THE ROLE OF THERAPY-INDUCED CD8$^+$ T CELLS IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

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THE ROLE OF THERAPY-INDUCED CD8$^+$ T CELLS IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

by

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THE ROLE OF THERAPY-INDUCED CD8$^+$ T CELLS IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

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Murine experimental autoimmune encephalomyelitis (EAE) is an induced, autoimmune, demyelinating disease model for human multiple sclerosis (MS). Glatiramer acetate (GA) is an approved immunomodulatory therapy for MS that was discovered through studies in EAE. It is thought that the main mode of action of GA is the induction of a Th1 to Th2 shift in CD4$^+$ T cells. We have shown that, in human MS, GA therapy induces an upregulation of cytotoxic, suppressor CD8$^+$ T cell responses, suggesting that these cells are integrally involved in mediating the immune effects of this drug. In this study, we show that

GA induces robust $CD8^+$ T cell responses even in mice. Whereas GA immunization ameliorates the clinical and histologic severity of EAE in wild type C57BL/6 mice, such protection is not observed in CD8(-/-) mice, indicating that $CD8^+$ T cells are required in mediating the effects of GA. Moreover, adoptive transfer of $CD8^+$ T cells from GA-immunized (but not OVA-immunized) mice results in amelioration of EAE in CD8(-/-) and wildtype mice, suggesting therapeutic potential for GA-specific $CD8^+$ T cells. These cells appear to mediate their effects through a cytotoxic/suppressor mechanism, similar to our findings in human MS. In addition, GA treatment results in decreased myelin oligodendrocyte glycoprotein (MOG)-specific $CD4^+$ T cell responses. In conclusion, our studies provide strong evidence that the *in vivo* induction of immune modulatory $CD8^+$ T cells is an essential step in mediating therapeutic protection during autoimmune demyelination.

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ABBREVIATIONS

μg: micrograms

μl: microliters

μM: micromolar

Ab: antibody

Ag: antigen

APC: antigen presenting cells

APC: allophycocyanin

APL: altered peptide ligand

BSA: bovine serum albumin

CD: cluster of differentiation

cDNA: complementary DNA

CFA: complete Freund's adjuvant

CFSE: 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester

CNS: central nervous system

Con A: conconavalin A

COP: copaxone or copolymer -1

CTL: cytotoxic T lymphocyte

DC: dendritic cell

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

EDTA: ethylenediaminetetra acetic acid

ELISA: enzyme linked immunosorbant assay

ETOH: ethanol

FACS: fluorescence activated cell sorter

FBS: fetal bovine serum

FITC: fluorescein isothiocyanate

FoxP3: forkhead box P3

GA: glatiramer acetate

HBSS: Hanks Balanced Salt Solution

HRP: horseradish peroxidase

IFN-γ: interferon gamma

Ig: immunoglobulin

IkB: inhibitor kappa B

IL: interleukin

IL-2: interleukin 2

IL-4: interleukin 4

IL-10: interleukin 10

IL-12Rβ2: interleukin 12 receptor beta 2

IL-23R: interleukin 23 receptor

i.p.: intraperitoneally

i..v.: intravenous

kDa: kilodalton

LNC: lymph node cells

LPS: lipopolysaccharide

LT: lymphotoxin

MAP kinase: mitogen activated protein kinase

MBP: myelin basic protein

MHC: major histocompatibility complex

ml: milliliter

MMP: matrix metalloproteinase

MOG: myelin oligodendrocyte glycoprotein

mRNA: messenger RNA

MS: multiple sclerosis

NFkB: nuclear factor kappa B

NK: natural killer

NKT: natural killer T cells

NO: nitric oxide

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phycoerythrin

PerCP: peridinin-chlorophyll-protein

PLP: proteolipid protein

PMA: phorbol myristate acetate

PML: progressive multifocal leukoencephalopathy

PT: pertussis toxin

r: recombinant

RA: rheumatoid arthritis

RAG: recombinase activating gene

RNAi: RNA interference

RT-PCR: reverse transcriptase polymerase chain reaction

s.c. subcutaneous

SEB: staphylococcal enterotoxin B

SEM: standard error of the mean

siRNA: small interfering RNA

STAT: signal transducer and activator of transcription

T-bet: T-box expressed in T cells

TCR: T cell receptor

TE: Tris EDTA

Tg.: transgenic

TGF-β: transforming growth factor beta

Th: T helper

Th1: T helper 1

Th2: T helper 2

Th17: T helper 17

TLR: toll like receptor

TNF-α: tumor necrosis factor alpha

Treg: regulatory T cell

VCAM: vascular cell adhesion molecule

WT: wildtype

Chapter I: Introduction

Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS that affects 250,000 – 350,000 people in the United States and 3 million people worldwide [1]. MS presents in patients with varying symptoms including vision loss or double vision, limb weakness, pain, fatigue, and cognitive dysfunction. MS is the most common disabling disease in young adults and is three times more common in women. Typically MS has onset between late adolescence and 30 years of age [2]. MS is diagnosed by its clinical symptoms and is confirmed by MRI imaging. MS patients will exhibit lesions or "plaques" in the brain in increasing frequency and severity during a symptomatic attack. The main clinical forms of MS are relapsing remitting, primary progressive, and secondary progressive MS. Relapsing remitting MS (RRMS) is the most common form of the disease and appears in 75% of MS patients. There are many treatments for MS symptoms although there is no cure for the disease.

MS was first described in the 19th century when physicians observed scarred tissue or plaques in the brains and spinal cords of autopsied patients that exhibited the symptoms we now know characterizes MS. These multiple scars (sclerosis in Latin) were actually areas of demyelination. MS pathology is attributed to activated autoreactive effector T cells that cross the blood-brain barrier and are reactivated within the CNS. These autoreactive T cells release proinflammatory cytokines and start an immune cascade of events leading to destruction of the myelin sheath. Myelin sheath demyelination occurs by a) T cells and macrophage-mediated demyelination, b) antibody-mediated demyelination, c) oligodendrogliopathy and oligodendrocyte apoptosis, and/or d) primary oligodendrocyte degeneration [3]. In a healthy individual, T regulatory cells prevent autoreactive T cells from causing autoimmunity and induce peripheral tolerance.

The etiology of MS is unknown although some studies suggest a genetic and/or environmental component. There is a concordance in the occurrence of MS of 30% in monozygotic twins and 3% in siblings with parents that have MS [4]. The human leukocyte antigen (HLA) – DR1501 and HLA-DQ0601 alleles are associated with a marked increase in susceptibility to developing MS [5]. The environmental theory is that the incidence of MS increases with direct relationship to latitude. The prevalence of MS is higher in the northern U.S. versus that observed in southern states although no specific environmental factor has been proven to cause MS. Migration studies show that individuals who grow up in a higher risk area are able to acquire a lower risk if they move to a low risk before adolescence [6, 7].

Molecular mimicry by viral or bacterial proteins could also play a role in initiating autoimmunity by autoreactive T cells [8, 9]. One study has shown that a viral peptide from cytomegalovirus (CMV) can activate autoreactive T cell clones [10]. Epstein-Barr virus (EBV), *Chlamydia pneumonia*, hepatitis B, and *Haemophilus influenza* all have epitopes that are cross-reactive with autoreactive T cells [11-14]. EBV has a 100% incidence rate in MS patients and a 90% incidence rate in healthy individuals although this does not prove etiology of MS [15].

MS is a major focus of many research centers around the world. Although significant strides have been made in the understanding of this disease, many questions remain unanswered. Animal models of autoimmune demyelination provide a methodology for dissecting the immunological and pathological mechanisms of MS.

Diagnosing Multiple Sclerosis

The first diagnostic criteria for MS were described by Jean-Martin Charcot in his *Lecons du mardi* in 1868 [16]. Charcot described the symptoms of his housemaid as nystagmus,

intention tremor, and scanning speech. In 1931, Sydney Allison devised four categories to describe disseminated sclerosis (DS) occurrence in Wales: a) typical cases; b) early cases in which DS is a likely diagnosis; c) deceased or untraceable cases; and d) doubtful cases due to inconclusive symptoms [17]. DS was the term used for MS in the United Kingdom. In 1954, Allison and Millar published the first diagnostic approach by dividing patients into three categories: early, probable, and possible DS [18]. Patients put in the early category were required to have a history of symptoms that fit a list of symptoms known to be associated with DS. Patients in the probable category had a current physical manifestation of the disease such as remitting attacks. The possible category was reserved for cases that only had potential to be called DS based on unclear presentation.

Diagnostic criteria for MS were published by Broman et al. in 1965. This group put into place formal diagnostic criteria that included age of onset, symptom significance, multiplicity of lesions, number of relapses, and familial occurrence of MS. This study also described electrophoretic examination of cerebral spinal fluid (CSF) protein. Broman reported the fundamental criteria for diagnosing MS- dissemination in time and in space [19].

Another major breakthrough was published the same year by George Schumacher who defined only a single category of clinically definite MS that could be included in clinical trials [20]. Schumacher's criteria for clinically definite MS are: signs of dysfunction of CNS (not symptoms), predominate white matter damage at two or more sites, two or more attacks that are at least 1 day in duration separated by at least 6 months or progression over 6 months, onset age 10-50 years, diagnosis by a neurologist, and signs/symptoms cannot be explained by another disease. Schumacher emphasized that because no specific diagnostic tests were available,

patients could only be considered to be highly probable to have MS and the responsibility remained on the experienced physician to make the final diagnosis [20].

A number of other studies were published in the 1970s that attempted to elucidate the diagnostic criteria for MS. McAlpine, Lumsden, and Acheson published their book in 1972 that classified MS cases as either latent probable, probable, and possible MS and called for more evidence of past MS [21]. The McAlpine, Lumsden, and Acheson criteria were sometimes used as an alternative to the Schumacher criteria during this time period. In 1976, Augustus Rose published his MS diagnostic scheme, which divided cases into either probable MS and possible MS based on the number of episodes and sites affected [22]. William McDonald and Anthony Halliday published their criteria in 1977, but very importantly stated the only way to prove MS was by biopsy or autopsy [23].

The Schumacher criteria were modified in 1980 when Bauer published his global investigation. This report was the first to add CSF oligoclonal bands into the diagnostic criteria [24]. In late 1979, Charles Poser designed a numerical scoring system to classify MS cases by assigning a value to signs and symptoms based on its frequency in a patient's history. Poser attempted to sort through the inconsistent terminology from all the previous reports and decrease the amount of subjective judgment [25]. Poser et al. updated their criteria in 1983 and divided cases into two groups, clinically definite MS and probable MS, each was then subdivided into clinical and laboratory supported groups [26]. Laboratory supported groups were based on the presence of CSF oligoclonal bands and increased immunoglobulin G (IgG) production.

Magnetic resonance imaging (MRI) was introduced in 1981 by Young et al. and was quickly embraced to help in diagnosing neurological diseases including MS [27]. MRI is a powerful tool that helps clinicians view the occurrence of lesions and can be tracked during the

course of disease. In 2001, McDonald et al. published new diagnostic criteria for MS which included a MRI scheme and criteria for diagnosing primary progressive MS [28]. The McDonald criteria permits for an accurate diagnosis 83% of the time before a second attack [29]. It has been reported that the McDonald criteria identified three times as many clinical definite MS cases compared with the Poser criteria in patients that were followed after their first attack [29, 30].

Typically, when patients present with a single attack, evidence of dissemination in space and in time is required for a diagnosis of MS. For dissemination in space, the presence of three of the four MRI diagnostic criteria that Barkhof et al. developed is required [31]. If three of these criteria are not met, two or more T2 lesions and a positive CSF oligoclonal band sample with increased IgG production will meet the dissemination in space definition [31, 32]. For dissemination in time, MRI can show an original enhancing lesion 3 months after an initial attack or if MRI shows a new T2 lesion 30 days or more after initial clinical onset. If a patient presents with multiple attacks with involvement of more than one area of the CNS, then a MS diagnosis is complete with proof of dissemination in time [28, 30, 31].

It is important to exclude other diagnoses as well. In older patients, vascular disease may be confused for MS, but a positive MRI of the spinal cord should exclude cerebrovascular disease [33]. Even with a negative spinal cord MRI, MS could be diagnosed with delayed visual evoked potentials or increased IgG production. In pediatric cases, the most common diagnostic doubt is from acute disseminated encephalomyelitis (ADEM). However, ADEM patients are more likely to have multifocal involvement, pseudoencephalitic presentation, or involvement of both optic nerves [34, 35].

Experimental Autoimmune Encephalomyelitis

Experimental Autoimmune Encephalomyelitis (EAE) is a well-characterized murine model of MS. EAE-like illness was initially described in patients involved in vaccine studies who received vaccines made from spinal cord from rabbits infected with rabies. Louis Pasteur's vaccine induced encephalomyelitis post-vaccination in patients [36]. This induced encephalomyelitis was reproducible in rabbits and monkeys following repeated injections of neural tissue [37, 38]. The necessity of the repeated injections was eliminated when complete Freund's adjuvant (CFA) was discovered [39, 40]. CFA is composed of mineral oil with *Mycobacterium* and is emulsified with a myelin-related antigen to induce disease. Pertussis toxin (PT) is also injected to increase the disease incidence [41, 42].

EAE induction in mice and rats is now a very common method of studying autoimmune demyelination. There are two major ways to induce murine EAE, active or passive immunization. Active EAE can be induced in several different strains of mice including B10.PL, SJL/J and C57BL/6 by injecting myelin related antigens emulsified in CFA and then injecting with PT at Day 0 and Day 2. B10.PL mice will get acute disease, but recover completely while SJL/J mice show a relapsing remitting form of EAE even without the PT requirement. In C57BL/6 mice, myelin oligodendrocyte glycoprotein (MOG) immunodominant peptide 33-55 or whole bovine myelin basic protein (MBP) is used to induce a chronic form of the disease [43-45]. Active EAE has an induction phase where in response to the antigen/CFA injection, autoreactive T cells are activated and expand in the peripheral lymph nodes. In the effector phase, these activated and expanded T cells cross the blood brain barrier resulting in the clinical signs of disease. Chronic EAE has pathological features similar to what is observed in the CNS

of MS patients. Both MS and EAE feature perivascular infiltration of mononuclear cells and CNS lesions [43].

Mice that have been immunized can in turn be used for adoptive transfer of EAE. Bulk cell suspensions from draining lymph nodes from immunized mice are stimulated *in vitro* with myelin related antigen for 3 days and then myelin-specific $CD4^+$ T cells are adoptively transferred into naïve recipient mice thereby inducing passive disease. Passive EAE only has an effector phase, as myelin-specific T cells were directly injected into the mice [46, 47].

