG 35-55/CFA (A)or OVA 323-339/CFA (B) 30 days prior were used as suppressor cells. Responder cells were stained with CFSE and mixed with suppressors at the ratios indicated. Mixtures were then incubated for five days in the presence of the indicated antigens. Suppression was then measured by change in proliferation as seen by flow cytometry. Data shown is representative of three independent experiments.

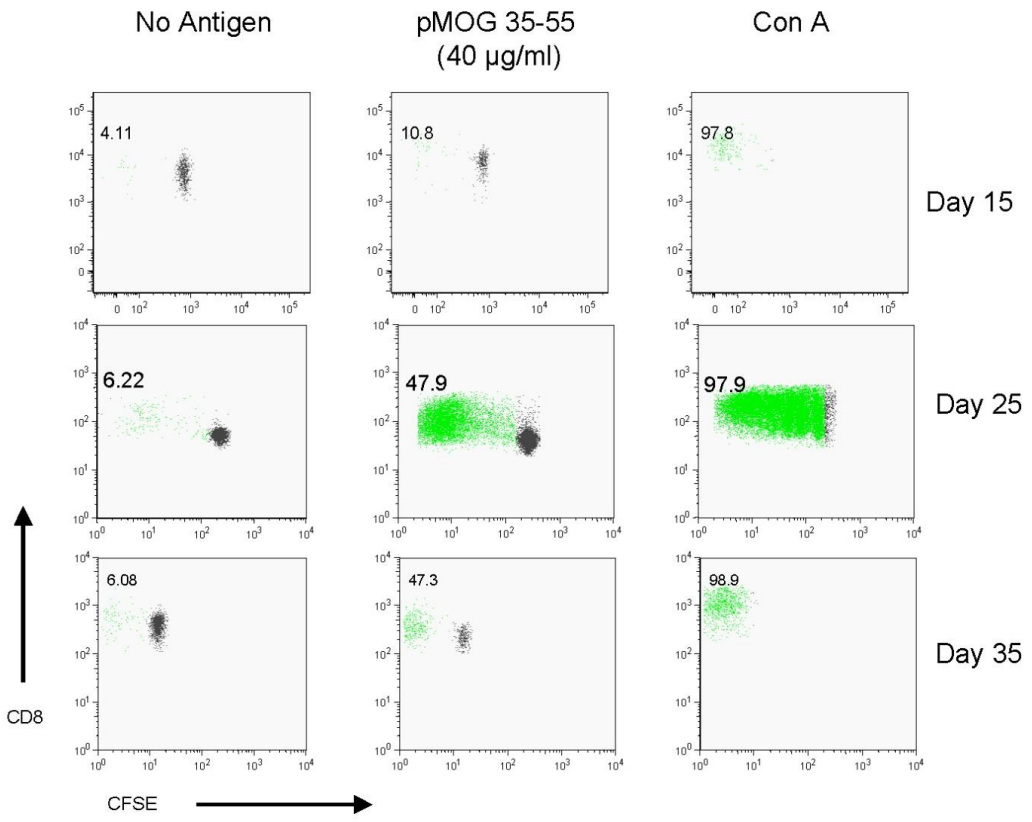
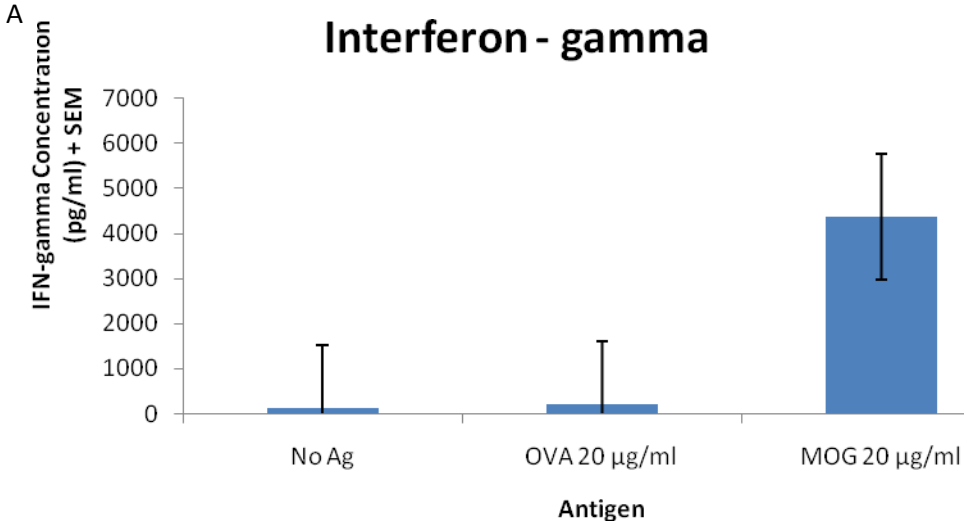


Figure 16: Autoreactive CD8+ T cells Are Most Prevalent During Maintenance Phase of Disease.

A



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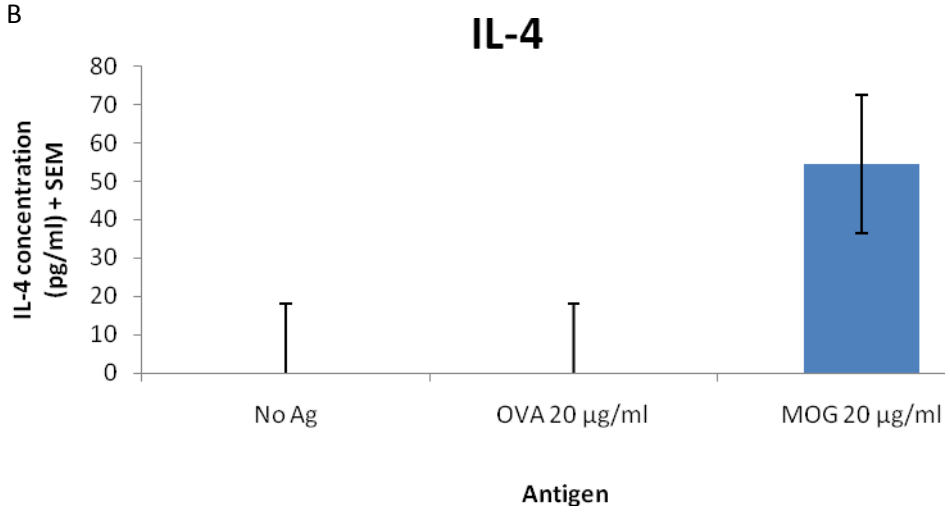


Figure 17: MOG-stimulated CD8+ T Cells Produce IFN-γ and IL-4.

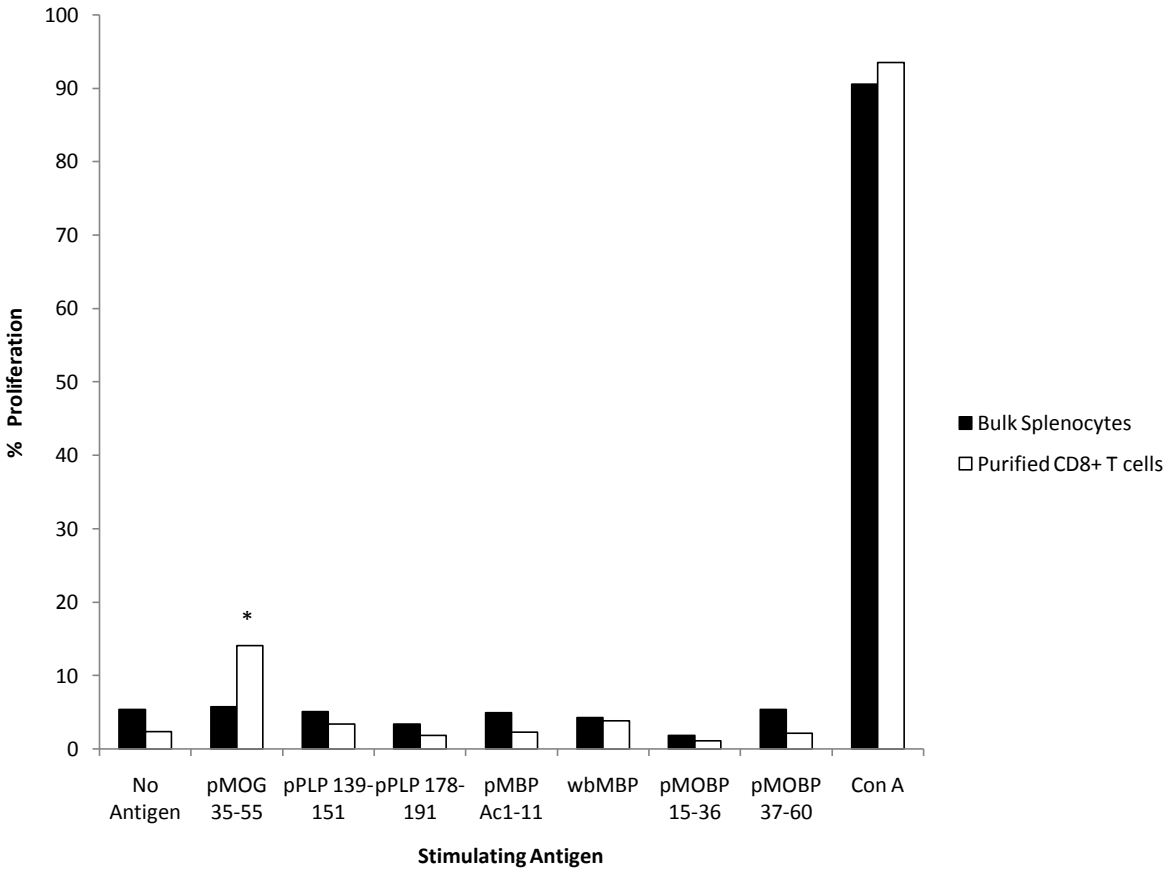


Figure 18: MOG-specific CD8+ T Cell Response Does Not Spread to Different Epitopes.