EAE onset will vary by strain, myelin antigen, and injection scheme. In C57BL/6 mice, disease onset typically occurs between day 10 and day 12 with peak disease around day 15 or 16. To evaluate EAE, mice are assessed using a clinical scoring system as shown in Table 1. A score of 0 is a mouse with no clinical signs of EAE. A clinical score of 1 is a limp tail while a clinical 2 is a mouse with mild hind limb weakness. A grade of 3 is given if the mouse exhibits severe hind limb weakness. A clinical 4 is hind limb paralysis. A score of 5 is given to an animal that also has forelimb paralysis. A mouse that dies from EAE is given a clinical score of 6 [48, 49].

Table 1: Clinical Grading Scale for EAE

Clinical Score	Clinical Symptoms
0	**Normal**
1	**Limp tail**
2	**Hind limb weakness**
3	**Moderate to severe hind limb weakness**
4	**Complete hind limb paralysis**
5	**Quadriplegia**
6	**Moribund or death to EAE**

T cells in EAE and MS

In EAE, $CD4^+$ T cells have been shown to be important to the induction of disease through adoptive transfer and CD4+ depletion studies [50, 51]. CD4 depletion immediately prior to immunization prevented disease entirely. In EAE, Th1 T cells secrete interferon- γ (IFN-γ) and tumor necrosis factor (TNF-α), which in turn activate macrophages and microglia to start the autoimmune cascade of tissue destruction and demyelination [52]. IFN-γ also enhances adhesion molecules on endothelial cells. These adhesion molecules help allow mononuclear cell infiltration into the CNS that are characteristic of both MS and EAE lesions [52]. In EAE and MS, there has been contradictory evidence regarding the role of the inflammatory cytokine IFN-γ. IFN-γ and IFN-γ receptor (-/-) mice get more severe EAE; moreover, anti-IFN-γ antibodies exacerbate disease when given early in the disease course [53-58]. In MS patients, however, injected IFN-γ actually exacerbated disease [59]. Conversely, also in MS, recent clinical trials with anti-IFN-γ receptor antibody have shown a decrease in disease progression [60]. This suggests that IFN-γ may have dual roles, play a stronger role at certain time points, or have a different role in humans than in mice. Other Th1 cytokines believed to be involved in EAE pathogenesis are lymphotoxin α (LT- α), IL-1β, and IL-6. LT-α, which has direct effects on oligodendrocytes, can be neutralized with antibodies, which will prevent and reduce severe EAE [61]. IL-1β and IL-6 are now also considered Th17 cytokines as explained below.

MS has always been characterized as a Th1 $CD4^+$ T cell-mediated disease. However, recent publications in the mouse model have shown involvement of the Th17 $CD4^+$ subset which release the cytokine IL-17. Previously it was believed that the IFN-γ inducing cytokine IL-12 was important for EAE disease induction. This belief changed when knockouts of the IL-12 subunits, p40 and p35 yielded different results. p40 (-/-) mice are resistant to EAE and p35 (-/-)

mice are not, but it turns out p40 is also shared with IL-23. IL-23 is essential for EAE disease, not IL-12. IL-23 induces the cytokine IL-17 which plays a crucial role in EAE [62-64]. Now some cytokines are classified as Th17-associated including IL-1β, IL-6, IL-17, and IL-23.

In addition to the disease-enhancing Th1 and Th17 cytokines, T cells also secrete Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13. Th2 cytokines are often considered helpful in ameliorating EAE. IL-4 has been shown to mitigate EAE when administered to diseased mice. IL-4-deficient mice get more severe EAE, as IL-4 is essential for the production of a Th2 response [65, 66]. IL-10 also ameliorates EAE when administered *in vivo*. Like IL-4, IL-10-deficient mice also are prone to severe EAE [67-69]. In addition, transforming growth factor- β (TGF-β) is an important regulatory cytokine. TGF-β will down regulate MHC class II and inhibit T cell activation [70-73].

$CD8^+$ T cells in EAE and MS

$CD8^+$ T cells are mainly known for their roles in viral immunity and tumor surveillance. $CD8^+$ T cells can kill through direct lysis of virally infected cells or through secretion of TNF-α and IFN-γ. The memory response of $CD8^+$ T cells allows for a stronger response following re-infection with the same antigen. The role of $CD8^+$ T cells in autoimmunity has remained controversial over the years. In the 1980s the suppressor T cell was a debated topic before opposition won out. The suppressor T cell did not return for a decade until Sakaguchi et al. showed that by reconstituting nude mice with CD25 depleted $CD4^+$ T cells, spontaneous autoimmunity could be induced. Sakaguchi also showed that replenishing the $CD4^+CD25^+$ population could prevent autoimmunity [74].

As mentioned earlier, EAE is mainly a $CD4^+$ T cell disease. Since EAE can be passively transferred by encephalitogenic $CD4^+$ T cells, it is conceivable why $CD8^+$ T cells were largely ignored as playing a major part is the disease. In MS and EAE there is evidence for both a pathogenic and a regulatory role for $CD8^+$ T cells. In support of a pathogenic role for $CD8^+$ T cells, it was observed in MS that $CD8^+$ T cells actually outnumbered $CD4^+$ T cells in MS lesions [43, 52, 75]. The number of $CD8^+$ T cells and macrophages also correlates with the amount of axonal damage in MS lesions [76, 77]. There are also studies that show $CD8^+$ T cells kill oligodendrocytes and neural cells directly [77, 78]. From the animal studies, several groups have shown a role for $CD8^+$ T cells in the pathogenesis of disease. In 2001, Huseby et al. showed that adoptive transfer of $CD8^+$ T cells clones into wildtype and SCID C3H mice could induce EAE [79]. The EAE that developed was atypical in pathology and exhibited lesions in brain. In typical EAE, lesions are found throughout the spinal cord. Also in 2001, another study was published by Sun et al, but in C57BL/6 mice using the MOG 35-55 peptide. This study took autoreactive $CD8^+$ T cells and adoptively transferred them into naïve or RAG (-/-) mice inducing EAE. Very interestingly, this study also showed that EAE could not be induced by transferring autoreactive $CD8^+$ T cells into β2-microglobulin gene-deleted recipients [80].

In 2005, Ford et al. showed that MOG-reactive CD8+ T cells could be adoptively transferred into naïve C57BL/6 mice or SCID mice and induce EAE [81]. This group also identified a MHC class I ($H\text{-}2D^b$) restricted epitope (MOG 37-46). MHC class I ($H\text{-}2D^b$) tetramers can be used to identify these MOG-specific $CD8^+$ T cells in the CNS.

In 1992, Koh et al. published a study that supported both a pathogenic and a regulatory role for $CD8^+$ T cells. They showed that CD8 (-/-) mice suffered more relapses from EAE than littermate controls (pathogenic role). However, they also showed that although disease incidence

and onset was the same, EAE in CD8 (-/-) mice was milder and exhibited less mortality compared to wildtype mice [82]. These observations indicate a contradictory regulatory role for CD8+ T cells though it is possible CD8+ T cells play a different role at different times of disease.

In DBA/1 CD4 and CD8 (-/-) mice, other key observations were made. In both sets of knockouts, susceptibility to EAE was decreased. However, when $CD4^+$ T Cells were depleted from CD8 (-/-) mice, this protected from EAE at a much higher rate than the reverse situation [83]. This indicated a regulatory role for $CD8^+$ T cells. Jiang et al. also showed that $CD8^+$ T cells have a regulatory role through CD8 depletion antibody experiments. Using B10.PL mice, $CD8^+$ T cells were depleted prior to immunization and showed no change in disease incidence, onset, or severity [84]. They concluded that because disease incidence or severity did not decrease that the $CD8^+$ T cells did not have a pathogenic role. When they CD8 depleted later in the disease course, during the recovery phase, resistance to secondary disease was lost. This indicates that the $CD8^+$ T cells have a regulatory role. Additional studies done by Montero et al. showed that CD8 depletion prior to disease induction results in more severe EAE again supporting a regulatory role for $CD8^+$ T cells [85].

In 2004, Hu et al. published a study that may have implications with our current work. This group showed that Qa-1 (-/-) mice had increased susceptibility to proteolipid protein (PLP)-peptide induced EAE. This study showed that Qa-1-dependent and restricted $CD8^+$ T cells have inhibitory activity that works directly on pathogenic $CD4^+$ T cells [86]. This may provide a mechanistic explanation for some of our experimental observations that will be discussed later.

MS Therapies

There are currently several FDA-approved therapies available for MS patients. IFN-β-1a and IFN-β-1b are Type I interferon treatments for relapsing remitting MS (RRMS). IFN-β-1a (Avonex and Rebif) is derived from Chinese Hamster Ovary cells (CHO) transfected with the human IFN-β gene. Avonex is injected once a week, intramuscularly. Rebif is injected three times a week, but subcutaneously. IFN-β-1a, like natural IFN is glcoslylated containing a single N-linked carbohydrate moiety [87, 88]. IFN-β-1b (Betaseron) is used weekly as a subcutaneous injection. IFN-β-1b is mass produced from *E.coli* with the gene for human IFN-β without the carbohydrate side chains. IFN-β is believed to work by enhancing regulatory suppressor T cell activity, reduction of Th1/Th17 cytokines, and down modulation of antigen presenting cells [89, 90]. IFN-β acts through the IFN-β receptor and decreases activation of autoreactive T cells. IFN-β inhibits MHC class II expression and induces secretion of anti-inflammatory cytokines. IFN-β also inhibits MMP-9 protease secretion and blocks leukocyte migration to the CNS which reduces the number of lesions.

Natalizumab (Tysabri) is a monoclonal antibody that binds to α4-integrin thereby preventing migration of T cells across the endothelium into inflamed parenchymal tissue [91-93]. Natalizumab has been shown to decrease the $CD4^+$ and $CD8^+$ T cells, CD19+ B cells, and plasma cells in the cerebral spinal fluid of MS patients. Natalizumab now carries a warning because there is an increased risk of progressive multifocal leukoencephalopathy (PML), a brain infection caused by the JC virus which can lead to death [94].

Several other immunosuppressive drugs exist for the treatment of MS. Mitoxantrone (Novantrone) is a synthetic antineoplastic anthracenedione used intravenously every three months. Mitoxantrone has been observed to inhbit B cell, T cell, and macrophage activation [95-

97]. Methotrexate (Rheumatrex), azathioprine (Imuran), cyclophosphamide (Cytoxan), cladribine (Leustatin), and intravenous immunoglobulin G (IVIg) are immunosuppressive agents with some observed success, but they are generally not as effective or prescribed as IFN or glatiramer acetate for the treatment of MS [98-106].

Glatiramer Acetate (GA; Copaxone)

Glatiramer acetate (GA, Copaxone, COP, YEAK) is a random copolymer made up of four amino acids, L-glutamic acid, L-alanine, L-tyrosine, and L-lysine, at an average molar ratio of 0.141: 0.427: 0.095: 0.338. GA has an average molecular weight of 5,000-9,000 daltons and is 40-100 residues in length. GA was discovered in 1971 by Sela and Teitalbaum when they were trying to synthesize a copolymer similar to myelin basic protein. To their surprise, GA actually inhibits EAE in several animal models [107-110]. GA eventually proved its *in vivo* efficacy in clinical trials by reducing the number of relapses and decreasing the severity of relapses [111-113]. GA is the second most prescribed FDA-approved drug for relapsing remitting multiple sclerosis (RRMS) and is administered in a daily subcutaneous dose of 20mg [112-114].

The mechanism of action of GA is not completely understood although several theories exist. The predominant dogma is that GA induces a Th1 to Th2 shift in the $CD4^+$ T cells and then suppresses by bystander inhibition of the Th1 response [115-117]. Some studies have shown that GA directly competes with myelin related antigens for MHC binding [118]. It has also been suggested that GA cross-reacts with MBP [117]. Follow-up studies have shown that not only does GA competitively inhibit MHC binding, but will even displace antigens that are already bound. Specifically, GA was confirmed to bind to HLA-DR as confirmed by its inhibition with

the relevant anti-MHC class II antibodies or analogs thereby inhibiting T cell responses [119]. It is also believed that GA works by inducing clonal anergy and deletion of T cells [120, 121].

Other groups have shown that GA may have effects on antigen presenting cells [122-125]. Weber et al. has shown that GA induces type II monocytes which are defined by their cytokine profile of increased IL-10 and TGF-β, and decreased IL-12 and TNF-α. These type II monocytes were able to ameliorate EAE after adoptive transfer even when specific for a foreign antigen indicating that GA specificity and self-antigen recognition were not required for this effect [123]. Vieira et al. has shown that dendritic cells exposed to GA are not able to secrete the major Th1 polarizing factor IL-12p70. These DC induce IL-4 secreting Th2 T cells when exposed to GA and increased IL-10 [126].

Hong et al. reported that GA induced the conversion of CD4+ CD25- T cells to CD4+ CD25+ regulatory cells through Foxp3 expression. The formation of these T regulatory cells was dependent on IFN-γ, as Tregs were not formed in the GA-treated IFN-γ (-/-) [125]. GA also induces GA-reactive T cells to produce brain-derived neurotrophic factor (BDNF) for neuroprotective effects [127].

As previously mentioned, because MS has been thought of as a $CD4^+$ T cell mediated and regulated disease, the majority of previous MS and EAE studies have focused on the $CD4^+$ T cell subset. Our lab has shown that untreated MS patients have a deficient $CD8^+$ T cell response to GA compared to healthy individuals. Following daily GA therapy, the $CD8^+$ T cell response is restored to levels comparable to healthy adults [128]. **We hypothesized that GA-induced $CD8^+$ T cells play a vital role in mediating the immunomodulatory effect of the drug.** The necessity or sufficiency of these cells in the therapeutic modulation of autoimmune demyelination is best studied in the murine system with the availability of adoptive transfer and

knockout models. To address our hypothesis, our first specific aim was to determine if a GA-specific response was present in mice and characterize the response. Our second aim was to determine the *in vivo* relevance of these GA-induced $CD8^+$ T cells and determine the mechanism of action.

In this study we show the presence of a $CD8^+$ T cell response to GA and then elucidate the regulatory role of therapy-induced $CD8^+$ T cells during the modulation of EAE. We show the first evidence that GA-induced $CD8^+$ T cells are required for the clinical benefit of GA therapy, characterize their phenotype during protection from severe EAE, and evaluate their functional profile.

Chapter II: Methodology

Mice

C57BL/6 female mice were purchased from Taconic (Hudson, NY) and the UT Southwestern Mouse Breeding Core Facility (Dallas, TX). B6.129S2-Cd8a (CD8 -/-) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were housed and bred in the UT Southwestern Medical Center Animal Resource Center in accordance with the regulations of the Institutional Animal Care and Use Committee (IACUC).

Peptides

MOG 35-55 (MEVGWYRSPFSRVVHLYRNGK) and OVA 323-339 (ISQAVHAAHAEINEAGR) were purchased from the Protein Chemistry Technology Center, UTSWMC (Dallas, TX). Peptides were reconstituted in PBS according to manufacturer's guidelines at a working concentration of 4 µg/µl.

Active EAE Induction

7-10 week old C57BL/6 mice were immunized subcutaneously at two injection sites with 200 µg MOG 35-55 emulsified in complete Freund's adjuvant (CFA) (Difco) supplemented with 4 mg/ml Mycobacterium tuberculosis (MT) (H37Ra, DIFCO, Detroit, MI) in a final volume of 0.1 mL. On days 0 and 2, C57BL/6 mice were administered 250 ng pertussis toxin in 0.1 ml of PBS (List Biological Laboratories) intraperitoneally. Clinical disease severity was monitored daily and scored according to the following EAE scale: 0, no clinical disease; 1, limp tail; 2, moderate hind limb weakness; 3, severe hind limb weakness and/or partial hind limb paralysis; 4, complete

hind limb paralysis; 5, quadriplegia; 6, death. All groups were represented across multiple cages and all clinical scoring was performed in a blinded manner.

Glatiramer Acetate Immunization

GA (TEVA Neuroscience) was graciously donated by the Multiple Sclerosis Center at St. Paul Hospital (Dallas, TX). 1 or 2 mg GA was emulsified in incomplete Freund's adjuvant (IFA) (Difco) and PBS at a final volume of 0.2 ml and injected at 3 subcutaneous sites. 20 µg GA in 0.1 ml PBS was administered at 1 subcutaneous site for daily treatment regimens.

Passive EAE Induction

C57BL/6 mice were immunized with MOG 35-55 as described above. Splenocytes were harvested either 10 or 25 days post-immunization. These cells were then incubated for 72 hours at 37ºC in EAE 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 β-mercaptoethanol. MOG 35-55 (20 µg/ml) and mouse rIL-2 (10 pg/ml) were also added to the 72 hour stimulation. Live cells were then separated using a differential density gradient centrification (Ficoll-Paque Plus) (GE Healthcare, Sweden). $CD4^+$ T cells were purified by positive selection using CD4(L3T4) microbeads (Miltenyi Biotech, Germany) as per manufacturer's protocol with a purity of >94%. 4 – 10 x 10^6 $CD4^+$ T cells were injected intravenously into naïve, wild-type C57BL/6 female mice and monitored for disease according to the previously outlined clinical scoring system.

Protective Adoptive Transfer

Female C57BL/6 mice were immunized with GA or OVA 323-339 as described above. 20-25 days post-immunization, splenocytes were stimulated with either GA or OVA 323-339 (20 μg/ml) and mouse rIL-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-Paque gradient. $CD8^+$ cells were purified by positive selection using CD8a(Ly-2) microbeads (Miltenyi Biotech, Germany) with a purity of >94%. 2.5 – 10 x 10^6 CD8+ cells from the GA-immunized mice or the OVA-immunized mice were then injected into naïve, wild-type C57BL/6 mice or naïve, B6.129S2-Cd8a (-/-) mice via tail vein (day -1). 24 hours later (day 0), these mice were then immunized with MOG 35-55/CFA. Mice received pertussis toxin on days 0 and 2. Mice were randomized and monitored for clinical symptoms of EAE in a blinded manner.

Therapeutic adoptive transfer experiments

$CD8^+$ cells from GA-immunized and OVA-immunized mice were isolated and stimulated in the same manner as described above. C57BL/6 or B6.129S2-Cd8a (-/-) mice that had been previously immunized with MOG 35-55/CFA were normalized according to disease score and divided into groups. GA or OVA-induced $CD8^+$ cells were then injected via tail vein into each group. Mice were monitored daily for clinical symptoms of EAE in a blinded manner.

Adoptive Co-Transfer of $CD4^+$ and $CD8^+$ T Cells

$CD4^+$ cells: Lymph node cells and splenocytes were harvested from C57BL/6 mice 10 days post-immunization with MOG/CFA. Cell suspensions were stimulated with MOG 35-55 (20 μg/ml) and mouse rIL-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-Paque gradient. $CD4^+$ cells were purified by positive selection using CD4 (L3T4) microbeads (Miltenyi Biotech, Germany) with a purity of >94%. $CD8^+$ cells: Bulk splenocytes were also isolated from C57BL/6 mice 20 days post-immunization with GA or OVA/IFA, cultured, and isolated as described above. MOG-specific $CD4^+$ T cells were then mixed with either GA or OVA-induced $CD8^+$ T cells at a 1:0.5 or 1:0 ratio. Each group was then intravenously transferred into naïve, wild-type C57BL/6 mice and in a blinded manner monitored for clinical disease.

Tritiated Thymidine Incorporation Proliferation Assays

Lymph node cells and/or splenocytes were harvested from GA, OVA, or MOG immunized mice. $CD8^+$ cells were magnetically separated using the CD8a T Cell Isolation Kit, for negative selection or CD8a (Ly-2) microbeads, for positive selection (Miltenyi Biotech, Germany), as per manufacturer's protocols. Purity following isolation was consistently >94% pure. Bulk cells or $CD8^+$, or CD8 depleted cells were then incubated in 96-well plates for 72 hours at a concentration of 400,000 cells per well for bulk cells and 200,000 - 250,000 cells per well for $CD8^+$ cells at a final volume of 0.2 mL. Irradiated splenocytes from naïve mice or immunized mice were used as antigen presenting cells (APC) at a ratio of 1:4 or 1:5 ($CD8^+$ cells: APCs.) After 72 hours in culture, cells were pulsed with 0.5 µCi/well of [^{3}H]methyl-thymidine for 18-24 hours. On day 5, cells were harvested and analyzed for incorporation of radioactivity using a Wallac Betaplate counter (Wallac, Gaithersburg, MD). Results are expressed in CPM.

CFSE-Based Proliferation Assays

5- (and 6-) carboxyfluorescien diacetate succinimidyl ester (CFSE)-dilution assays were preformed as previously described [129]. For bulk cultures, 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 CFSE followed by addition of serum and two PBS washes. Stained cells were incubated at 1×10^6 cells/ml of culture media plus antigens. For $CD8^+$ proliferation, $CD8^+$ cells were isolated before staining using the positive or negative selection protocol described above. $CD8^+$ cells were suspended at 0.5-1 x 10^6 cells/ml of culture media. Irradiated, CD8-depleted splenocytes (3500 rads) from mice immunized with OVA 323-339/CFA or from naïve mice were used as APCs at a 1:5 ratio. 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–conjugated (APC-conjugate) anti-CD4 (Caltag/Invitrogen, Carlsbad, CA). Cells were washed and fixed in 1% paraformaldehyde (BD Biosciences). Flow cytometric data were acquired using a BD FACSort four-color flow cytometer using BD CellQuest software or BD LSR II using FACSDiva software. For analysis, FlowJo software (TreeStar, Ashland, OR) was used to gate on lymphocytes via forward and side scatter, and further on the $CD4^+/CD8^-$ or $CD8^+/CD4^-$ populations. The mean background proliferation was calculated based on the proliferating fractions in media alone. Proliferation was considered significant if the ΔPF (% proliferation in sample - % proliferation in background) was >1 and the stimulation index (SI = % proliferation in sample / % proliferation in background) was >2.

$CD8^+$ T cell Depletion

The 2.43 hybridoma cell line (ATCC, Rockville, MD) was a generous gift from Dr. Michael Bennett, UT-Southwestern Medical Center. This line secretes rat monoclonal antibody that is reactive with the CD8 surface marker [130]. Supernatants from the cell culture were saved at -20°C until they were purified in bulk by high performance liquid chromatography (HPLC) as previously described using a protein G affinity column [131]. 200 µg anti-CD8 was injected at day -11 and -8 and 44 µg were injected at -5 to deplete the CD8+ T cell population for 20 to 30 days. In a separate experiment, 200 µg were injected at day -1, day 6, and day 13 during EAE disease. The day -1 group was subsequently injected with a 100 µg anti-CD8 before sudden death of two mice prevented further boosters injections.

Histology

Mice were injected at day -10 with 2 mg GA or OVA/IFA. At day 0 mice were immunized with MOG/CFA to induce EAE and monitored for clinical symptoms. At day 28, mice were euthanized and brain and spinal cord sections were paraffin embedded in cassettes in collaboration with the UT-Southwestern Pathology Core. To assess immune cell infiltration, paraffin sections were stained with hematoxylin and eosin (H&E) and with luxol fast blue to examine demyelination. LFB staining was carried out as described by Kim et al [132]. Two researchers analyzed each tissue section for each mouse in a blinded manner. The LFB negative area, represents white matter demyelination was expressed as a % of demyelination.

In vitro Cytotoxicity Assay

This assay was adapted from human MS studies from our laboratory [133]. Wildtype mice were injected with 200µg GA/CFA at day 0. At day 12, mice were euthanized and splenocytes were stimulated in vitro with GA (20µg/mL) for 7 days. At day 19, CD8+ T cells were purified by negative selection (Effectors). In parallel, at day 18, naïve mice were euthanized and splenocytes were incubated overnight in GA (20µg/mL) and ConA (0.5µg/mL). After culture, cells are CFSE stained (Targets). Target cells are plated in a 96-well U bottom plate at 5000 cell per well in triplicate for each condition. Effector and Target populations are incubated together at different ratios 0:1; 1:1; 4:1; 16:1; 64:1 in a final volume of 0.2mL per well. The plates were incubated for 24 hours before fluorescent allophycocyanin beads (BD Biosciences) are added as an external number control. The cells were harvested and run on a BD FACSCalibur flow cytometer. The number of live, CFSE bright cells was quantified and normalized to the number of beads in each tube.

% Killing is calculated as:

$$\left[1 - \frac{\text{(Live CFSE target cells per 1000 beads for effector:target ratio)}}{\text{(Live CFSE target cell per 1000 beads for no effector cell well)}}\right] \times 100$$

The positive control was a redirected lysis assay with P815 cells, a mastocytoma cell line that expresses high levels of Fc receptor. P815 cells are incubated with anti-CD3 (OKT3) (1 µg/mL) and then $CD8^+$ T cells were added at the indicated ratios. The anti-CD3 binds to the Fc receptors on the P815 cells which in turn activate the effector cells via CD3 resulting in redirected lysis of the target cells [134].

Suppression Assay

Responder cells came from 4 sources: CD8 (-/-) mice were injected with MOG, GA, or OVA/CFA (200 µg) and euthanized at day 10 or CD8 depleted cells from C57BL/6 wildtype mice that were at day 17 of EAE disease. Suppressor cells were from C57BL/6 mice that were immunized with 1 mg GA or OVA/IFA 20 days prior. A third set of GA suppressor cells came from mice that were injected with 1 mg GA/IFA 35 days prior. $CD8^+$ T cells from this group were purified by positive selection. Responders and suppressors were set-up at increasing ratios (1:0, 1:0.1, 1:0.2, 1:0.5), incubated for 5 days with either no antigen, OVA, GA, (20 µg/mL) or ConA (0.5 µg/mL). Cells were harvested, stained with phycoerythrin-conjugated (PE-conjugated) anti-CD8 and allophycocyanin–conjugated (APC-conjugate) anti-CD4. Cells were washed and fixed in 1% paraformaldehyde. Flow cytometric data were acquired using a BD LSR II using FACSDiva software. For analysis, FlowJo v6 software was used to gate on lymphocytes via forward and side scatter, and further on the $CD4^+/CD8^-$ or $CD8^+/CD4^-$ populations.

% suppression was calculated by:

$$\frac{\text{(No Ag\% Proliferation – New Ratio \% Proliferation)}}{\text{(No Ag\% Proliferation)}} \times 100$$

ELISA Cytokine Detection Assay

ELISA assays were performed in 96-well plates that were purchased pre-coated or coated overnight in our laboratory. All ELISA experiments were preformed on mice that were 10 or 20 days post immunization (day 0). For cytokine analysis of $CD8^+$ T cells, supernatants were taken from the following cultures at 48, 72, and 120 hours: purified $CD8^+$ T cells from a GA-immunized mouse + CD8-depleted APCs from a OVA-immunized mouse; $CD8^+$ T cells from a

MOG-immunized mouse that had been protected from severe EAE by daily GA/PBS injection + CD8-depleted APCs from a OVA-immunized mouse; and $CD8^+$ T cells from a MOG-immunized mouse that had been injected with PBS control. Samples were analyzed in duplicate for the presence of IFN-γ or IL-10 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 $CD11b^+$ cells were measured in a similar fashion. $CD11b^+$ cells were purified by $CD11b^+$ microbeads (positive selection) (Miltenyi Biotech, Germany) from harvested splenocytes from mice that were treated with 150 μg GA for 7 days. $CD11b^+$ cells were incubated in 48-well plates at a concentration of $1x10^6$ cells/ml with no antigen, IFN-γ (10 units/ml), IFN-γ (100 units/ml), or lipopolysaccharide (1 mg/ml). Supernatants were collected at 48, 72 and 120 hours and stored at -80°C. 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 were then washed five times; detection antibody is added, and 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. 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 or IL-10. Statistical analysis was performed using Microsoft Excel software.

Quantitative Real-Time PCR assay

C57BL/6 mice were immunized with GA or OVA/IFA peptide for 20 days. Splenocytes were harvested and stimulated in an *in vitro* culture for 72 hours with GA or OVA (20 µg/ml) and mouse rIL-2 (10 pg/ml). Dead cells were removed by ficoll gradient and $CD8^+$ cells were purified by positive selection using CD8a(Ly-2) microbeads (Miltenyi Biotech, Germany). Purity was >94%. Total RNA was extracted from $1x10^6$ $CD8^+$ cells using RNeasy Mini Kit (Qiagen, Valencia, CA) and was reverse transcribed to cDNA using Ready-To-Go T-primed First Strand kit (Amersham, Piscataway, NJ) as per manufacturer's protocol. Quantitative real-time PCR assays were performed in 25 ul reaction volumes using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on the MX3000p thermocycler (Stratagene, La Jolla, CA) Primer pairs as listed in Table 2, (Invitrogen) were used to evaluate expression level of each protein relative to b-actin, a house keeping gene. The thermal protocol condition was: 95°C for 10 minutes x 1; 95°C for 45 seconds, 57°C for 1 minute, 72°C for 1 minute x 40 cycles, 95°C for 3 minutes. The SYBR Green fluorescence was measured at the extension phase of the reaction. The specificity of the product was confirmed by dissociation curve analysis as well as visualization of product size on agarose gels. The data were normalized to β-actin and were expressed as the mean relative expression of the indicated molecules.