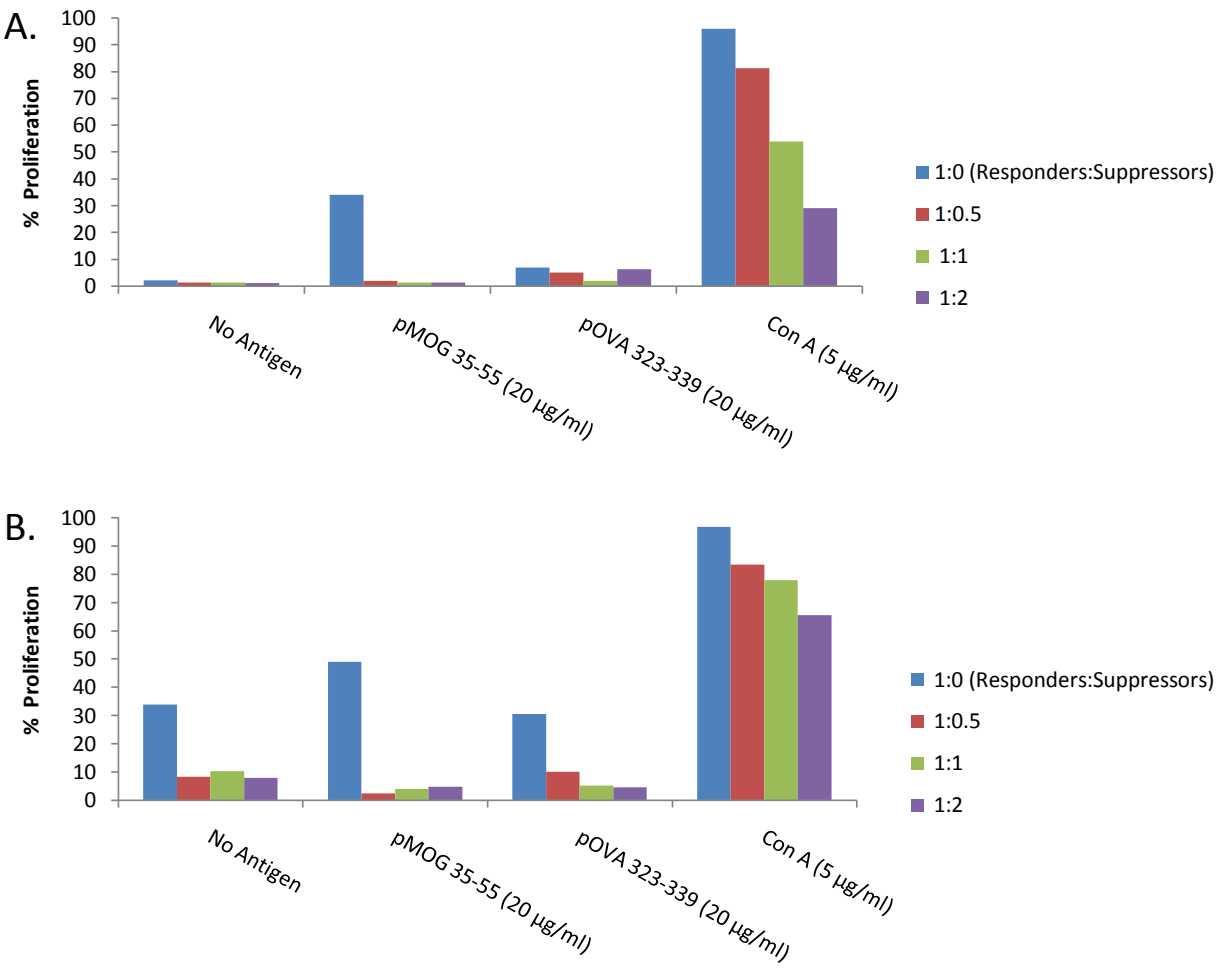


Figure 19: CD8+ T cells Suppress in a Non-Antigen-Specific Manner.

Chapter VII: Mechanism of CD8+ T Cell Regulation - Influence on Antigen Presenting Cells

Our observations from the adoptive transfer studies also indicated that CD8+ T cells may have a possible regulatory effect on APCs. We observed that when MOG-specific CD8+ T cells were injected before the mice were immunized to induce active disease, the disease was ameliorated. One hypothesis of this observation is that the CD8+ T cells were able to somehow disrupt the ability of the APCs to activate encephalitogenic CD4+ T cells. There is evidence of CD8+ T cells having this ability in other disease models (134). Specifically, Najafian, et.al., showed that CD8+CD28- T cells were able to suppress the ability of APCs to induce activation and cytokine production by CD4+ T cells by suppressing the expression of costimulatory molecules. These cells, however, were not shown to act in an antigen-specific manner. We hypothesized that the autoreactive, antigen-specific CD8+ T cells that we found in our experiments could function in a similar manner and that could offer insight into their ability to ameliorate actively induced disease.

In order to investigate the mechanism whereby antigen-specific CD8+ T cells could ameliorate actively induced disease (as we saw in our adoptive transfer experiments), we repeated the assays described above in which CD8+ T cells were injected one day prior to active disease induction. 15 days following immunization, mice were sacrificed and cells from the lymph nodes and spleen were pooled. We also pooled bulk cells from disease control mice. CD4+ T cells were purified from these pooled cells by positive selection through a magnetic column. These cells were then stained with CFSE. The CD4-depleted subsets were stained with a second dye, CMTPX. This is another intracellular dye that binds to intracellular proteins and can be used to track populations, but unlike CFSE, it does not appear to dilute with serial

proliferation (personal observations). We therefore can use it in our assay to flow cytometrically gate out unwanted populations when analyzing proliferation. We then set up our assay so that each group of CD4+ T cells (disease control, mice injected with CD8+ T cells from OVA-immunized mice and mice injected with CD8+ T cells from MOG-immunized mice) was incubated with each group of CD4-depleted APCs. Then, using CD4+ T cells and CD4-depleted APCs from disease controls as our reference, we measured any changes in proliferation, either in the T cells themselves, or caused by APCs from one of the test groups. We found that CD4+ T cells from all groups proliferated significantly in the presence of APCs from control mice. However, we did note that when CD4+ T cells from disease controls were incubated with APCs from the mice that received the CD8+ T cells from the MOG-immunized mice, they did not proliferate in response to MOG stimulation (Figure 20). In fact, proliferation was even hindered in response to Con A as well. These results suggest that the APCs (CD4-depleted SpCs) from the mice that received the MOG-specific CD8+ T cells were somehow rendered unable to stimulate proliferation. The mechanism that underlies this regulation has yet to be determined and will require further study.

In the same study, we sought to determine if the CD8+ T cells had an effect on the cytokines secreted by APCs during active disease induction. CD4-depleted SpCs from each group (disease control, mice that received CD8+ T cells from OVA-immunized mice and mice that received CD8+ T cells from MOG-immunized mice) were incubated in 48-well plates in the presence of no antigen, lipopolysaccharide (a TLR4 stimulator), or one of two concentrations of interferon-γ (IFN-γ, 10 units or 100 units). Supernatants from these cultures were taken at 48, 72, and 120 hours and frozen down. For cytokine detection, supernatants from 72 hours were thawed and ELISA assays were performed to detect those outlined in the figure 21. As seen in

the figure, it appears that the APCs from the protected mice produce higher levels of IL-10 and TNF-α in response to LPS. This difference does not appear when the APCs are incubated with IFN-γ. These results suggest that the influence of the autoreactive CD8+ T cells on the APC population may affect its cytokine profile. It is therefore possible that these CD8+ T cell affect the APCs ability to stimulate CD4+ T cell proliferation both through costimulatory molecule expression as well as alteration of cytokine production. These hypotheses require further study to determine the exact mechanism. There are possible explanations as to why there is no difference in cytokine expression when the APCs are exposed to IFN-γ. Perhaps the concentration of IFN-γ was insufficient to activate the APCs. Another possibility is that this pathway is disrupted by the CD8+ T cells, but not the TLR4 pathway. Further studies would need to be conducted in order to determine the reasoning behind this observation.

Chapter VII Figure Legends

Figure 20: CD8+ T Cells Negatively Influence APC Ability to Stimulate CD4+ T Cells. CD4+ T cells and APCs (CD4-depleted, irradiated SpC) were obtained from disease control mice, diseased mice that had been injected with MOG-reactive CD8+ T cells or diseased mice that had been injected with OVA-reactive T cells. CFSE-stained CD4+ T cells and APCs from different groups were mixed as indicated and cultured in the presence of indicated antigens. Following five days of culture, CD4+ T cells were fluorescently labeled and proliferation was measured by flow cytometry. Data shown is representative of two independent experiments. * indicates significant decrease in proliferation, as determined by $\Delta SI > 2$.

Figure 21: CD8+ Ts Cells Alter Cytokine Expression by APCs. Splenocytes were harvested from mice 15 days post immunization from our protection adoptive transfer experiment (see figure 13). CD4-depleted splenocytes were cultured in 48-well plates at a concentration of $1x10^6$ cells/ml/well in the presence of the antigen indicated. Supernatants from these cultures were collected at 48, 72 and 96 hours of culture. These supernatants were then used in ELISA assays to observe production of IL-10 (A), IL-12 (B) or TNF-α (C). Concentration of sample was determined by comparing the optical density of the sample to that of a standard curve. "Disease Control" groups were immunized mice that had not received any CD8+ T cells prior to immunization. "Protected" APCs are from MOG-immunized mice that received activated MOG-specific CD8+ T cells one day prior to immunization. "Peptide

Control" APCs are from immunized mice that received activated OVA-specific CD8+ T cells one day prior to immunization.

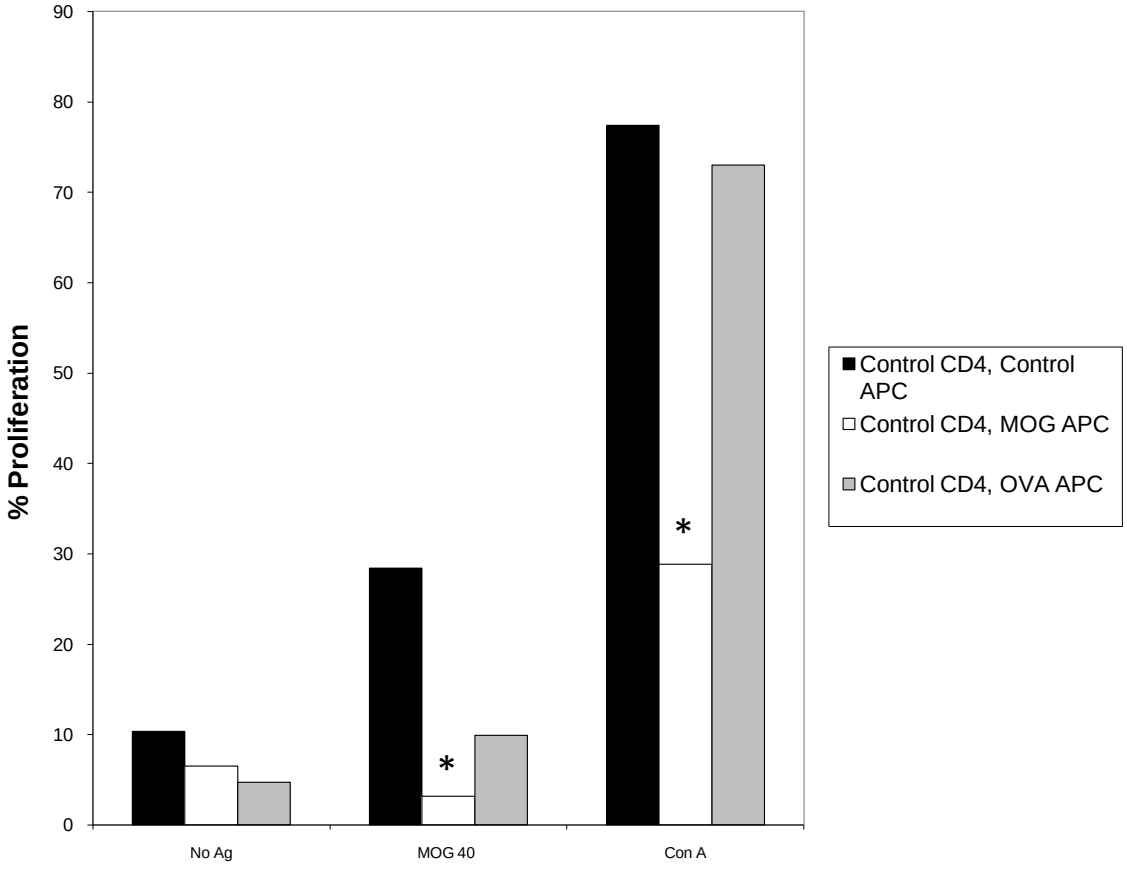


Figure 20: CD8+ T Cells Negatively Influence APC Ability to Stimulate CD4+ T Cells.