Table 2: Primers used in RT-PCR Assay

Cytokine	Forward Primer	Reverse Primer
β-actin [135]	GTGGGCCGCTCT AGGCACCAA	CTCTTTGATGTC ACGCACGATTTC
IL-10 [135]	CAGAGCCACATG CTCCTA	GGAGTCGGTTAG CAGTATG
IFN-γ [135]	AGCAACAGCAAG GCGAAAAA	AGCTCATTGAAT GCTTGGCG
TNF-α [135]	CATCTTCTCAAA ATTCGAGTGACAA	TGGGAGTAGACA AGGTACAACCC

Chapter III: Establishing the EAE Model

EAE is a well established model of autoimmune demyelination. Although universally accepted, variations in disease induction protocol exist from laboratory to laboratory and it was essential to establish the model in our own animal facilities. The EAE model exists in several different strains including B10.PL, SJL/J, C3H, and C57BL/6. We chose to optimize the C57BL/6 model due to the availability of knockout mouse strains and vast amounts of literature in this strain. Previous attempts to establish the C57BL/6 mouse model in our lab had been unsuccessful. These attempts included injecting 200 μg or 400 μg MOG 35-55 emulsified in complete Freund's adjuvant or incomplete Freund's adjuvant and injecting in either 3 or 4 subcutaneous sites. Pertussis toxin was also injected at Day 0 and Day 2 intraperitoneally at dosages ranging from 250 to 500 ng.

Attempts had also been made to acquire MOG 35-55 antigen from various different sources coupled with PT injections at day 0 and day 2, but again without disease incidence. We received advice from Dr. Amy Lovett-Racke, Ohio State University, that injecting in 2 sites instead of 3 or 4 would be likely to induce disease. We injected MOG 35-55 in CFA supplemented with *Mycobaterium tuberculosis* (4 mg/mL) in 2 subcutaneous sites and injected 250 ng PT intraperitoneally at day 0 and day 2. This experimental design gave us 100% incidence. A typical EAE disease curve is shown in Figure 1.

All additional experimental attempts resulted in almost 100% incidence of disease. Disease onset typically occurred between day 10 and day 12 with peak disease occurring at approximately day 15. EAE disease in C57BL/6 mice has been reported to be typically a chronic disease [43-45]. We routinely saw chronic disease (Figure 1), but in some experiments we also saw some degree of remission. Mice that peaked to a score of 3 or 4 typically recovered to grade

1 or 2, with residual chronic disability. Most experiments were typically followed until 45-60 days post disease induction.

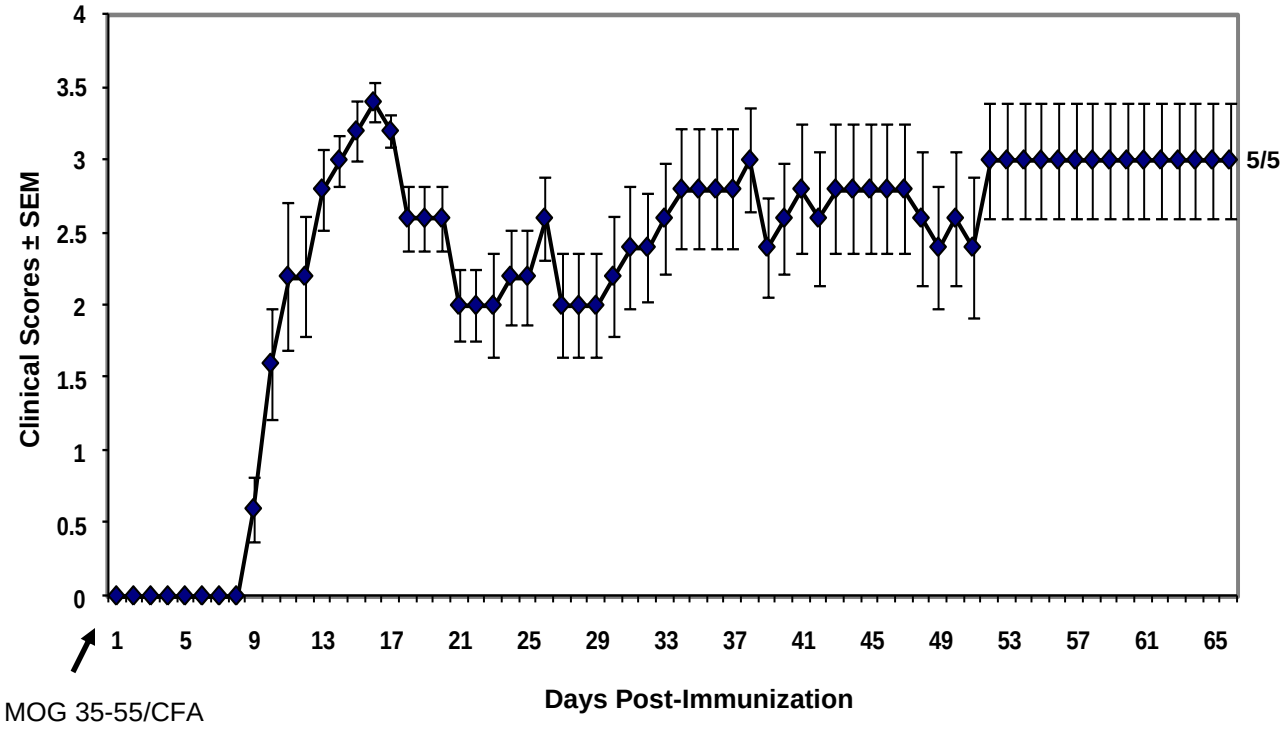

Figure 1: MOG (33-55)/CFA EAE disease in C57BL/6 mice. C57BL/6 wildtype female mice were immunized with 200 µg myelin oligodendrocyte glycoprotein (MOG) (35-55) emulsified in CFA supplemented with *M. tube*rculosis (4 mg/mL) at day 0. 250 ng pertussis toxin (PT) was injected at day 0 and day 2. Mice were scored for clinical disease until day 65. The graph shows mean clinical score +/- SEM of five mice.

Chapter IV: Glatiramer Acetate-Reactive $CD8^+$ T cells

As previously mentioned, because MS has been thought of as a $CD4^+$ T cell mediated and regulated disease, the majority of previous MS and EAE studies have focused on the $CD4^+$ T cell subset. Published studies from our lab have shown that human MS patients have a deficient $CD8^+$ T cell response to GA. Over the course of daily GA therapy, the $CD8^+$ T cell response to GA is restored to levels seen in healthy individuals [128]. Taken together, **we hypothesized that GA-induced $CD8^+$ T cells play a vital role in mediating the immunomodulatory effect of the drug.**

In the mouse model, there have been no previous reports of a $CD8^+$ T cell response to GA. One possibility was that a GA-specific response by $CD8^+$ T cells does not exist, which would be another difference between the human and mouse immune system. It was also possible that a $CD8^+$ T cell response to GA has not been previously reported because of the differences in drug administration. In humans, GA is given as a daily aqueous solution while in mice GA is administered emulsified in IFA at one injection time point.

Our first experiment was designed to optimize the methods of measuring a GA bulk response in the lymph nodes and spleen using different methods of GA administration. We injected 4 different groups of mice: 200 µg GA/CFA, 2 mg GA/IFA, 20 µg GA/PBS daily for 9 days, or 100 µg GA/PBS daily for 9 days. At day 10 post immunization we took lymph node cells (LNC) and splenocytes (SpC) and performed thymidine incorporation assays and CFSE (5' and 6' carboxyfluorescein diacetate succinimidyl ester)–based proliferation assays to determine if a GA-specific response was present. Our thymidine incorporation results show that LNC have a stronger proliferative response to GA (Figure 2) than SpC at day 10 post-immunization (Figure 3). The group that was injected with 200 µg GA/CFA predictably had a stronger proliferative

response than the other three groups due to the CFA adjuvant. Group 4 which was injected with 100 µg COP/PBS daily for 9 days did not elicit a proliferative response in the lymph node cells and very little response from the splenic cells.

GA induces not only a $CD4^+$ T cell response, but also a $CD8^+$ T cell response

Now that we had observed a bulk GA-specific response in the lymph nodes and spleen, we sought to determine if a $CD8^+$ T cell to GA existed. We injected 4 groups: 200 µg GA/CFA, 2 mg GA/IFA, 20 µg GA/PBS daily, and PBS/CFA + GA/PBS daily. We reasoned that in our new group the PBS/CFA might induce an inflammatory response initially and the daily GA/PBS might elicit a stronger antigen specific response in the context of an activated immune system. At day 10 post immunization we euthanized all 4 groups and performed thymidine and CFSE assays on bulk and $CD8^+$ purified T cells + irradiated antigen presenting cells (APC). In this experimental setup we did not process the lymph node and spleens separately to increase the starting population of bulk cells. In all four groups a clear antigen-specific dose-dependent bulk response was observed (Figure 4). The 200 µg GA/CFA, and 2 mg GA/IFA group did elicit a stronger response due to the use of an adjuvant and a higher concentration of GA. We confirmed the lymphocyte proliferation results with our CFSE flow-based proliferation assay. An advantage of the CFSE assay is that even in a bulk culture the responding cells can be phenotyped with antibodies. We found that GA induces not only a $CD4^+$ T cell response, but also a $CD8^+$ T cell response (Figure 5).

To determine the specificity of the observed response we purified the $CD8^+$ T cells by negative selection. This untouched $CD8^+$ population was combined with irradiated APCs at a 1:5 ratio and used in our thymidine proliferation assay. Figure 6.1 shows a $CD8^+$ T cell dose-

dependent response to GA in both the 200 µg GA/CFA and the 2 mg GA/IFA groups. The 200 µg GA/CFA group has a stronger response due to the CFA adjuvant. The 20 µg GA/PBS daily did not show a CD8 response to GA. The PBS/CFA + GA/PBS daily group showed a $CD8^+$ T cell proliferative response to GA (Figure 6.2), but not as strong as the CFA and IFA groups. These results confirm that a $CD8^+$ T cell response to GA does exist in the mouse model.

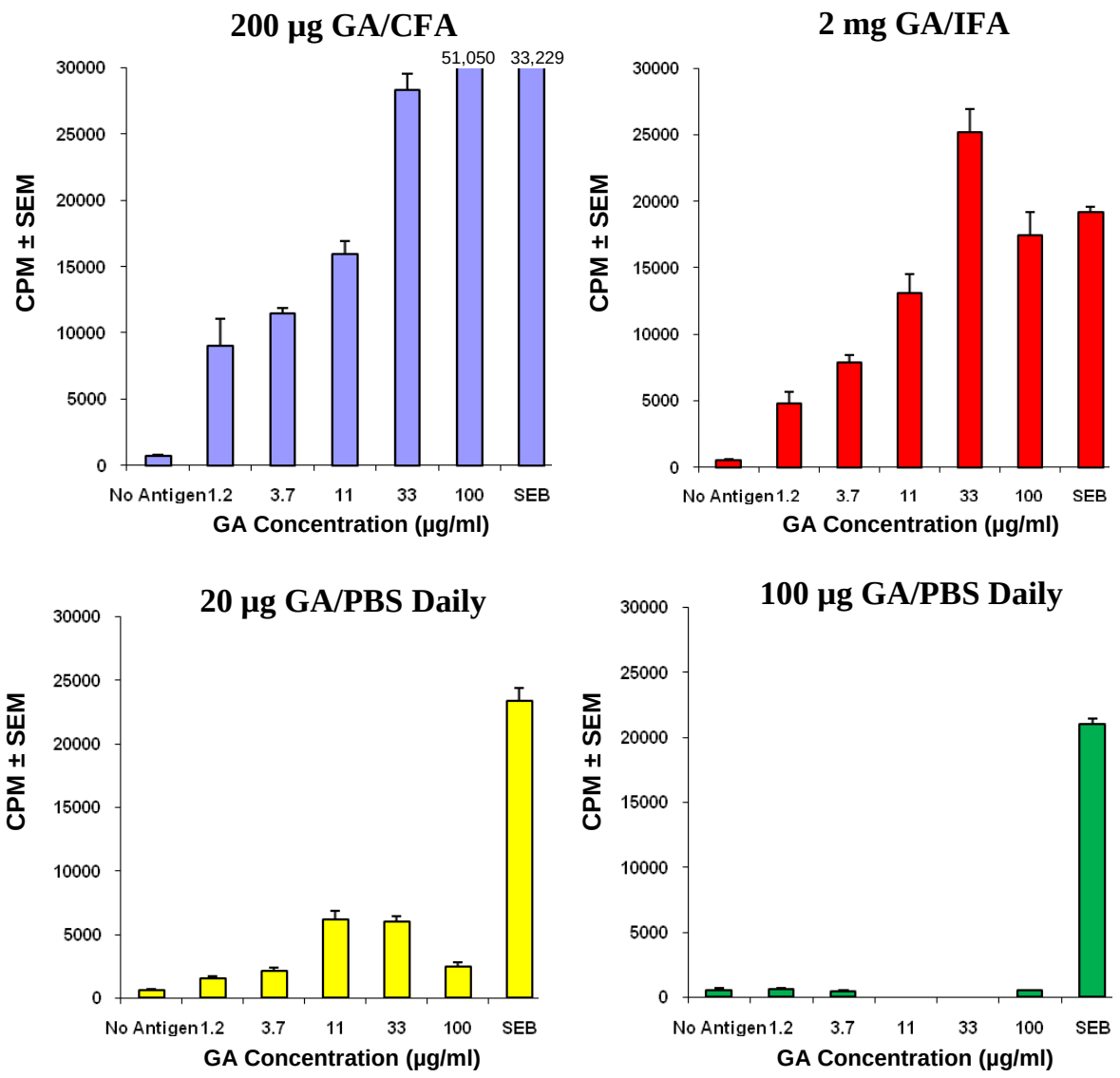

Figure 2: Dose-dependent proliferation of bulk lymph node cells to GA following different methods of *in vivo* GA administration. LNC were isolated from female wildtype C57BL/6 mice at day 10 following different methods of GA administration: 200 µg /CFA; 2 mg/IFA; 20 µg daily GA/PBS; or 100 µg daily GA/PBS. Cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of GA, or staphylococcal enterotoxin B (SEB). Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for isotope incorporation. This figure is representative of 3 independent experiments.