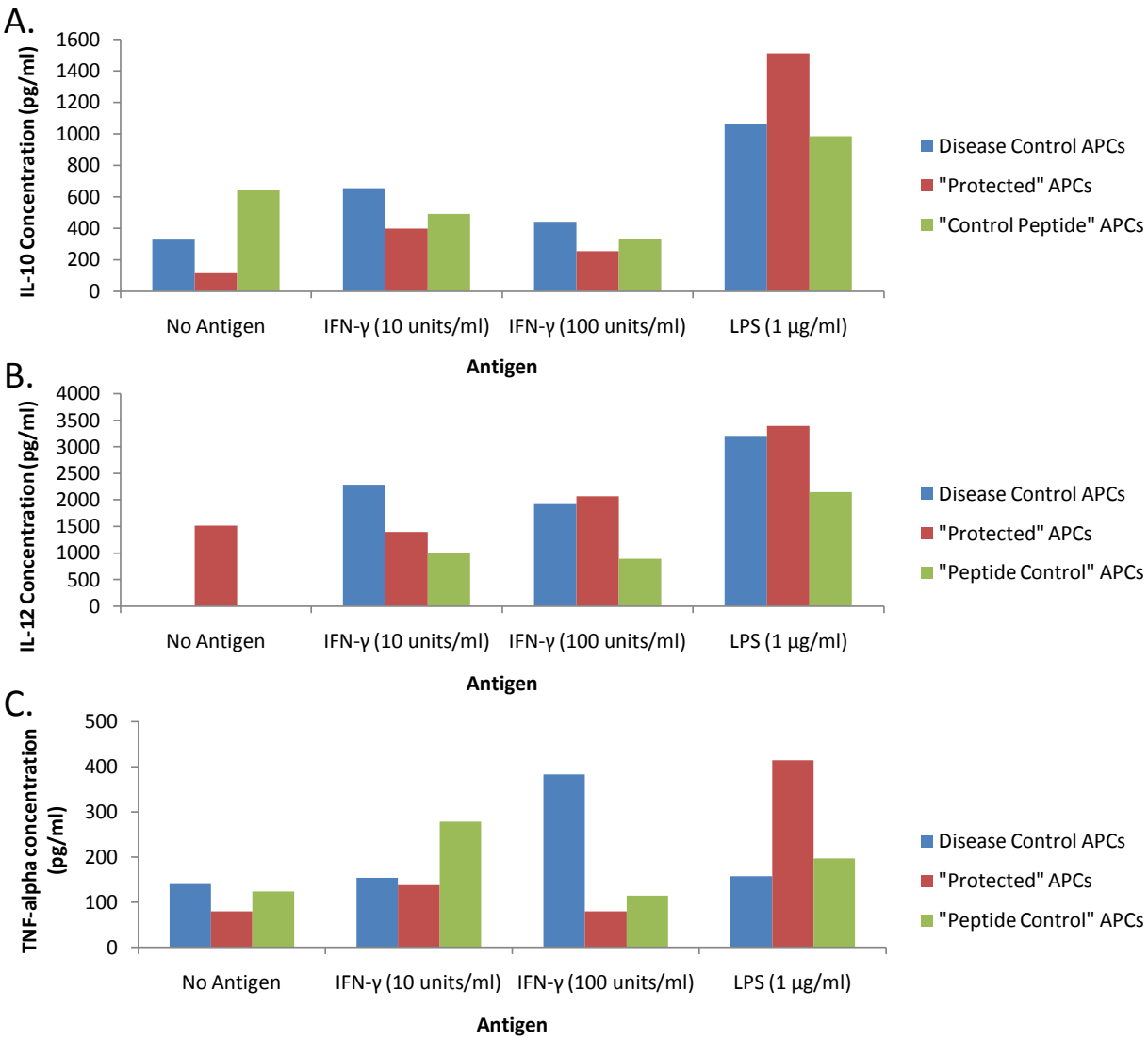


Figure 21: CD8+ Ts Cells Alter Cytokine Expression by APCs.

Chapter VIII: Mechanism of CD8+ T Cell Regulation – *in vivo* Killing Assays

Another mechanism that we hypothesized might be involved in the regulation of disease by CD8+ T cells is that of antigen-specific killing. It is widely known that CD8+ T cells use this mechanism in defense of both viral infection as well as altered-self tumor cells. In autoimmunity, our lab has shown that CD8+ T cells play a mechanistic role in the therapeutic effect of glatiramer acetate, one of the drugs used to treat MS. In *in vitro* studies, these cells showed the ability to kill drug-loaded target cells in an antigen specific manner. We hypothesized that CD8+ T cells in EAE may have the same ability.

In order to study the killing ability of these cells, we developed a novel killing assay using CFSE along with another intracellular dye, CMTPX. This assay was based on the FATAL assay developed by Sheehy and colleagues (150). In short, the assay determines the killing ability of CD8+ T cells by injecting stained target populations into immunized mice. By loading target cells with different antigens, we can conclude that killing is antigen-specific. This assay also gives us the ability to determine the killing in an in vivo environment, which allows us to more accurately observe mechanism in the biological setting.

In our first group of experiments, we sought to determine if we could detect different stained populations of cells. We pooled LNC and SpC from a mouse and stained them with four different concentrations of CFSE (0.25 μM, 0.125 μM, 0.05 μM and 0.0125 μM). An aliquot of each concentration was then mixed together. All of the individual populations, as well as the mixed population, were incubated overnight to simulate antigen loading. Cells were then fixed and analyzed by flow cytometry, each population individually and also the mixed population, as well as a non-stained control. Analysis of the populations revealed that each of the populations

could be detected individually. When combined, the cells with the two highest concentrations of CFSE were distinguishable, but the cells with the two lowest concentrations of CFSE blended together, making them distinguishable from the other populations, but not distinguishable from each other. We therefore proceeded to future experiments using three different concentrations of CFSE to use as target populations.

Our next goal was find if these populations could be recovered after being injected into a mouse. To this end, mouse splenocytes were stained with three different concentrations of CFSE (0.25 μM, 0.125 μM and 0.025 μM). An aliquot of cells was set aside and incubated for two days, after which it was fixed for analysis by flow cytometry. The remaining cells were mixed at a 1:1:1 ratio and injected intravenously into a naïve mouse. 2 days later, the mouse was sacrificed and SpC were harvested and fixed in paraformaldehyde. Cells were then analyzed by flow cytometry to see if the target populations could be detected and compared to the populations that were not injected. Analysis revealed three different recoverable populations from the in vitro culture of the cells. The mixed populations from the in vitro culture, however, failed to show the cells stained with the lowest concentration of CFSE. Since the population was detectable individually, we concluded that there must have been an error in transferring the cells into the mixed culture. Analysis also revealed the ability to recover the populations with the two highest concentrations of CFSE from the mouse (Figure 22a). The population with the lowest concentration of CFSE blended into the background of the unstained splenocytes and was unrecoverable. From the results of this testing, we determined that the 0.0125μM CFSE concentration was too low to use in this assay.

We also tested a second intracellular dye, CMTPX, to determine if we could combine the dyes and recover additional populations. Naïve SpC were used as target cells. They were

stained with either 0.25 μM CFSE ± CMTPX or 0.05 μM CFSE ± CMTPX. Cells were then mixed in equal ratios and injected into MOG-immunized mice. Two days later, the SpC were harvested from the injected mice and recovery was assessed. As was seen with CFSE alone, we were able to distinguish the different concentrations of CFSE, but now we were also able to detect the cells that were costained with CMTPX (Figure 22b). The results of this test allowed us to establish a protocol for recovering four different populations from an injected mouse. It also assured us that, when naïve target cells are injected into immunized mice, the populations all traffic through the spleen and are recoverable without having to harvest additional organs..

Now that we had our parameters set for detectable, reproducible results, we sought to discover the in vivo killing capacity of cells in pMOG 35-55-immunized mice. We harvested splenocytes from naïve B6 mice and set up two different antigen-loading conditions: pMOG 35-55 and pOVA 323-339 (control for antigen specificity). In our laboratory, a similar in vitro killing assay had been developed for use in studying MS. For this assay, the cells showed increased killing when the targets were incubated in the presence of Con A. For this reason, we decided to repeat our in vivo setup, but this time we included Con A (10 μg/ml) during the target loading incubation period. Following incubation, cells from the MOG and OVA cultures were mixed in a 1:1 ratio. The mixture was then injected via tail vein into MOG-immunized (day 40 post-immunization) or naive mice (Figure 22a). The results of this experiment confirmed that MOG-loaded target cells are specifically killed in a MOG-immunized mouse. We also observed that killing is increased when the cells are incubated with Con A. This suggests that activated cells are killed more efficiently than naïve target cells. This may be an important clue into the mechanism of killing. In a confirmatory experiment, we repeated the assay using mice immunized with PBS/CFA as controls, instead of naïve mice. This control would confirm for us

that the loss of target cells that we were observing was indeed by antigen-specific killing and not caused by general inflammation due to the presence of an adjuvant. The results of this assay (Figure 22b) confirmed that the loss of target cells that we observed was, in fact, due to antigen-specific killing. In a third set of control experiments, we injected MOG- and OVA-loaded target cells into OVA-immunized mice to ensure that the loss of MOG-loaded target cells was not a matter of alternative trafficking. We were able to recover both MOG-loaded and OVA-loaded target cells from the spleens of the OVA-immunized mice (data not shown). This confirmed that target cells loaded with the immunizing antigen were not alternatively trafficking to other organs and causing the appearance of killing.

Now that we had established that MOG-immunized mice could specifically kill MOG-loaded target cells, we decided to confirm that this killing was effected by CD8+ T cells. To answer this question, we decided to perform this *in vivo* killing assay using immunized CD8 knockout mice for the killer cells. If MOG-immunized CD8 knockout mice failed to kill MOG-loaded target cells, then we could conclude that killing may be CD8+ T cell mediated. Naïve SpC were harvested and then stained with one of the following combinations of intracellular dye: 0.05 μM CFSE, 0.25 μM CFSE, 0.05 μM CFSE + CMTPX, 0.25 μM CFSE + CMTPX. CMTPX is an intracellular dye that is used to track cells in a manner similar to CFSE. For the purposes of this assay, the addition of the second dye allowed us to study four populations within a mouse instead of just two populations. This availability allowed us to control for the different CFSE concentrations in the mice (in the event that the CFSE showed to be cytotoxic at a certain concentration). It also gave us another parameter by which we could measure percent killing in the given mouse. All of the groups were then loaded with either MOG 35-55 or OVA 323-339 in the presence of Con A. Following loading, all of the groups were mixed in a 1:1:1:1 ratio and

injected into either MOG-immunized or PBS/CFA immunized mice that were either wild type or CD8 knockout. 2 days later, SpC were harvested and killing was analyzed by measuring recovery of populations by flow cytometry. In this assay, we showed that while MOG-loaded target cells are killed in an antigen-specific manner in MOG-immunized wild type mice, this killing is significantly diminished in the CD8 knockout setting (Figure 23). This evidence suggests that the majority of the killing effected in the wild type mice is through CD8+ T cells. It is reasonable to believe that the small amounts of killing seen in the knockout setting may be due to "leaky" production of CD8+ T cells or by NK cells reacting to minor histocompatibility differences between the target cells and the host, although this killing would be expected to be seen in an antigen-nonspecific manner since NK cells are part of the innate immune system and do not recognize specific antigens. It is also possible that this observed killing is due to CD4+ T cells that express killing function (151). The exact mechanism of this residual killing remains to be determined.