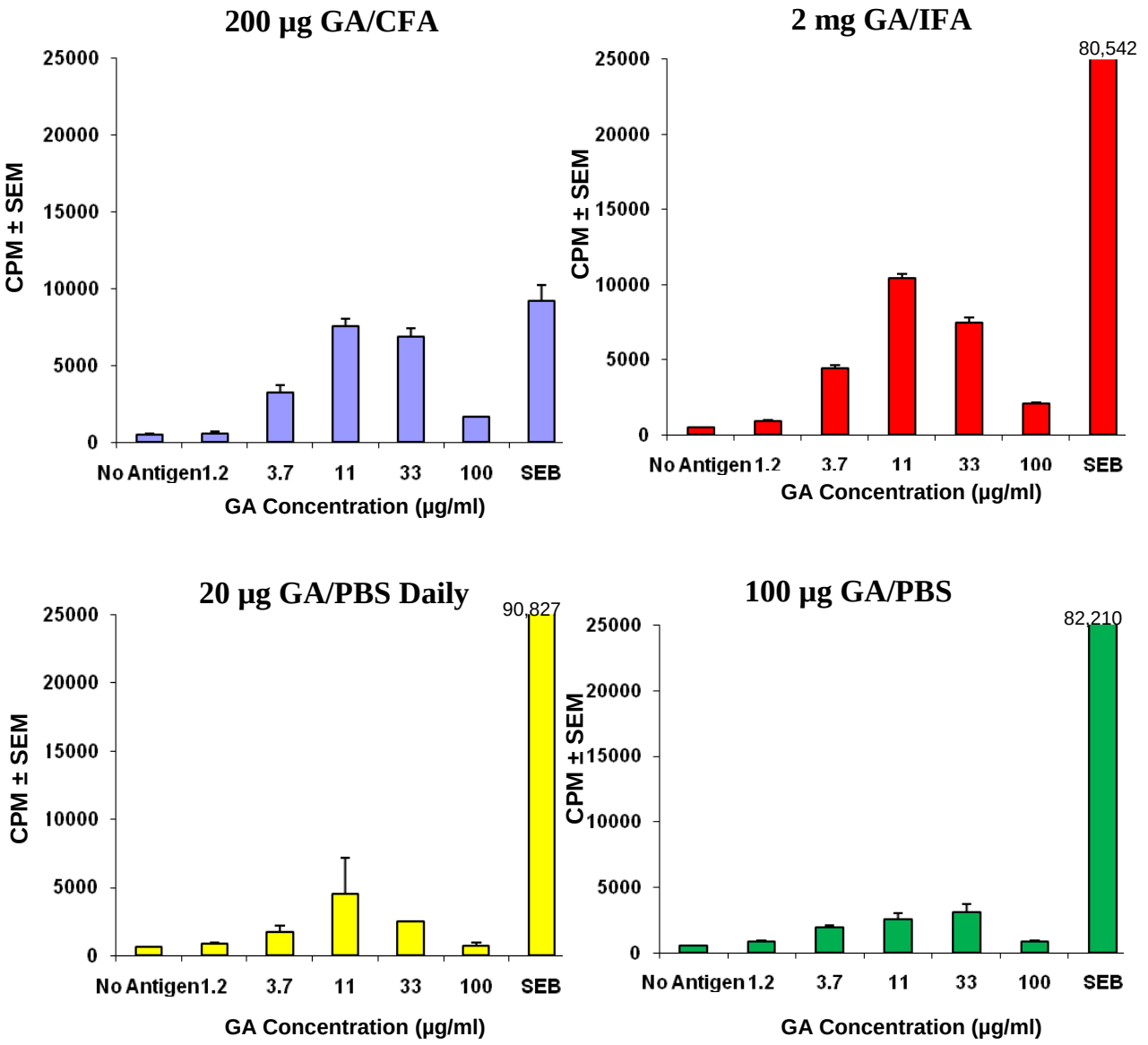

Figure 3: Dose-dependent proliferation of bulk spleen cells to GA following different methods of *in vivo* GA administration. SpC were isolated from C57BL/6 mice at Day 10 following different methods of GA administration: 200 µg/CFA; 2 mg/IFA; 20 µg daily GA/PBS; or 100 µg daily GA/PBS. Cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of GA, or SEB. Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. This figure is representative of 3 independent experiments.

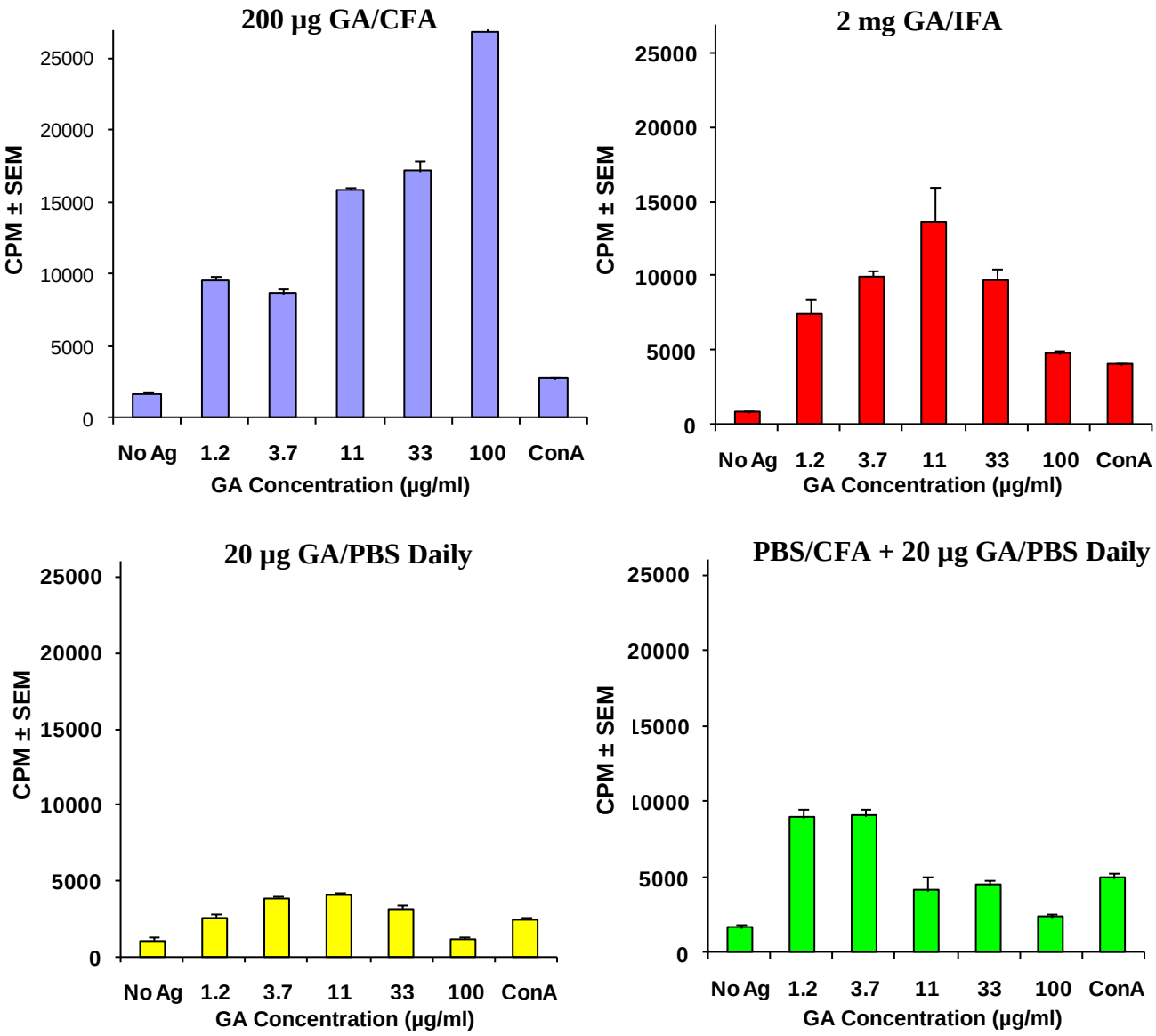

Figure 4: Dose-dependent proliferation of all bulk lymphocytes to GA following different methods of *in vivo* GA administration. Bulk cells were isolated from C57BL/6 mice at day 10 following 200 µg GA/CFA; 2 mg GA/IFA; 20 µg GA/PBS daily; or PBS/CFA at day 0 followed by 20 µg GA/PBS daily. Cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of GA, or ConA. Cells were pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. This figure is representative of 7 independent experiments.

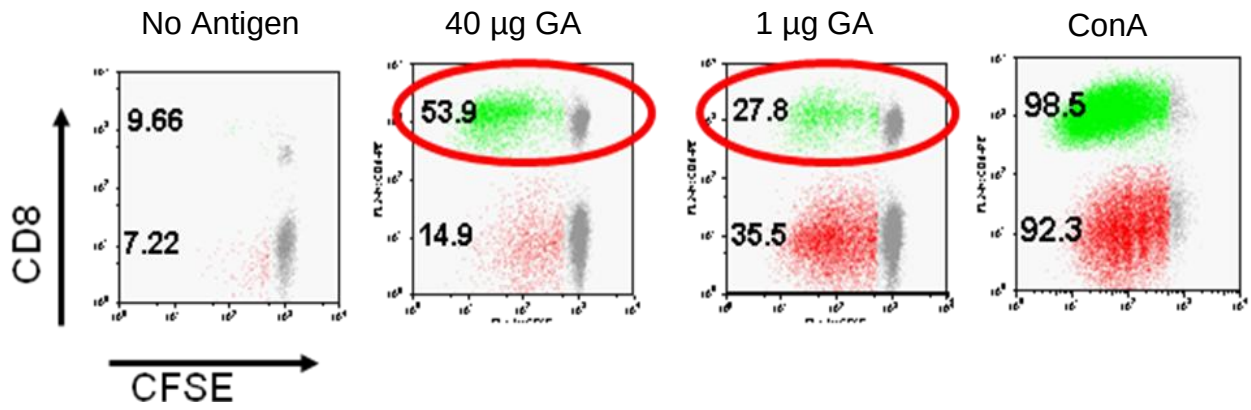

Figure 5: CD8$^+$ and CD4$^+$ specific proliferation to GA in a CFSE assay. Bulk cells were harvested from spleens and lymph nodes 10 days after injection with GA/IFA. Cells were incubated for 5 days with no antigen, GA, or ConA; stained with anti-CD4 and anti-CD8 flow antibodies; and run on a BD FACSort or BD LSRII. The data represent gated CD4$^+$/CD8$^-$ (red) or CD8$^+$/CD4$^-$ T cells (green). CFSE staining is shown on the *x* axis and CD8 staining is on the *y* axis. The gray populations represent nondividing cells. The numbers next to the green or red populations are the proliferating fraction of CD4$^+$ and CD8$^+$ T cells. This figure is representative of 10 independent experiments.

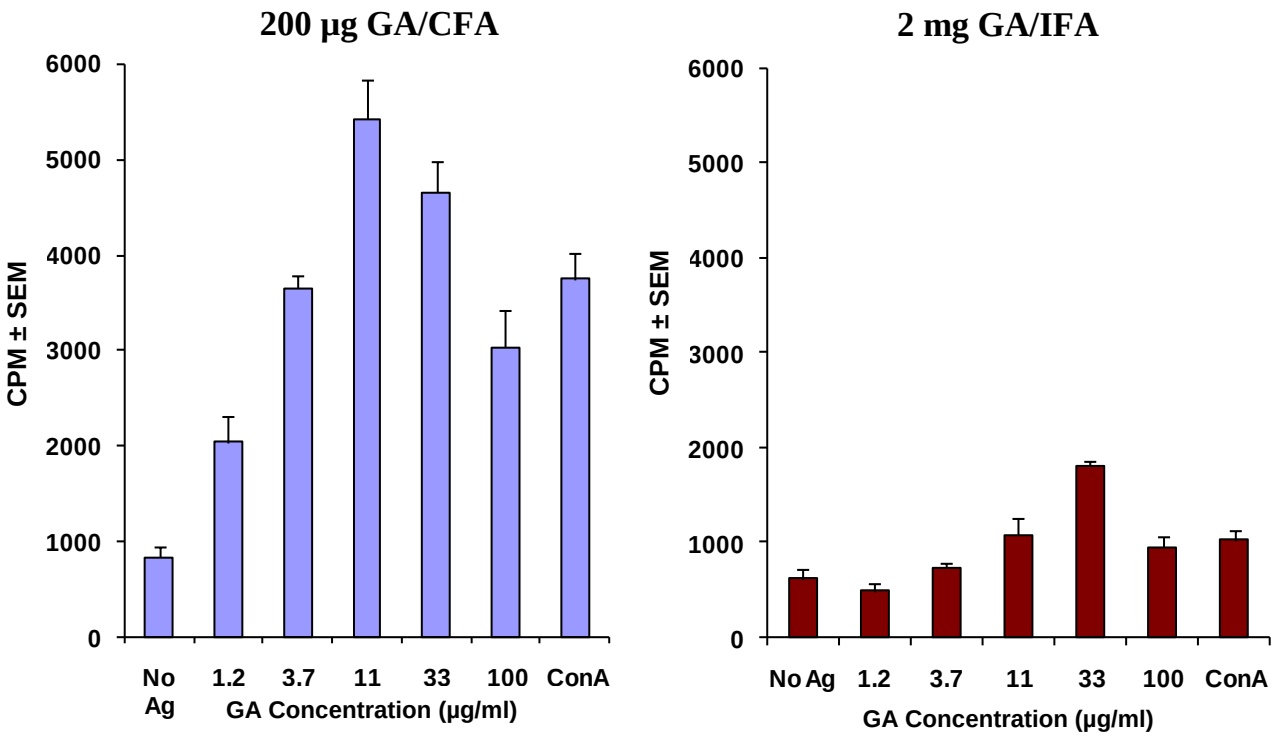

Figure 6.1: GA elicits a CD8$^+$ specific response following immunization with GA and adjuvant. CD8$^+$ T cells were isolated by negative selection from C57BL/6 mice injected with 200 µg GA/CFA or 2 mg GA/IFA 10 days prior. Untouched CD8$^+$ T cells were combined with x-irradiated APCs (3500 rads) at a 1:5 ratio in a 96-well plate in quadruplicate with no antigen, increasing concentrations of GA or ConA for 72 hours. After 72 hours, each condition was pulsed with tritiated thymidine and analyzed for thymidine incorporation 18 hours later. Data are expressed as counts per minute ± SEM. This figure is representative of 6 independent experiments.

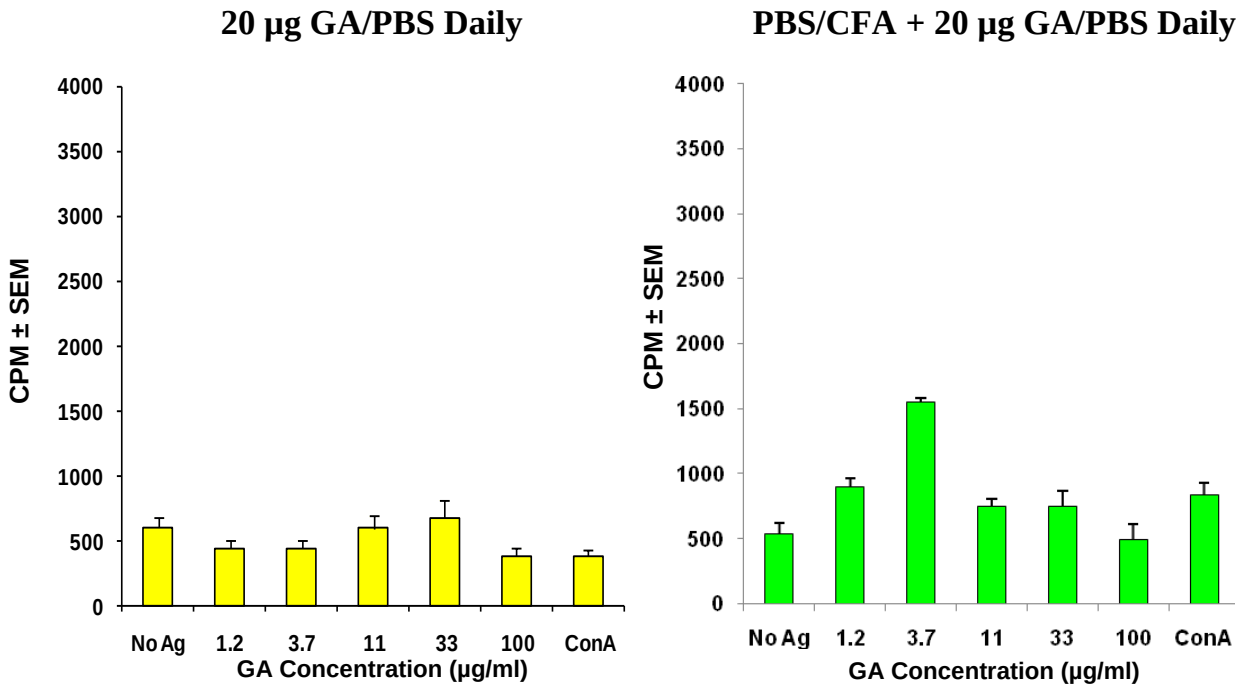

Figure 6.2: GA elicits a CD8$^+$ specific response following immunization with daily GA in an activated immune system (CFA immunized). CD8$^+$ T cells were isolated by negative selection from C57BL/6 mice that had been injected with 20 µg GA/PBS daily or PBS/CFA + 20 µg GA/PBS daily 10 days prior. Untouched CD8$^+$ T cells were combined with x-irradiated APCs (3500 rads) at a 1:5 ratio in a 96-well plate in quadruplicate with no antigen, increasing concentrations of GA or ConA for 72 hours. After 72 hours, each condition was pulsed with tritiated thymidine and analyzed for thymidine incorporation 18 hours later. Data is expressed as counts per minute ± SEM. This figure is representative of 5 independent experiments.

Chapter V: *In vivo* relevance of GA reactive CD8+ T cells

Now that we had observed that there is a GA-specific $CD8^+$ T cell response *in vitro* we wanted to determine if these $CD8^+$ T cells had an *in vi*vo relevance during autoimmune demyelination. GA has been shown to protect mice from EAE when administered ten days prior to MOG/CFA disease induction. Our first task was to replicate the published literature showing GA protection. We immunized wildtype female C57BL/6 mice with 2 mg GA or ovalbumin (OVA) 323-339 emulsified in IFA. Ten days later we immunized all mice subcutaneously with MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng pertussis toxin was also injected on day 0 and day 2. As seen in Figure 7, 2 mg GA/IFA protected wildtype mice from severe EAE while the control peptide (OVA) did not. Average clinical disease score was significant on days 14 to 17 ($p<0.05$). This protective result was reproducible at both 1 mg and 2 mg GA/IFA dosages over numerous experiments ($n>10$). Table 3 illustrates the peak severity and disease onset of one representative experiment.

Daily aqueous GA treatment is effective in mice similar to human MS

Since GA is administered to humans as a daily aqueous solution, we wanted to determine if disease protection in mice could be replicated by a different method of GA administration other than as an emulsion in IFA. In a blinded "pre-disease" experiment, we induced EAE with MOG/CFA as described above, but at day 2 we started treating half the mice with a daily 20 µg GA/PBS injection. The remaining mice received an injection control. Both groups were treated daily from day 2 to day 15. We evaluated the mice for clinical EAE symptoms until day 30. Results revealed that the 20 µg GA/PBS daily injection delayed disease onset by an average of 3 days and prevented severe EAE compared with the injection control (Figure 8). Table 4 illustrates the peak severity and disease onset of one representative experiment.

We then wanted to determine if diseased mice could be treated with daily GA/PBS once symptoms had already started, as such therapeutic treatments would be more relevant to human MS. Twelve mice were immunized to induce disease. Once the estimated peak clinical scores were reached, (day 11 in the first experiment), mice were divided into two groups normalized by average disease score. In a blinded manner, one group received a daily 20 µg GA/PBS injection and the other received an injection control from days 11 to day 24. Mice were evaluated for clinical disease until day 30. Daily GA/PBS immunizations ameliorated severe clinical symptoms when treatment was started at peak disease (day 11) and continued until day 24 (Figure 9). Table 5 illustrates the peak disease severity after day 15 (after therapy commenced + 4 days) and the disease trough for the same time frame.

A similar experimental design was used to evaluate the effectiveness of the 20 µg GA/PBS daily injection when treatment was started post-peak disease at day 16 and continued until day 29. Similar to the previous injection regimen, daily GA/PBS reduced the severity of established EAE compared to the injection control (Figure 10). Table 6 illustrates the peak disease severity after day 19 (after therapy commenced + 4 days) and the disease trough for the same time frame.

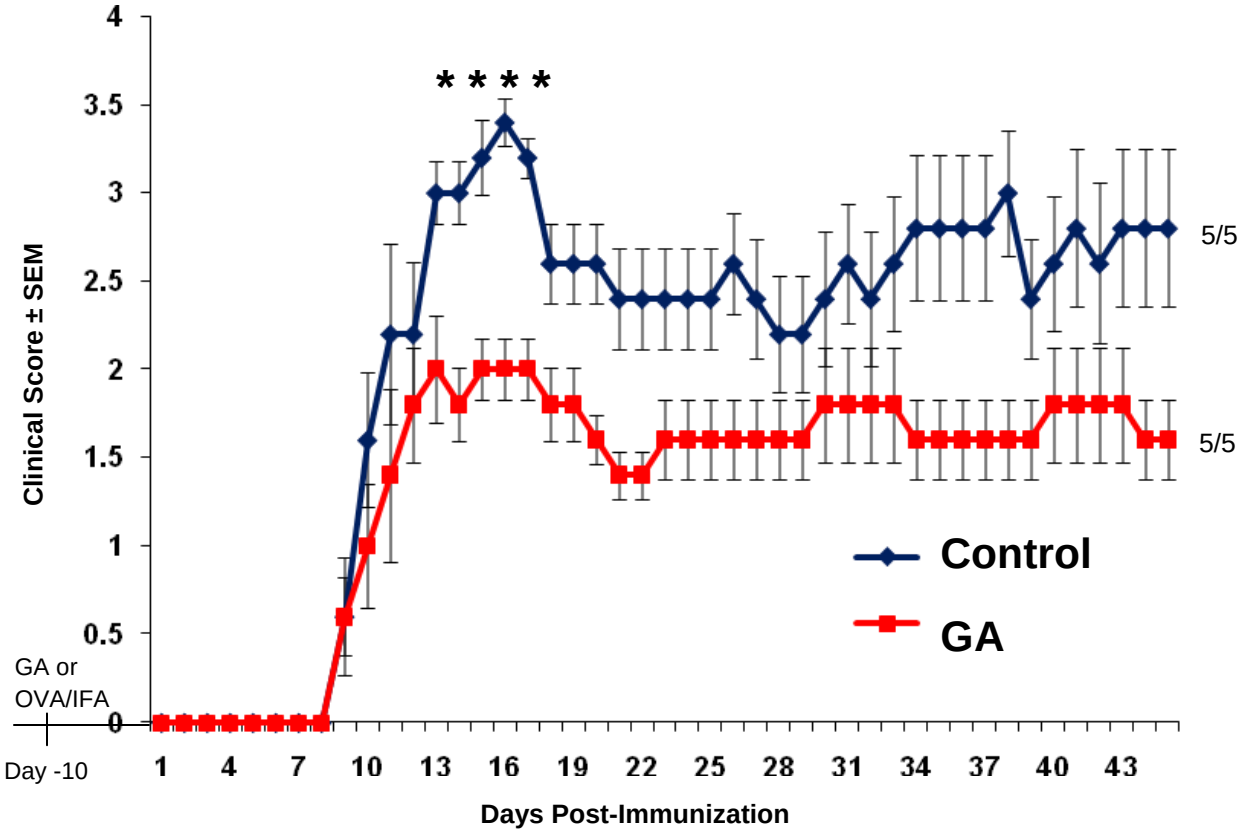

Figure 7: GA/IFA protects against Severe EAE when injected 10 days prior to disease induction. Female wildtype C57BL/6 mice were immunized subcutaneously with either 2 mg GA or OVA 323-339 emulsified in IFA. Ten days later all mice were immunized with MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected intraperitoneally at the time of EAE induction and 48 hours later. Mice were evaluated in a blinded fashion for 45 days. This figure is representative of 10 independent experiments. ***** indicates $p<0.05$

Table 3. Peak Disease and Disease Onset: GA/IFA Treated Vs. Control

	Incidence	**Peak Severity *$_{p= 0.051}$**	**Disease Onset**
Control	**5/5**	**4.0 +/- 0.00 ***	**10.6 +/- 0.45**
GA/IFA	**5/5**	**2.6 +/- 0.28 ***	**11.0 +/- 0.35**

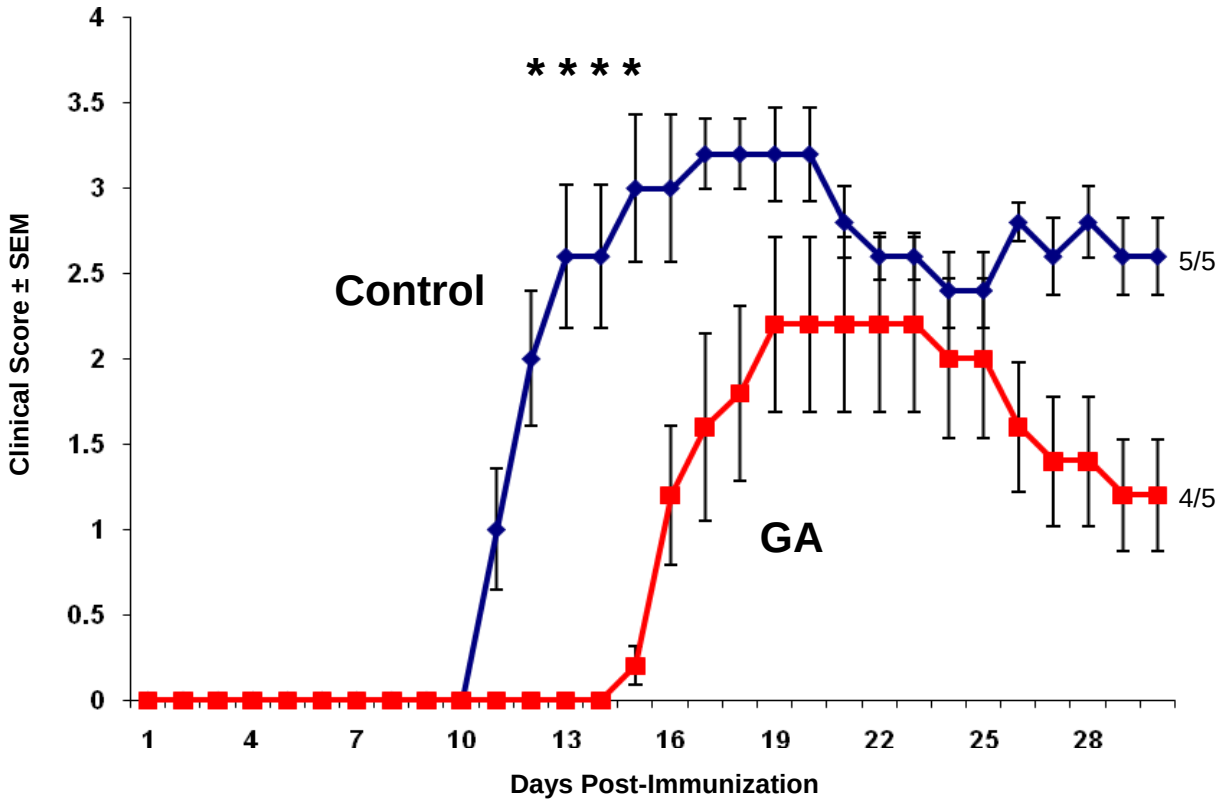

Figure 8: Treating before clinical onset of symptoms with daily GA/PBS prevents severe EAE. Female wildtype C57BL/6 mice were immunized with 200 µg MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. From day 2 to day 15 half the mice were treated subcutaneously with 20 µg GA/PBS daily or injection control. Mice were scored for clinical disease until day 30 in a blinded manner. The graph shows mean clinical score ± SEM of five mice. This figure is representative of 5 independent experiments. ***** indicates $p<0.05$

Table 4: Peak Disease and Disease Onset: GA-Treated Days 2 to 15 Vs. Control

	Incidence	Peak Severity	Disease Onset
Control	**5/5**	**4.0 +/- 0.00**	**12.6 +/- 0.63**
GA Treated D2 to 15	**4/5**	**2.8 +/- 0.41**	**20 +/- 2.48**