Another question that we sought to answer concerned which specific subset(s) of target cells, if any, were the target of the antigen-specific killing. If there was a specific subset of cells that was preferentially targeted by the CD8+ T cells, then that may give us a hint as to the mechanism of regulation that we had observed. We decided to broadly characterize subsets into CD4+ T cells and APCs (defined for our purposes as CD4-depleted splenocytes). Naïve splenocytes were harvested and CD4+ T cells were purified by positive selection through a magnetic column. Both subsets were then stained with one of the following combinations of intracellular dye: 0.05 μM CFSE, 0.25 μM CFSE, 0.05 μM CFSE + CMTPX, 0.25 μM CFSE + CMTPX. All of the groups were then loaded with either MOG 35-55 or OVA 323-339 in the presence of Con A. Following loading, all of the groups were mixed in a 1:1:1:1 ratio and

injected into either MOG-immunized or PBS/CFA immunized mice. 2 days later, population recovery was analyzed by flow cytometry and percent killing was determined. The results of this assay showed conclusively that the CD4-depleted subset (APCs) are targeted for destruction in the MOG-immunized mouse when they are loaded with MOG 35-55 (Figure 24a). The CD4+ population also showed likely killing as well, although the low cell numbers make conclusions difficult. In order to resolve this issue, we repeated the assay, but this time the cells were stained and loaded in bulk cultures, then separated following the overnight loading process. This procedure increased the numbers of CD4+ T cells available to inject into the mice. The results of this assay conclude that CD4+ T cells are also killed in an antigen-specific manner when loaded with pMOG 35-55, although not to the extent that we see in the APC subset (Figure 24 a, b). From these results we can conclude that both CD4+ T cells, as well as CD4-depleted cells, can be targeted for destruction by the autoreactive CD8+ T cells. This suggests that the presentation of the antigen to the CD8+ T cell must be effected by an antigen found on the surface of activated CD4+ T cells, as well as APCs. However, the exact mechanism of killing still remains to be determined.

Chapter VIII Figure Legends

Figure 22: Recovery of Stained Populations from MOG-Immunized Mice. A. Cells were stained with two different concentrations of CFSE (0.25 μM, 0.125 μM), mixed in equal ratios and injected via tail vein into MOG-immunized mice. Two days later, the SpC from the mice were harvested and population recovery was analyzed by flow cytometry. B. SpC were stained with the one of the two highest CFSE concentrations listed above and also ± CMTPX. All four stained populations were then injected into MOG-immunized mice and recovery was assessed two days later as described in part A.

Figure 23: MOG-Immunized Mice Kill MOG-Loaded Target Cells in an Antigen-Specific Manner. A. Splenocytes were harvested from naïve, wild-type B6 mice. They were then stained with one of two concentrations (HI or LO) of CFSE. The cells that were stained with the HI concentration were incubated overnight in the presence of MOG35-55 antigen and ConA. The cells that received the LO concentration were incubated for the same time period with OVA323-339 antigen and ConA. Following incubation, cells were ficolled and mixed together in a 1:1 ratio. They were then injected via tail vein into naïve mice or mice that were 25 days post-immunization with MOG35-55/CFA. 2 days following tail vein injection, the mice were sacrificed and the splenocytes were harvested. The cells were then analyzed by flow cytometry. Percent killing was calculated to the normalized ratio found in the naïve control mice. This data

is representative of 6 different experiments. B. Graphic representation of normalized killing seen using PBS/CFA-immunized mice for controls.

Figure 24: Specific Killing of MOG-Loaded Target Cells is CD8+ T Cell Dependent. Splenocytes from naïve B6 mice were harvested. and stained with CFSE (± CMTPX) as described. Cells were then incubated overnight with either MOG35-55 or OVA323-339 antigen and ConA. Following incubation, the cells were mixed into a single suspension in a 1:1:1:1 ratio (CFSE Hi, CMTPX+:CFSE Lo, CMTPX+:CFSE Hi, CMTPX-:CFSE Lo, CMTPX-). This suspension was injected via tail vein into mice that had been immunized with MOG35-55/CFA or PBS/CFA 25 days prior. 2 days later, the mice were sacrificed and the splenocytes were isolated. Killing was then determined by flow cytometric evaluation. This data is representative of 2 independent experiments.

Figure 25: CD4+ and CD4-Depleted Cells are Targeted for Destruction by CD8+ T Cells. Splenocytes from naïve B6 mice were harvested. CD4+ cells were isolated by magnetic sorting and both the CD4+ and CD4-depleted subsets were stained with CFSE as described above. Each CFSE dilution was then divided into two groups and one of the groups was further stained with CMTPX. Cells were then incubated overnight with either MOG35-55 or OVA323-339 antigen and ConA. Following incubation, the cells were mixed into a single suspension in a 1:1:1:1 ratio (CFSE Hi, CMTPX+:CFSE Lo, CMTPX+:CFSE Hi, CMTPX-:CFSE Lo, CMTPX-). This suspension was injected via tail vein into mice that had been immunized with MOG35-55/CFA or PBS/CFA 25 days prior. 2 days later, the mice were sacrificed and the splenocytes were

isolated. Killing was then determined by flow cytometric evaluation. This data is representative of 2 independent experiments. B. Graphic representation of normalized % killing.

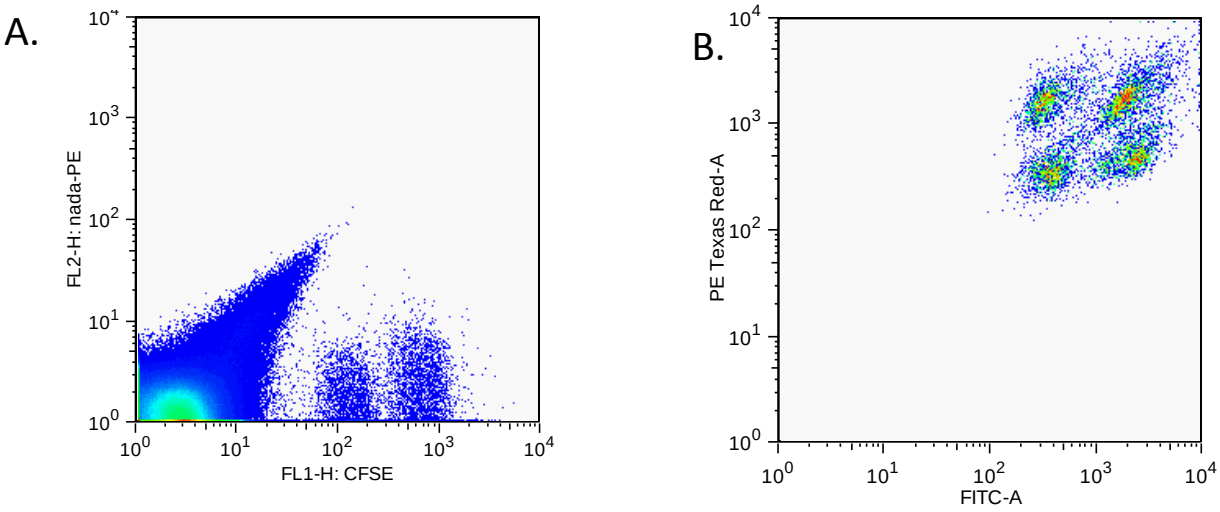
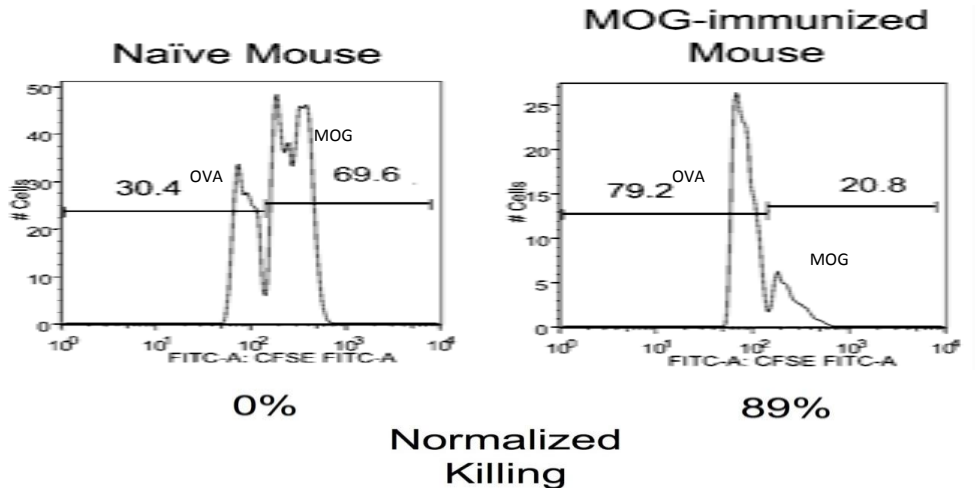


Figure 22: Recovery of Stained Populations from MOG-Immunized Mice.

A.



B.

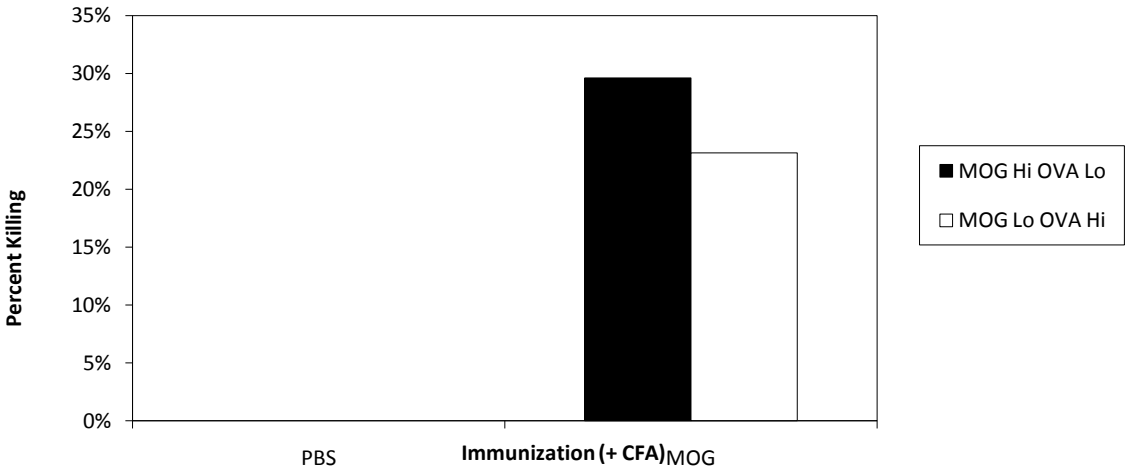


Figure 23: MOG-Immunized Mice Kill MOG-Loaded Target Cells in an Antigen-Specific Manner.