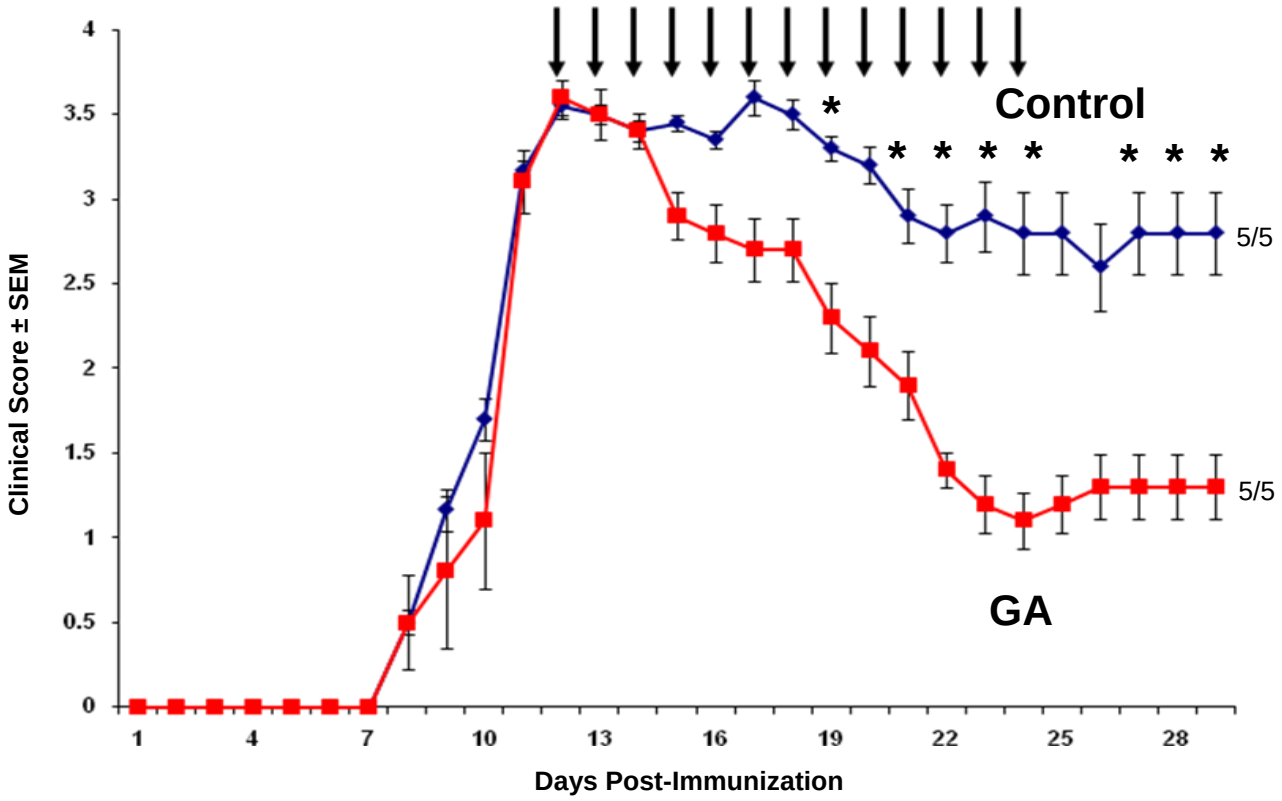

Figure 9: Treating with daily GA/PBS starting at peak disease reduces severe EAE. Female wildtype C57BL/6 mice were immunized with 200 µg MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. At day 11 mice were divided into 2 groups that were normalized for average disease score. From day 11 to day 24, one group of mice was treated subcutaneously with 20 µg GA/PBS daily and the other was given an injection control. Mice were scored for clinical disease until day 30 in a blinded manner. The graph shows mean clinical score ± SEM of five mice. This figure is representative of 3 independent experiments. * indicates $p<0.05$

Table 5: Peak and Trough Disease Post D15: GA-Treated Days 11 to 24 Vs. Control

	Incidence	Peak After Day 15 * p=0.048	Trough After Day 15 ** p= 0.029
Control	**5/5**	**3.7 +/- 0.11 ***	**2.6 +/- 0.26 ****
GA Treated D11-24	**5/5**	**3.0 +/- 0.13 ***	**1.1 +/- 0.16 ****

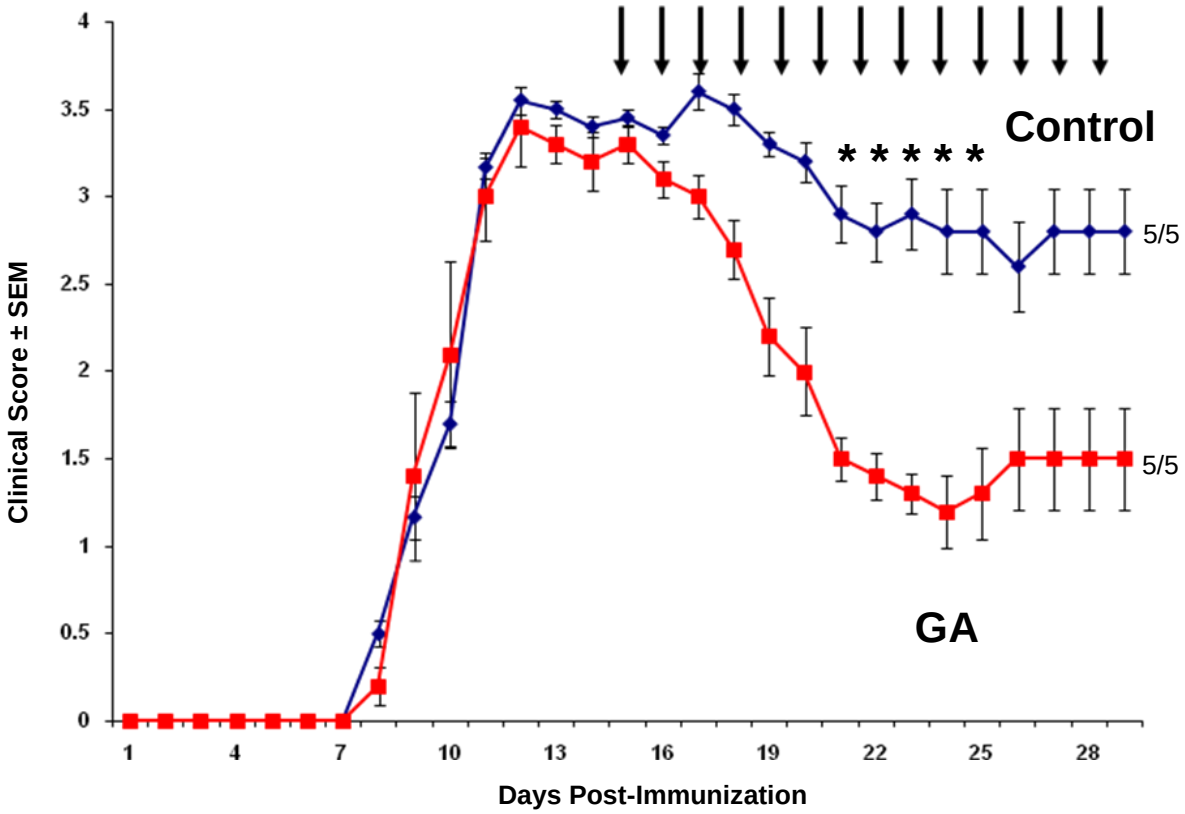

Figure 10: Treating with daily GA/PBS starting post-peak disease (day 16) reduces severe EAE. Female wildtype C57BL/6 mice were immunized with 200 µg MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. At day 16 mice were divided into 2 groups that were normalized for average disease score. From day 16 to day 29, one group of mice was treated subcutaneously with 20 µg GA/PBS daily and the other was given an injection control. Mice were scored for clinical disease until day 30 in a blinded manner. The graph shows mean clinical score ± SEM of five mice. This figure is representative of 3 independent experiments. * indicates $p<0.05$

Table 6: Peak and Trough Disease Post D19: GA-Treated Days 15 to 29 Vs. Control

	Incidence	Peak After Day 19 * p= 0.032	Trough After Day 19 ** p= 0.021
Control	**5/5**	**3.4 +/- 0.10 ***	**2.6 +/- 0.26 ****
GA-Treated D16-29	**5/5**	**2.0 +/- 0.25 ***	**1.0 +/- 0.13 ****

$CD8^+$ T cells are required for the *in vivo* efficacy of GA therapy

To determine the role of $CD8^+$ T cells in GA-mediated immune modulation we evaluated GA protection in the absence of $CD8^+$ T cells. We utilized two approaches to address this issue: 1) evaluate GA protection in CD8 (-/-) mice and 2) evaluate GA protection in wildtype mice following antibody depletion of $CD8^+$ T cells.

We injected CD8 (-/-) mice with either 2 mg GA or OVA/IFA 10 days prior to disease induction (day -10). At day 0, these CD8 (-/-) mice were immunized with MOG 35-55/CFA and PT as described above. Contrary to our results with wildtype mice, GA did not protect against severe EAE in the absence of $CD8^+$ T cells (Figure 11). In fact, GA/IFA immunized CD8 (-/-) got slightly more severe EAE than the control group. We further investigated the requirement of $CD8^+$ T cells using a method of GA administration more similar to humans.

Similar to the blinded pre-disease wildtype experiments, CD8 (-/-) mice were immunized with MOG/CFA to induce EAE at day 0 and then treated with either 20 µg GA/PBS daily or injection control from day 2 to day 15. Contrary to the wildtype experiments, daily GA did not prevent severe EAE in the absence of $CD8^+$ T cells (Figure 12). Of note, CD8 (-/-) mice in the pre-disease GA/PBS treatment group got worse EAE than the control group and resulted in a 20-40% mortality rate. The mortality rate of the control untreated CD8 (-/-) mice was 0%. Table 7 illustrates the peak disease and disease onset of one representative experiment. GA protection was also absent in the peak disease experimental setup further confirming that $CD8^+$ T cells are required for the therapeutic effects of GA (Figure 13).

To further support our conclusions from the CD8 (-/-) experiments suggesting that $CD8^+$ T cells are required for GA immune modulation, we depleted $CD8^+$ T cells from wildtype GA immunized mice and induced EAE. Normally this regimen confers protection against severe

disease (Figure 7). To acquire large quantities of CD8 depletion antibody, we set up the 2.43 hybridoma cell line (ATCC), generously donated by Dr. Michael Bennett. The hybridoma cell line was maintained for 8 weeks and supernatants containing the antibody were collected every 72 to 96 hours. The anti-CD8 antibody was purified by high performance liquid chromatography (HPLC) as described in the Methodology section above. 200 µg of anti-CD8 antibody or an injection control was injected at day –11 and day -8, and 44 µg at day -5 into wildtype mice. GA or control/IFA was injected at day -10. EAE was induced at day 0 with MOG/CFA and PT was injected at day 0 and 2. Clinical scores were evaluated for 45 days.

GA/IFA protection from severe EAE was observed in the group that was not $CD8^+$ T cell depleted. However, the GA/IFA immunized, $CD8^+$ T cell-depleted mice did not protect against severe EAE further confirming that CD8+ T cells are required for the efficacy of GA *in vivo*. The GA/IFA $CD8^+$ T cell-depleted group had a similar disease course to the group that was not immunized with GA (Figure 14). Flow analysis revealed that reconstitution of the $CD8^+$ T cell population occurred beginning at day 15, but $CD8^+$ T cells did not reach wildtype levels until day 30. This accounts for the observation that the GA/IFA $CD8^+$ T cell depleted group begins to show a slight recovery not observed in the control/IFA group after day 30. This indicates that while GA-reactive $CD8^+$ T cells may play some role in the induction phase of disease, they play a strong role in the effector phase. Table 8 illustrates the peak disease and post day 21 mean clinical disease for one representative experiment.

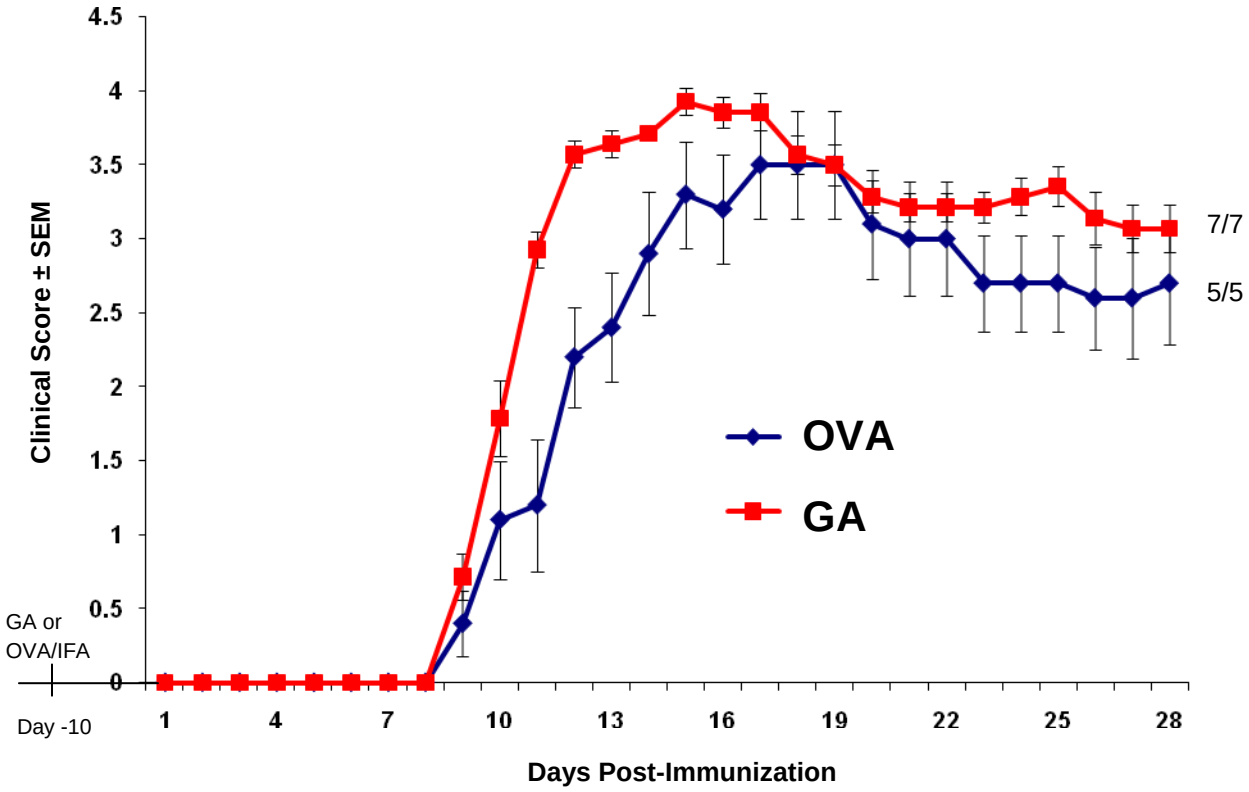

Figure 11: GA/IFA does not protect against severe EAE when injected 10 days prior to disease induction in the absence of CD8$^+$ T cells. Female CD8 (-/-) C57BL/6 mice were immunized subcutaneously with either 2 mg GA or OVA 323-339 emulsified in IFA. Ten days later all mice were immunized with MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected intraperitoneally at the time of EAE induction and 48 hours later. Mice were scored in a blinded manner until day 30. The graph represents the mean clinical score ± SEM of 7 GA/IFA mice and 5 control mice. This figure is representative of 4 independent experiments.