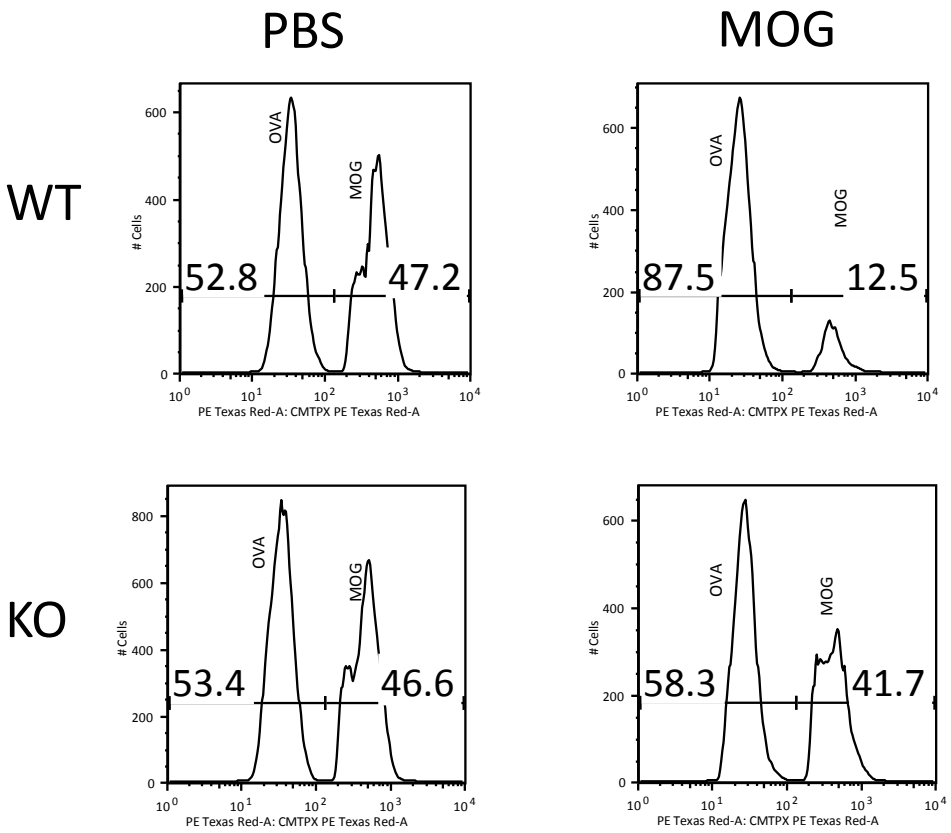
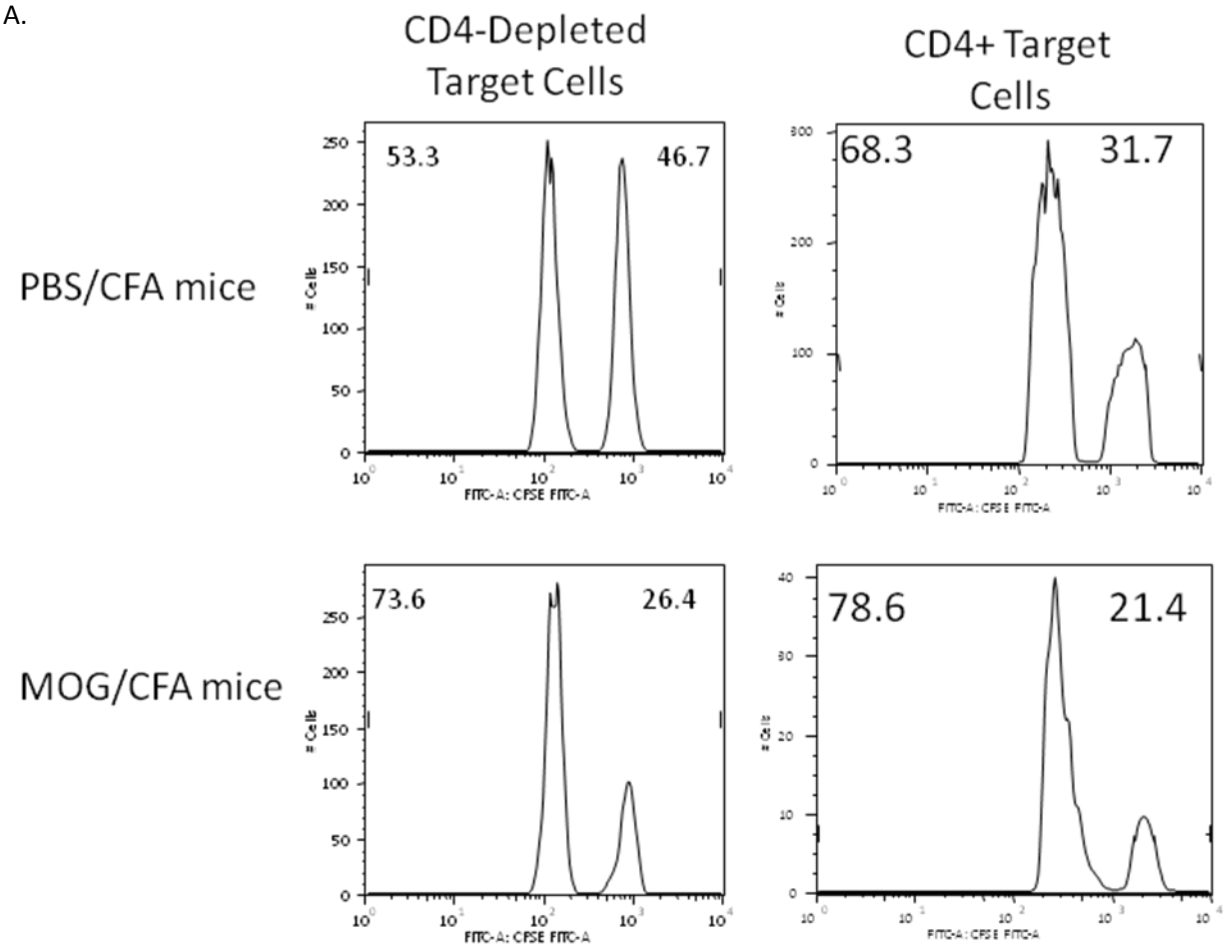
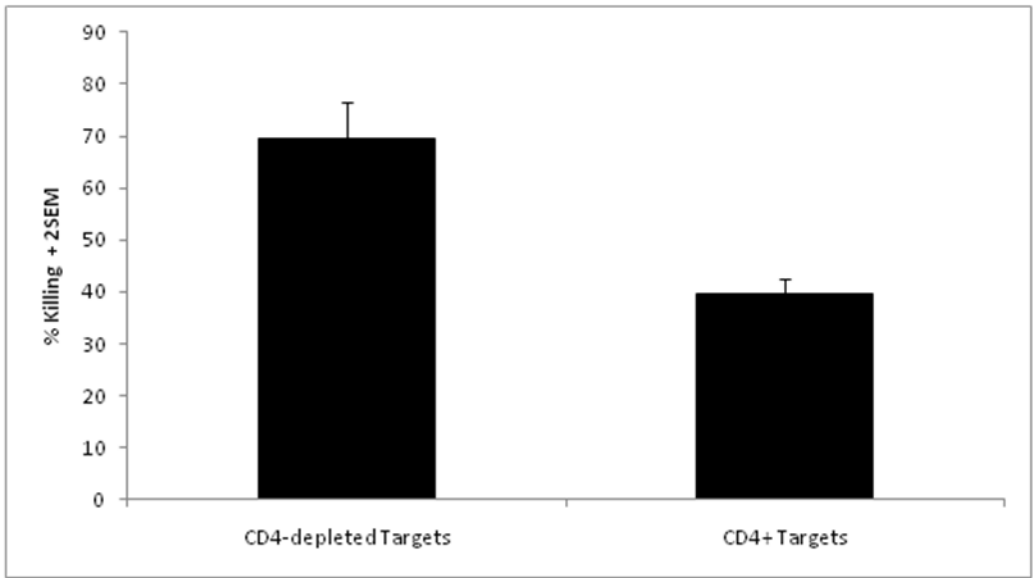


Figure 24: Specific Killing of MOG-Loaded Target Cells is CD8+ T Cell Dependent.

A.


CD4-Depleted Target Cells
CD4+ Target Cells
PBS/CFA mice
MOG/CFA mice
53.3
46.7
68.3
31.7
73.6
26.4
78.6
21.4
Cells
FITC-A: CFSE FITC-A

B.


% Killing + 2SEM
CD4-depleted Targets
CD4+ Targets

Chapter IX: Discussion

The role of CD8+ T cells in autoimmune demyelination processes, such as those seen in MS and EAE, has been both understudied and controversial. There have been multiple evidences produced for the pathogenic, as well as the regulatory, role of these cells within the context of disease. Because of the many different models of EAE that have been characterized, it is possible that multiple roles exist for this subset of lymphocytes. In our studies, we have characterized the role of CD8+ T cells in an EAE model in which evidence has suggested a pathogenic role for CD8+ T cells. We were able to show that, in this model, there exists a subset of CD8+ T cells that are both antigen-specific and autoreactive. However, our observations indicate that the role for these CD8+ T cells is not a pathogenic one, but rather a regulatory role. In several different adoptive transfer settings, we have shown that these cells are capable of ameliorating disease at different time points along the disease course, or prevent disease altogether. We have also shown that these cells possess the capacity to destroy target cells that are loaded with the immunizing antigen, and that this killing is mediated by CD8+ T cells. Observations in these adoptive transfer settings also indicate that these cells possess the ability to affect both APCs and antigen-specific CD4+ T cells in a non-lethal manner that prevents them from leading to proliferation of encephalitogenic T helper cells.

Immunological Tolerance

Because of the random nature through which our adaptive immune system develops its specificity, it is inevitable that there should develop T and B lymphocytes that will recognize and react to self proteins. If left unchecked, a full-fledged immune response against these self-antigens would develop, causing inflammatory damage to host tissues of every kind. In order to

prevent this, the body has developed tolerance mechanisms that eliminate and/or inhibit these autoreactive cells.

Historically, the most studied tolerance mechanisms are those that take place in the thymus, known as central tolerance mechanisms. Within this organ, immature T cells must pass a series of requirements before they are allowed to mature into functioning T cells and pass into the periphery. These requirements test the binding strength of the interaction between T cell receptors on the T cells and host MHC molecules loaded with a wide variety of self proteins. If the signal is too weak, the T cells don't receive proper survival signals and die by neglect. If the signal is too strong, the T cells are deleted. The end result is a surviving population of T cells that recognize self MHC molecules, without binding so strongly that they evoke a response to a self-antigen (152, 153).

However, it has been observed that, despite this stringent testing, there are autoreactive T cells found in the blood of all healthy individuals (31). This observation implies that there must be "peripheral tolerance" mechanisms in place as well to regulate these cells. There are many different kinds of peripheral mechanisms that have been described. Among them are sites with immunological privilege, which do not allow normal immune surveillance, clonal anergy mechanisms that shut autoreactive lymphocytes off through incomplete signaling interactions and clonal deletion (154, 155). One of the more recent tolerance mechanisms to be described is the presence of "regulatory" T cells (Tregs) that are able to suppress activation of other T cells, both specifically and non-specifically.

Most of the focus on these regulatory T cells has centered around specific Tregs of the CD4+ lineage (156). These cells have a characterized phenotype (CD4+CD25+) and have been

shown to suppress immune responses through the secretion of regulatory cytokines such as IL-10 and TGF-β.

CD8+ "Suppressor" T Cells

Despite the majority of Treg research today involving CD4+CD25+ Tregs, the concept of peripheral T cells being able to regulate the immune response actually originated in the CD8+ T cell subset. Gershon and colleagues first demonstrated the ability of T cells to transfer tolerance from one animal to another (114). Later, antigen-specific Ts cells were isolated in *in* vitro cultures (157) and that these cells were Lyt2+ (CD8+) (115). However, due to the inability to phenotype a specific suppressor CD8+ T cell population, research in this area came to a halt.

With modern technology and new techniques, however, interest in this field has been rekindled. There have now been several reports of the presence of specific regulatory CD8+ T cell populations (Ts cells) in both humans and animals. In characterizing these cells, many different phenotypes have been described, making this regulatory cell subset more diverse than the CD4+CD25+ Tregs that have been widely studied.

The Role of CD8+ T Cells in Autoimmune Demyelination

We have used modern technologies in our laboratory to dissect the role of the CD8+ T cell in the EAE setting. One of these technologies is flow cytometry. In earlier studies, T cell proliferation was measured in bulk proliferation assays with the assumption that the proliferation being seen was due to CD4+ T cells, an assumption founded on the observations made in adoptive transfer and knockout settings. These assumptions, however, could not account for the different populations in the bulk cultures and could therefore not appropriately answer the question of responding cell types. Flow cytometry has given us the power to observe the

different cell types that are causing the increased proliferation to autoantigens that we see in the EAE setting. As expected, we observed an increase in CD4+ T cell proliferation in an antigen-specific fashion. We also observed, however, that the CD8+ T cells were proliferating in an antigen-specific fashion. This supported previously published results that CD8+ T cells played a role in the neuroantigen response (127, 130).

The other advantage of the flow cytometry based assay is the ability to observe the total response of the CD8+ T cells over a given period. In the standard thymidine proliferation assays, the responding cells are pulsed with radioactive nucleotides for a relatively short amount of time (18-20 hours) before being observed for proliferation. The CFSE assay, however, allows us to observe proliferation over an extended period and therefore gains a clearer picture into the extent of autoreactivity of the CD8+ T cells.

We have shown that, in our model, CD8+ Ts cells seem to become activated around the time of peak disease, and do not seem to be present in the very early stages of disease. This correlates with results in other labs that indicate that they arise in later stages of disease course.

We also sought to determine if CD8+ T cells participate in the epitope spreading phenomenon in the same way as autoreactive CD4+ T cells (70, 71). This phenomenon was not observed in CD8+ cells or CD4+ cells in the B6 model of EAE that we have established in our laboratory. Preliminary studies did not reveal that it existed in other models either. However, we cannot conclude that other models do not display this phenomenon in the CD8+ T cell subset because we did not do confirmatory tests. We also can only conclude that the T cells in the B6 model did not spread to the antigens that we tested. This does not conclusively show that epitope spreading does not exist in this model. It is very conceivable that they spread to an entirely

different set of epitopes. The epitopes that we used were all derived in CD4+ T cell-driven models and have been shown to be presented on class II MHC. We tested these epitopes with the hypothesis that they would behave in a manner similar to MOG (with the ability to be presented to both class I and II MHC molecules). This assumption, however, has not been proven and may be the reason why we did not see epitope spreading in our experiments. Further studies must be undertaken to find true class I-binding epitopes and test them in the system. This pursuit is outside the scope of the current study and was therefore not undertaken.

Because of previously published results in the B6/MOG model of EAE (127, 128), we decided to first look at the possible pathogenic role of CD8+ T cells. Despite many attempts to induce disease with CD8+ T cells derived from a MOG-immunized mouse, we were unable to do so. There are many reasons as to why this may have been the case. Although we made every effort to follow the published protocols, there are variabilities in culture conditions from laboratory to laboratory that make it impossible to make an exact replication of an experiment. One must also take into account subtle differences in peptide production used for immunization and intra-strain differences of mice. These are differences that we noticed merely through our efforts to establish a mouse colony with reproducible active disease. It is plausible that they also may have been a reason why we could not adoptively transfer encephalitogenic CD8+ T cells.

We next decided to try a different approach to observe the pathogenic effects of these cells. Since we could not establish disease by merely adding CD8+ T cells, we hypothesized that we could augment actively induced disease, and thus prove a pathogenic effect. Our observations, however, were again contrary to the aforementioned studies. We observed that adoptive transfer of these CD8+ T cells delayed disease onset, decreased disease severity and promoted faster recovery. This observation suggested that CD8+ T cells play a regulatory role in

disease. This idea has been reported in other models (130, 131, 158). This is, we believe, the first evidence of an antigen-specific CD8+ T cell acting in a regulatory role in this model of EAE. We also showed that these cells are able to suppress active disease in immunized mice. Our analyses indicated that MOG-specific CD8+ T cells effectively protect mice from various stages of actively-induced disease. During these studies, we observed that cells that had been cultured for 72 hours with the immunizing antigen seemed to protect better than ex vivo cells that were injected into mice. This evidence correlates with other studies that suggest that CD8+ Ts cells only become apparent following antigenic restimulation (158, 159).

In another adoptive transfer setting, we observed that MOG-specific CD8+ T cells can protect mice from adoptively transferred CD4+ T cells. This further solidified our hypothesis that CD8+ T cells act in a regulatory manner in this disease model. Also, these observations gave us an insight into a possible mechanism of disease regulation. Since this was a purely adoptive transfer setting, disease is totally mediated by the effects of activated CD4+ T cells and not through presentation by APCs to naïve, antigen-specific CD4+ T cells. Therefore, the fact that the CD8+ T cells are able to suppress disease in this system indicates that they have a direct effect on the activated CD4+ T cells and/or their downstream disease processes.

Possible Mechanisms of CD8+ Ts Suppression of EAE

CD4+CD25+ T cells are typically thought to regulate immune responses through the secretion of cytokines such as IL-10 or TGF-β. There have also been observations that CD8+ T cells regulate immune responses through the secretion of IFN-γ (76-78). These early studies, however, did not explain the mechanism mediating the regulation. It is possible that the IFN-γ is stimulating CD8+ Ts cells, not being made by them. In another study, mice injected with OVA,

anti-4-1BB and poly I:C developed a peptide-specific CD8+ Ts cell (160). They also showed that these cells bound IFN-γ, which allowed them to mediate TGF-β-induced suppression. There has been relatively little research published on the possibility that CD8+ T cells regulate disease through killing.

CD8+ T cells are also known as "cytotoxic" T cells. This pseudonym refers to the fact that these cells are known to destroy other cells as part of the normal immune response. Their cytotoxic abilities have been well-characterized in anti-viral and anti-tumor responses. Because of this property, we sought to determine if the autoreactive CD8+ T cells that we observed expressed this killing ability. We observed, through an in vivo killing assay, that MOG-immunized mice have the ability to kill MOG-loaded target cells and that this killing is mediated by CD8+ T cells. We also showed that possible target cells include CD4+ T cells and CD4-depleted splenocytes. From these observations, we concluded that autoreactive CD8+ T cells have the ability to kill in an antigen-specific manner. This observation gives us a possible regulatory mechanism for these CD8+ Ts cells. If CD8+ Ts cells have the ability to kill cells that are expressing the specific auto-antigen on the cell surface, then they may be able to regulate their effects by eliminating them.

These observations coincide with observations that have been made in experiments involving the MHC molecule Qa-1. This molecule is a member of the MHC class Ib family and is known to interact with CD8, as well as the CD94/NKG2 family of proteins. It has been shown to bind to and present both self and non-self proteins (161, 162). Work has been shown that indicates CD8+ Ts cells interact through their TCR with CD4+ T cells and APCs in a regulatory manner when these cells express Qa-1 (163, 164). Further work indicates that the action taken by the CD8+ T cell depends on the peptide presented by the Qa-1 molecule. Qa-1 has been

shown to present portions of MHC class I leader peptide sequence, known as Qdm. If this molecule is presented, then the interaction is with CD94/NKG2A, CD94/NKG2C or CD94/NKG2E with the result being inhibition of cytolytic activity, regardless of the specificity of the TCR (165). Other studies have shown that if Qa-1 is loaded with specific Vβ peptide sequences from CD4+ T cells, then the interaction will be with TCR-dependent and the CD8+ T cell is activated, resulting in suppression of these CD4+ T cells (166) . Other proteins also shown to have this activating effect include self-proteins such as heat shock protein 60 and insulin as well as non-self proteins such as certain bacterially-derived proteins (167-169). Studies using a Qa-1 knockout mouse showed exaggerated CD4+ T cell responses to immunizing neuropeptides due to decreased CD8+ T cell-mediated suppression. These responses returned to normal levels when Qa-1 was reintroduced via viral vector (158).

This model shows many similarities to our observations. First, our observations indicate that a soluble, exogenous peptide is loaded into MHC class I complexes, since CD8+ T cells can be activated in response to incubation with exogenous activation. There have been many studies showing the phenomenon of cross-presentation (123, 125, 170). Furthermore, since it has been shown that soluble peptides can be loaded into the Qa-1 complex (168), it is plausible that exogenous pMOG 35-55 can be taken in through the external environment and presented on Qa-1 complexes. Also, we can hypothesize that these proteins will be loaded in such a way that they stimulate a clonal CD8+ T cell activation, similar to that seen with heat shock protein 60. Once the specific CD8+ T cells recognize their specific cognate peptide through the TCR-Qa-1 interaction, they would then be activated to direct the killing of the target cell. We also observed that the activation status of the target cells was important to their susceptibility to killing. When naïve, unstimulated T cells were used, the killing was drastically reduced compared to target

cells that had been activated with Con A. Further studies would need to be conducted in order to confirm this hypothesis. The lack of availability of a Qa-1 knockout mouse makes any in vivo studies difficult to perform.

In another group of experiments, we tested the possible effects of CD8+ Ts cells on the ability of APCs to activate CD4+ T cells and on the ability of CD4+ T cells to proliferate. Our results showed that APCs exposed to activated, auto-antigen specific CD8+ T cells were unable to activate CD4+ T cells as rigorously as control APCs. There are many mechanisms whereby they may effect this suppression. One type of CD8+ Ts cell, which has been termed the CD8+CD28- Ts cell, has been shown to cause downregulation of costimulatory molecules on the surface of APCs in a contact-dependent manner, which inhibits expansion and proliferation of T helper cells (171, 172). These Ts cells, however, were shown to function in an antigen-independent manner, whereas the suppressor cells that we observed to have this effect were antigen-specific.

Another type of CD8+CD28- Ts cell has been described to function by the secretion of IFN-γ and IL-6, which has been shown to inhibit CD4+ T cell expansion in a contact-independent manner (173). This is a possible mechanism for suppression in our assays. If this indeed is a mechanism utilized by the CD8+ Ts cells in our model, then the effect of these cytokines can be said to have a lasting effect, because the inhibition is seen even in the absence of the Ts cells or their cytokine influence. Again, this Ts cell phenotype has been shown in the past to function in an antigen-independent mechanism, which is different than our observations and may represent a different population of Ts cells. Further studies would need to be performed in order to confirm this mechanism.

CD8+ Ts Phenotypes

In a broad sense, CD8+ Ts cells can be classified into two categories: antigen-specific and antigen-nonspecific. In each of these categories, several different phenotypes has been described. Examples of non-specific CD8+ Ts phenotypes include CD8+CD25+ (174), CD8+CD28- (177) and CD8+CD122+ (135). Examples of antigen-specific CD8+ Ts cells include OVA-specific CD8+CD75s+ T cells in BALB/c and B6 mice (175) and Vβ peptide-specific CD8αα T cells in B10.PL mice (176). Most of these examples have been derived through in vitro manipulation following incubation with different cytokines and growth factors. Only the antigen-specific subsets were developed in vivo with antigen stimulation. The regulatory role that we propose for CD8+ T cells in the context of EAE follows this pattern of activation.

One of the more extensively studied CD8+ regulatory cell phenotypes is the CD8+CD28- phenotype. Even this characterization, however, is a broad interpretation, as several studies have shown differences in other surface molecules. These Ts cells have shown three different mechanisms of action (reviewed by Filaci, (177). It is interesting to note that these three subsets of CD8+ Ts cells were all generated through in vitro manipulation, not found through antigen-priming in a murine environment. It is also interesting to note that they do not appear to act in an antigen-specific manner. In contrast, our studies have shown the existence of an antigen-specific regulatory response in the CD8+ T cell subset. These cells did not require in vitro manipulation, but proliferated in response to antigen presence alone. This population of CD8+ Ts cells is therefore different in that aspect. However, we have shown evidence that antigen-specific CD8+ Ts cells exhibit regulatory functions on APCs in a similar manner, rendering them unable to

promote proliferation of antigen-specific CD4+ T cells. The mechanism of this inability will require further characterization.

Another group identified a subset of CD8+ T cells with a different molecular phenotype. Lee, et. al. identified a subset of naturally-occurring CD8+ Ts cells characterized by a CD8+CD122+ surface phenotype in MOG-induced C57BL/6 mice (135). They also showed that these cells, contrary to that seen in other described CD8+ Ts cells, downregulate the production of IFN-γ and produce the suppressor cytokine IL-10. Adoptive transfer of these cells into mice at the height of EAE disease was able to ameliorate disease symptoms. It was also shown that in vivo depletion of these cells led to increased disease. This evidence lead to the conclusion that, like the thymus-derived suppressor CD4+CD25+ T cells known as nTregs, there is a naturally-occurring, non-specific population of CD8+ Ts cells.

Our studies have not revealed a specific phenotype for the CD8+ Ts cells that we have observed. Because we use bulk CD8+ T cells for the adoptive transfer and in vivo killing assays that we perform, we hypothesize that the majority of the antigen-specific CD8+ T cells must be regulatory in nature. Since all of our assays include an in vitro activation step in which the cells are exposed to antigen, if pathogenic cells were present, one would hypothesize that they would proliferate along with the suppressor cells. If there is a pathogenic subset that exists, our experiments would suggest that it is either a minor subset or that they are also regulated by the suppressor effect that is produced. In either case, we do not believe that the regulatory subset of CD8+ T cells is a minor subset of the antigen-specific response.

We attempted to observe the presence of Foxp3 transcription factor in the CD8+ T cell subset, which has been observed in CD8+ regulatory cells in other models of disease and

pathology (178, 179). We did not, however, observe Foxp3 expression in the CD8+ T cell population in this model (data not shown).

As we continue to study CD8+ regulatory T cells in this model, it is important to continue to seek a specific phenotype. Since most of the other described CD8+ regulatory T cell subsets have been generated in vitro, we do not expect that autoreactive T cells, as we observe them, to share the same phenotype. Models that have produced antigen-specific regulatory CD8+ T cell phenotypes (CD8+CD75s+ Ts cells and CD8αα Tregs) were not established in an EAE model. Further mechanistic elucidation of the CD8+ Ts cells in our model may provide insights into specific phenotypes of these cells.

Cytokines and CD8+ Ts Cells

We also showed in our model that CD8+ T cells produce IFN-γ in an antigen-specific manner. This is yet another form by which these cells may be exerting a regulatory effect. Several different experimental models have shown that CD8+ Ts cells may exert their suppressor effects through the production of cytokines. For example, Myers and colleagues were able to classify a CD137 (41BB ligand)-dependent suppression that was mediated by CD8+ T cells and required the presence of IFN-γ (160). This IFN-γ production was shown to induce the immunosuppressive cytokine, TGF-β. IFN-γ expression was also shown to be crucial in the CD8+ Ts-mediated promotion of heart allografts in rats (180). This time, the IFN-γ was required for the production of a different suppressor molecule, indoleamine 2,3-dioxygenase. While the exact function of IFN-γ in our model has yet to be elicited, it is possible that it may follow the same regulatory patterns that have been shown by others.

Translation to Human Demyelination

Multiple Sclerosis has proven to be a complex disease with many cells having been shown to play a role in disease onset or recovery (reviewed by Lassmann, (181). Using EAE, we can attempt to resolve some of the possible roles of specific cell types in a more controlled environment. However, this same controlled environment makes it impossible to assume that all of the results that we see in the murine model can translate directly into the human disease. The fact that we use inbred strains of mice that have been selected for certain MHC haplotypes is one example of how the interpretation of experimental results must be viewed in a limited sense only. Having said this, the study of the model is still one of the most useful tools that we have to study autoimmune demyelination.

One of the broad conclusions that we can make from our studies is that autoreactive, antigen-specific CD8+ T cells are involved in various EAE models. We showed that they could be recovered from four different models of EAE which use a variety of different antigens and mouse strains with different MHC haplotypes. This evidence agrees with the observation made by our lab that neuroantigen-specific CD8+ T cells of different clonotypes can be observed in MS patients (31).

Our lab has also shown that CD8+ T cells play a role in the therapeutic suppression of encephalitogenic CD4+ T cells. We have shown that glatiramer acetate (GA) activates antigen-specific CD8+ T cells to kill GA-specific CD4+ T cells (140). This killing was shown to be mediated through the human Qa-1 cognate, HLA-E. This observation agrees with our observations that antigen-specific CD8+ Ts cells in EAE retain killing capacity and that they can

kill CD4+ T cells. It also agrees with the observations made by other labs that this "suppression by killing" mechanism has been shown to be Qa-1 dependent (167-170).

Along these lines, there have been further observations supporting the hypothesis of HLA-E-driven suppression in MS. It has been shown that CD8+ Ts cells that destroy myelin-specific CD4+ T cells are decreased during MS exacerbations (122). This decrease was especially noticeable in the CNS. Interestingly, this study also showed an upregulation of the surface marker CD94/NKG2A on CD8+ T cells during disease exacerbations. This would indicate that this marker is involved in the regulation of CD8+ Ts cells and could be partially responsible for the onset or progression of an inflammatory event. This evidence also coincides with observations made in the mouse model, in which these receptors have been shown to have the same function (182). This evidence could lead to possible therapeutic applications through the blockade of this surface receptor during inflammatory episodes.

Another possible clinical application is for the development of T cell vaccines (TCV). These vaccines are currently being developed and tested in the EAE model. Early observations showed that mice inoculated with encephalitogenic CD4+ T cells that had been weakened by irradiation were resistant to active disease induction. This resistance was shown to be mediated through CD8+ T cells (183). These TCV have been studied to limited extent in humans, and CD8+ Ts cells were isolated in this study as well (184). These vaccines are still being developed for widespread use, but offer a promising alternative to traditional vaccination efforts and also to non-infectious disease processes.

Proposed Model for Observed CD8+ Ts-mediated Suppression in EAE

Our observations have characterized the presence and functional role of CD8+ T cells. We have also shown evidence of several possible mechanisms of suppression effected by these cells. These mechanisms may work together to exert a global suppressive effect, or possibly work independently in a redundant manner to offer different regulatory checks in the bodies efforts to control peripheral autoreactivity. These observations lead to the possibility of several different modes of regulation by CD8+ Ts cells.

In our model, the process begins when the encephalitogenic peptide, in this case pMOG 35-55, is injected subcutaneously into the animal (Figure 25a). Because the antigen is emulsified in complete Freund's adjuvant, it incites inflammation in the local tissue. As part of the process, local antigen presenting cells (tissue histiocytes or dendritic cells) take in the antigen and begin to process it as they would other exogenous antigens. Meanwhile, they migrate to secondary lymphoid tissues where they come into contact with CD4+ T cells (Figure 25b).

Antigen-specific CD4+ T cells in the periphery become activated through antigen presentation on class II MHC expressed on the surface of the APC. We were able to show that autoantigen-specific CD4+ T cells do exist in the peripheral lymph tissue of immunized mice and that these cells were shown to proliferate to antigenic exposure as early as day 10 post-immunization (data not shown). This corroborates other studies indicating the presence of an encephalitogenic CD4+ T cell response (34, 81).

Once activated, these cells have the ability to pass through the blood-brain barrier into the CNS. Once inside the CNS, these cells are restimulated by resident APCs expressing the neuropeptide on class II MHC. Under the influence of APC-produced cytokines, these CD4+ Th

cells begin to produce inflammatory cytokines, specifically IL-17 (73, 74). This cytokine is involved in the recruitment of inflammatory cells, such as B cells and macrophages, which mediate the destruction of the myelin sheath and oligodendrocytes. This leads to the manifestation of symptoms seen in EAE.

CD8+ T cells are also activated in the peripheral lymphoid tissue (Figure 25c). This activation must be effected by a manner different than the normal presentation of exogenous peptide. One explanation is that the antigen is processed through a cross-presentation pathway that requires it to be released from endosomes into the cytoplasm (185) then loaded onto class Ia MHC, or potentially class Ib MHC, such as Qa-1. This receptor is upregulated on APCs that have been activated and has also been shown to have the ability to present soluble exogenous antigen (161, 168, 186, 187). This cross-presentation process could explain the apparent delay in the presence of activated CD8+ T cells that we observed in our kinetics assays. Since both cross-presentation and presentation through Qa-1 receptors would require additional processing, it is reasonable to suggest that the autoreactive CD8+ T cells are not primed as quickly as autoreactive CD4+ T cells, although there is no data to suggest that this is true. This apparent delay in CD8+ Ts cells could also help explain the normal disease course of these mice, which shows that the disease peaks early after disease induction, and is followed by a drop in disease severity before the chronic phase levels off, usually at a severity below the peak score (personal observation).

Once the CD8+ T cells become activated, they can also migrate to the CNS, where studies have shown that they, too, have the ability to expand (126, 129, 188). Our observations indicate that CD8+ T cells of a certain activation status seem to be better able to regulate, as seen in the fact that in vitro stimulated CD8+ T cells regulate disease better than ex vivo CD8+ Ts

cells. As they expand, they begin to exert their regulatory function. We have shown that these CD8+ Ts cells have the ability to kill cells that express the MOG antigen on their surface in an antigen-specific fashion. A likely theory that we propose is that activated APCs and CD4+ T cells within the CNS present antigen on an MHC class I complex such as the Qa-1 protein. This allows them to specifically target activated inflammatory cells for destruction. There have been several examples of CD8+ T cells killing antigen-loaded target cells in a TCR-Qa-1-dependant manner (163, 164, 186). This mechanism would account for the observations made by us that CD8+ T cells are able to ameliorate ongoing disease.

The overall effect of this mechanism would be two-fold. First, any APCs that are presenting the MOG antigen would be eliminated. Hypothetically, this would reduce presentation of the peptide to MOG-specific, encephalitogenic CD4+ T cells, thus preventing disease onset or progression. It would also specifically eliminate MOG-specific B cells, since they would most likely be presenting the peptide to which their B cell receptor specifically binds. The other function of the CD8+ T cells in this model would be to nonspecifically suppress activated CD4+ T cells. Assuming that any activated CD4+ T cell expresses MHC class Ia and Ib molecules, and that these molecules could potentially express the MOG35-55 peptide, it is plausible that this antigen-specific CD8+ T cell could effect a more global suppression, regardless of the CD4+ T cell specificity. The therapeutic effect is evident in our adoptive transfer model in that the CD8+ Ts cells can ameliorate autoreactive disease. For humans, however, the therapeutic application of this mechanism may come with unwanted side effects. Because of the controlled nature of the mouse model, the effects of globally suppressing activated CD4+ T cells cannot be fully appreciated.

These CD8+ T cells may also regulate disease through the production of IFN-γ. Other studies indicate that IFN-γ is involved in disease regulation (64, 76-78, 122, 160). We have shown that antigen-specific CD8+ Ts cells can also regulate APCs and CD4+ T cells in a non-lethal manner. Our observations are in accordance with these previous studies. This model system would tend to fit with the traditionally accepted mechanisms of the well-studied CD4+CD25+ T cells. Our observations indicate that the MOG-specific CD8+ T cells exert non-killing suppressive effects on the APC and CD4+ T cell subsets. There has been much evidence published indicating that CD8+ T cells have the ability to do this (134, 135, 177) but it is important to note, again, that these effects are by and large thought of as antigen-independent. These CD8+ Ts cells have been shown to cause dysfunction in the ability of APCs to stimulate proliferation in CD4+ T cells, and have also been shown to inhibit proliferation of antigen-specific CD4+ T cells. Because these studies were done in vitro following 15 days of in vivo exposure to antigen-specific CD8+ T cells, we can conclude that the mechanism of suppression is not killing in these cells; during incubation periods, cells were not exposed to active, primed CD8+ T cells. A possible model system is that CD8+ T cells interact in a contact dependent manner, causing the down-regulation of costimulatory markers found on the surface of APCs. Another possibility is that these CD8+ Ts cells effect changes in APCs through the secretion of cytokines. This could also be a mechanism whereby Ts cells induce a state of limited proliferation in CD4+ T cells. Further studies must be conducted in order to determine the exact mechanism of suppression in this setting. One conclusion that can be made is that the effect of these Ts cells on both APCs and CD4+ T cells does not require the continuous presence of CD8+ Ts cells. This suggests that the Ts cells bind to their targets and effect downstream changes

within the target cells to downregulate activation signals or secrete cytokines that have similar effects.

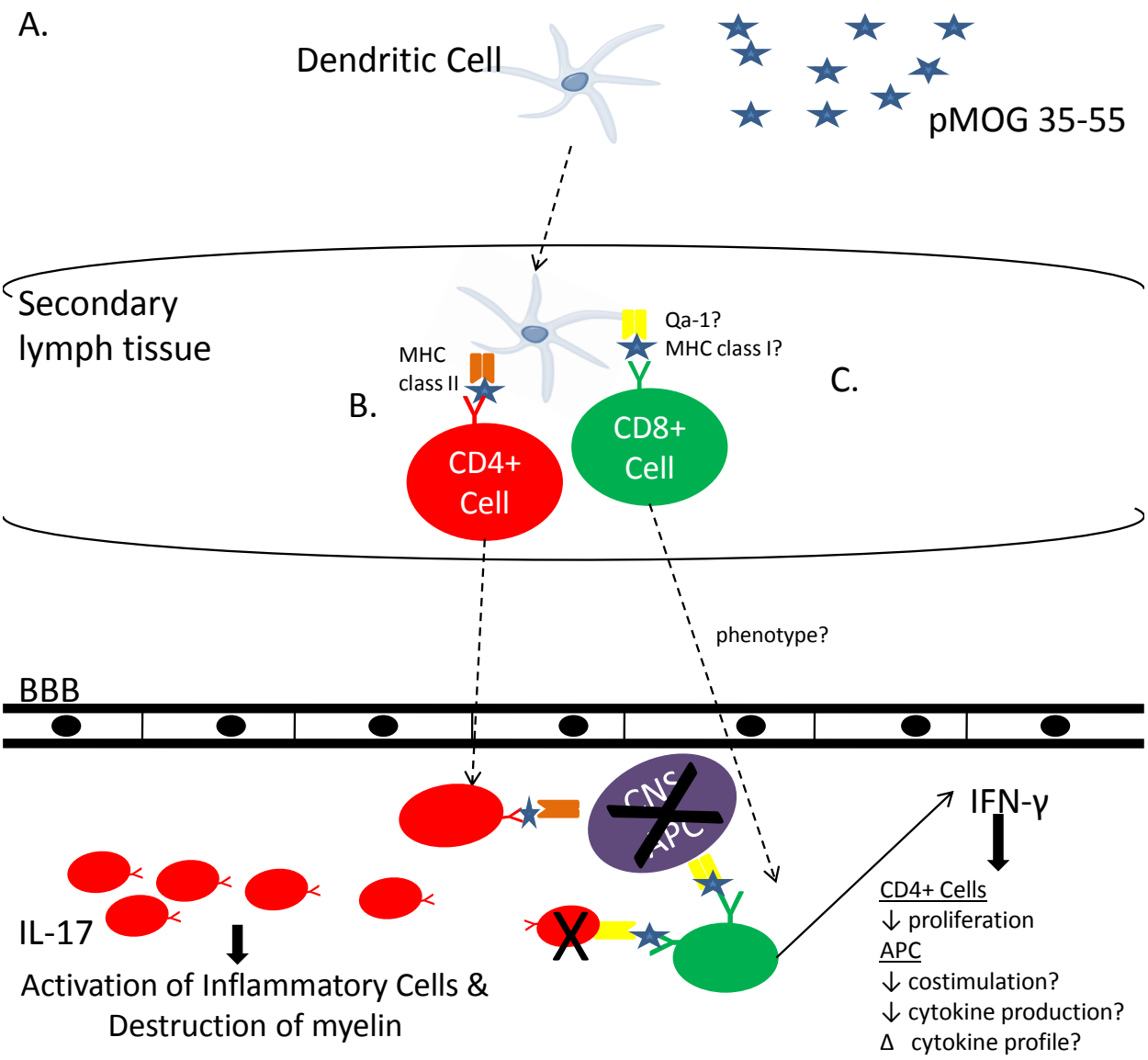
It is also possible that regulatory CD8+ T cells are able to exert regulatory mechanisms in the periphery as well. We know that activated T lymphocytes have the ability to traffic into and out of tissues. Our adoptive transfer experiments also indicate that activated CD8+ T cells can prevent disease in both the active immunization setting as well as the adoptive transfer setting. In some of these cases, the mice did exhibit symptoms (albeit to a lesser extent than controls). This indicates that some encephalitogenic CD4+ T cells were able to migrate to the CNS. We are unable to say for certain that the regulation in these cases takes place in the periphery or if both populations are able to infiltrate the CNS with the suppression occurring intrathecally, but either situation is a possibility. The fact that antigen-specific CD8+ T cells are able to prevent encephalitogenic CD4+ T cells from causing disease in the adoptive transfer setting also supports either possibility.

An interesting phenomenon has been observed in the setting of the anterior chamber of the eye. Like MS and EAE, it has been shown that protection involves many different cell types (reviewed by Niederkorn, (189). In this model, it has been shown that resident APCs capture antigens in the anterior chamber of the eye, then migrate to the spleen, where they induce many different immune cell to become activated. These APCs release fragments of the captured antigens into the splenic environment, which are then endocytosed by B cells specific for the epitopes. These antigen-specific B cells can then expand and capture more antigen, which they can then present to both CD4+ and CD8+ T cells (190). These T cells then differentiate in to CD4+ Tregs and CD8+ Ts cells.

It is possible that this same type of presentation exists in the autoimmune demyelination setting as well. We know that, like the eye, the CNS is an immune-privileged site, with the blood-brain barrier tightly restricting traffic into and out of the CNS. We also know that APCs within the CNS are found at the sites of lesions (52). We also know that oligoclonal bands are found within the CNS of MS patients, indicating the expansion of antigen-specific B cells (86). Our studies have shown that antigen-specific T cells, both CD4+ and CD8+, are found in the spleens of diseased mice, and that these CD8+ T cells exhibit regulatory properties. It is therefore plausible that this same process of migrating APCs leading to development of regulatory CD8+ T cells occurs in the autoimmune demyelination setting. This hypothesis, however, remains to be proven in this model.

Chapter IX Figure Legends

Figure 26: Proposed Model of Immune Regulation by Antigen-Specific CD8+ T Cells: A. pMOG 35-55 is injected in an emulsion subcutaneously into the mouse. Proximal dendritic cells pick up the antigen for processing and migrate to secondary lymph tissue (regional lymph nodes or spleen). B. Once inside the secondary lymph tissue, the dendritic cell presents the processed antigen on MHC class II to circulating CD4+ Th cells. These cells then become activated and migrate from the tissue to the blood stream. Their activation status allows them to cross the blood-brain barrier and enter the CNS. Once inside, these cells are restimulated by resident APCs, which induces them to expand and produce inflammatory cytokines such as IL-17. These cytokines then help to recruit and activate other cells, such as B cells and macrophages, which mediate the destruction of myelin, causing the symptoms. C. CD8+ T cells are also activated in the periphery, with an apparent delay (possibly due to differences in presentation, delay in cross-presentation, or activation status of the APC). Once activated, these cells also migrate to the CNS. There they target activated CD4+ T cells and activated APCs for destruction. These CD8+ T cells also produce IFN-γ, which also appears to play a regulatory function by affecting the ability of CD4+ T cells to proliferate and by affecting the ability of APCs to stimulate CD4+ T cells. These mechanisms help the CD8+ T cells to regulate the disease process in this model.


A.
Dendritic Cell
pMOG 35-55
Secondary
lymph tissue
B.
MHC
class II
Qa-1?
MHC class I?
C.
CD4+
Cell
CD8+
Cell
phenotype?
BBB
IFN-γ
CD4+ Cells
↓ proliferation
APC
↓ costimulation?
↓ cytokine production?
Δ cytokine profile?
IL-17
Activation of Inflammatory Cells &
Destruction of myelin

Chapter XI: Future Directions

Our study has confirmed the presence of antigen-specific, autoreactive CD8+ T cells in the EAE model of autoimmune demyelination. We found these cells not only in the B6/MOG 35-55 model, but several other models as well. Through a series of adoptive transfer studies, we showed that these MOG-specific CD8+ T cells do not act in a pathogenic fashion. To the contrary, these cells exhibited a regulatory function in four different adoptive transfer settings. Functionally, we have shown that these cells exhibit a killing function. This killing function has the ability to target both MOG-loaded CD4+ T cells, as well as CD4-depleted splenocytes. In vivo killing of these target cells has been shown to be mediated by CD8+ T cells. We have also shown that these cells have the ability to down-regulate ability of APCs to stimulate CD4+ T cell proliferation. They also appear to have the ability to inhibit proliferation of MOG-specific CD4+ T cells.

There are still many questions left to answer concerning the functions and mechanisms of regulation by these antigen-specific CD8+ Ts cells. More studies need to be conducted in order to determine if the antigen-specific Ts cells have a specific phenotype that would allow us to differentiate them from other autoreactive CD8+ T cells, if there are other cells that do not serve the same function. It would also be interesting to test if there is a difference in phenotype among the cells that kill and the cells that regulate APCs and CD4+ T cells in a non-killing mechanism. Phenotyping the cells would allow us to separate these cells and/or use knockout mice to study more specific mechanisms. Another possible property by which we could separate these cells is

through identification of the α and β chains of the TCR. If these cells have a specific pattern of expression in the TCR, this may also be useful for further characterization.

Another area in which more questions arise is in the killing function of these cells. Specifically, the question of involvement of the Qa-1 receptor is important to explore. Testing this hypothesis in vivo would require a Qa-1 knockout mouse, which is not currently available for wide use. An alternative test would be to develop an in vitro killing assay using cells that lack Qa-1 expression on the surface when activated or by using anti-Qa-1 blocking antibodies. As with all in vitro tests, it would be subject to interpretation owing to the fact that culture conditions do not exactly mimic in vivo conditions. However, it would allow us to gain some insight into the killing ability and mechanism of the CD8+ Ts cells. Further analysis could also be done to further characterize the ability of these Ts cells to kill different APCs. Our studies showed CD4-depleted splenocytes were specifically killed. This population, however, contains multiple cell types and could be further separated. This may give further insight into the mechanism of disease regulation as well.

The mechanism by which these cells regulate in a non-killing manner can also be further elucidated. Specific tests to be done include phenotyping of the dysregulated APCs to find if these cells have decreased expression of cell surface markers such as CD80 or CD86. Cytokine profiling of these cells will also elucidate what, if any, dysfunction may be present. These findings could then be further studied to elucidate changes effected in the APC (transcriptional pathways, protein expression, etc.). The CD4+ T cells could also be evaluated in the same manner to elucidate the mechanism by which these cells are inhibited as well.

For the purposes of further studies of the mechanism of these cells, it would also be beneficial to pursue the creation of a TCR transgenic mouse. Our efforts to establish a clonal line were unsuccessful, but this effort should be further pursued. Establishing a clonal line would allow the elucidation of the specific TCR sequence for the autoreactive CD8+ Ts cell. Once a clone with known regulatory function was isolated, then that sequence could be used to create a TCR transgenic mouse. If this mouse were then bred to a RAG knockout mouse, then theoretically all of the functional T cells would be specific for the MOG 35-55 antigen. This mouse could then be tested for susceptibility to disease by active induction or adoptive transfer. This mouse could also be used as a depot of antigen-specific cells for further testing. It would also be available for breeding to ask other questions.

Characterizing the functionality of these cells and the mechanisms by which they suppress are a beneficial pursuit. Considering the history of studies in this disease, it is critical that we understand the role of all of the cell populations involved in the pathogenesis and regulation of the disease. In pursuing regulatory mechanisms, these studies could potentially lead to new therapeutic approaches such as T cell vaccines or cytokine therapy that could increase and/or optimize Ts cells within the body. These approaches have the potential of controlling autoimmune demyelination and ameliorating the suffering of people with this disease.

Chapter XII: Bibliography

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