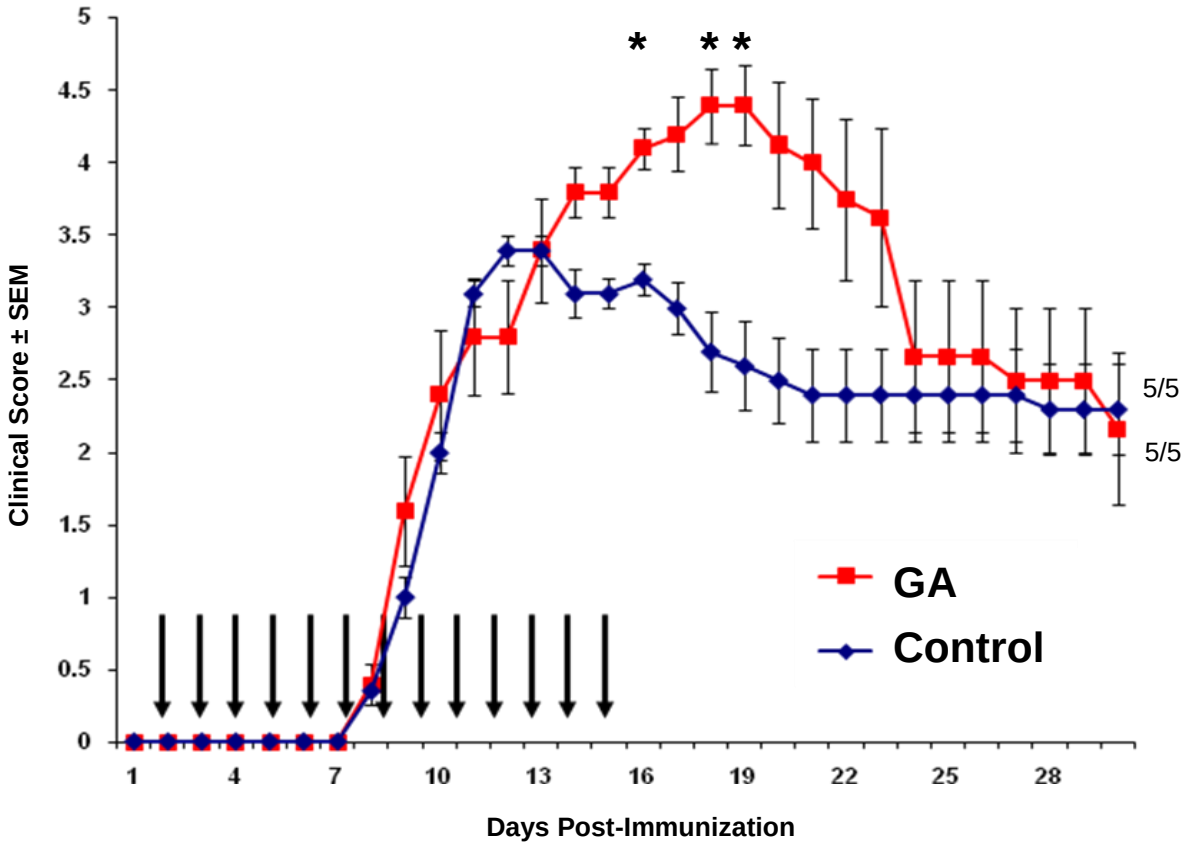

Figure 12: Treating before clinical onset of symptoms with daily GA/PBS does not prevent severe EAE in the absence of CD8$^+$ T cells. Female CD8 (-/-) mice were immunized with 200 µg MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. From day 2 to day 15 half the mice were treated subcutaneously with 20 µg GA/PBS daily or injection control. Mice were scored for clinical disease until day 30 in a blinded manner. The graph shows mean clinical score ± SEM of 5 mice. This figure is representative of 3 independent experiments. * indicates $p<0.05$

Table 7: Peak Severity and Disease Onset: CD8 (-/-) GA-Treated Days 2 to 15 Vs. Control

	Incidence	Peak Severity * p= 0.042	Disease Onset
CD8 (-/-) Control	**5/5**	**3.5 +/- 0.09 ***	**10.2 +/- 0.21**
CD8 (-/-) GA 2-15	**5/5**	**4.9 +/- 0.27 ***	**9.6 +/- 0.52**

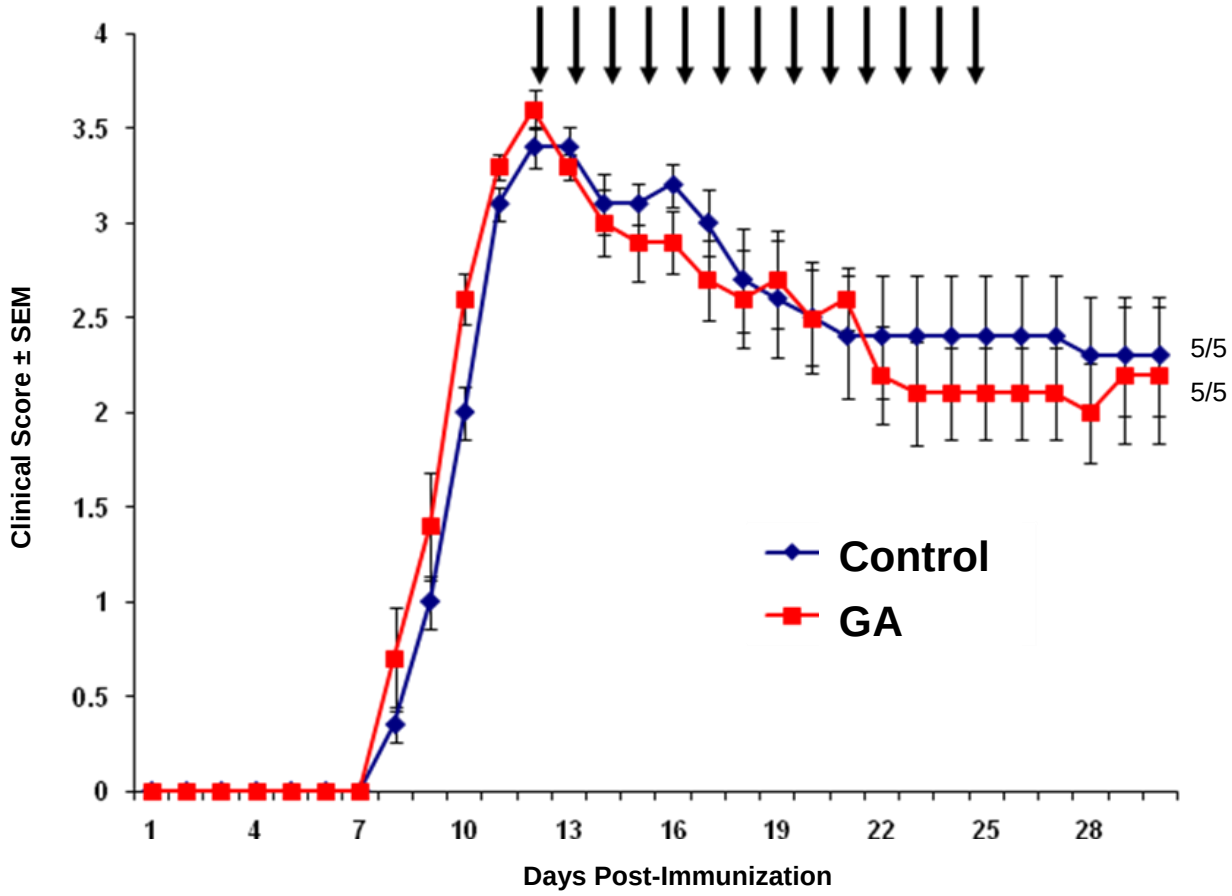

Figure 13: Treating with daily GA/PBS starting at peak disease does not reduce severe EAE in the absence of CD8$^+$ T cells. Female CD8(-/-) mice were immunized with 200 µg MOG 35-55/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. At day 11 mice were divided into 2 groups that were normalized for average disease score. From day 11 to day 24, one group of mice was treated subcutaneously with 20 µg GA/PBS daily and the other was given an injection control. Mice were scored for clinical disease until day 30 in a blinded manner. The graph shows mean clinical score ± SEM of five mice. This figure is representative of 3 independent experiments. $p>0.05$ in all categories.

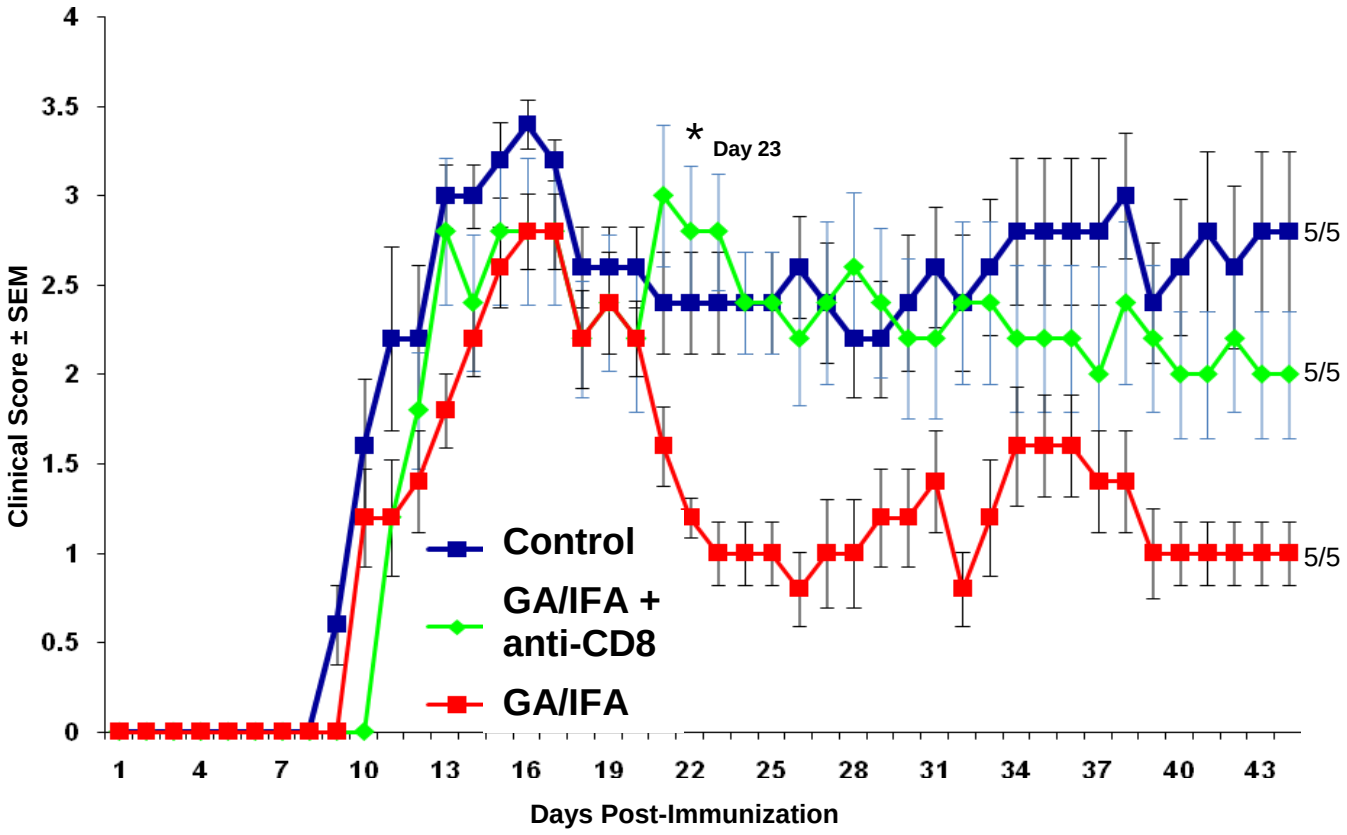

Figure 14: CD8$^+$ T cells are required for GA to prevent severe EAE. Female wildtype C57BL/6 mice were injected on Day -11, -8, and -5 with an anti-CD8 antibody (2.43 hybridoma). On Day -10, mice were injected with either 2 mg GA/IFA or control/IFA. EAE was induced on Day 0 with 200 µg MOG 35-55/CFA + 250 ng PT on days 0 and 2. Mice were evaluated for clinical symptoms until day 45 in a blinded manner. The graph shows mean clinical score ± SEM of five mice. This figure is representative of 2 independent experiments. * indicates $p<0.05$

Table 8: Peak Disease and Post Day 21 Mean: GA/CD8-Depleted Vs. GA

	Incidence	Peak Severity * p= 0.055	Post Day 21 Mean
GA/CD8-Depleted	**5/5**	**4.0 +/- 0.18 ***	**3.2 +/- 0.41**
GA/IFA	**5/5**	**3.0 +/- 0.18 ***	**2.0 +/- 0.25**

GA treated mice have less demyelination than control mice

As previously mentioned, mononuclear cell infiltration is seen by histology in MS lesions. Previous studies have reported MS-like lesions and demyelination in the EAE disease model [43]. We investigated whether or not GA treatment would affect lesions and demyelination in our model to correlate with the clinical observation. Two wildtype C57BL/6 groups of mice received either a 2 mg GA or control/IFA injection at day -10. EAE was induced at day 0 by MOG 35-55/CFA immunization and PT at day 0 and day 2 as described above. Typical GA protection was observed (Figure 7) and at day 28 mice from both groups were euthanized and brain and spinal cord sections were fixed in paraformaldahyde and processed for H&E staining and luxol fast blue (LFB) staining. In a blinded manner, two researchers then analyzed each tissue slice for infiltration, demyelination, and lesions (Figures 15 and 16). LFB stains myelin, thus % demyelination is calculated based on the disappearance of the blue LFB stain. We observed that GA-treated mice had less demyelination in the spinal cord than control mice (Figure 17). This result was reproducible (n=4).

GA does not protect against demyelination in the absence of $CD8^+$ T cells

Influenced by our previous findings, we investigated the role of $CD8^+$ T cells in GA protection from demyelination. Two groups of CD8 (-/-) mice were injected with either 2 mg GA or control/IFA. EAE was induced at day 0 by MOG 35-55/CFA immunization and PT at day 0 and day 2 as described above. We did not observe protection against EAE as expected (Figure 11) and mice were euthanized at day 28. Similar to wildtype groups, each mouse was processed individually, brains and spinal cords were sectioned, and H&E and LFB staining was performed. Two blinded researchers analyzed each tissue slice for infiltration, demyelination, and lesions (Figures 18 and 19). Our results indicate that GA does not protect from demyelination in the

absence of $CD8^+$ T cells (Figure 20). This result was reproducible (n=4). The degree of inflammatory cell infiltration in the GA-treated and control mice was also similar.

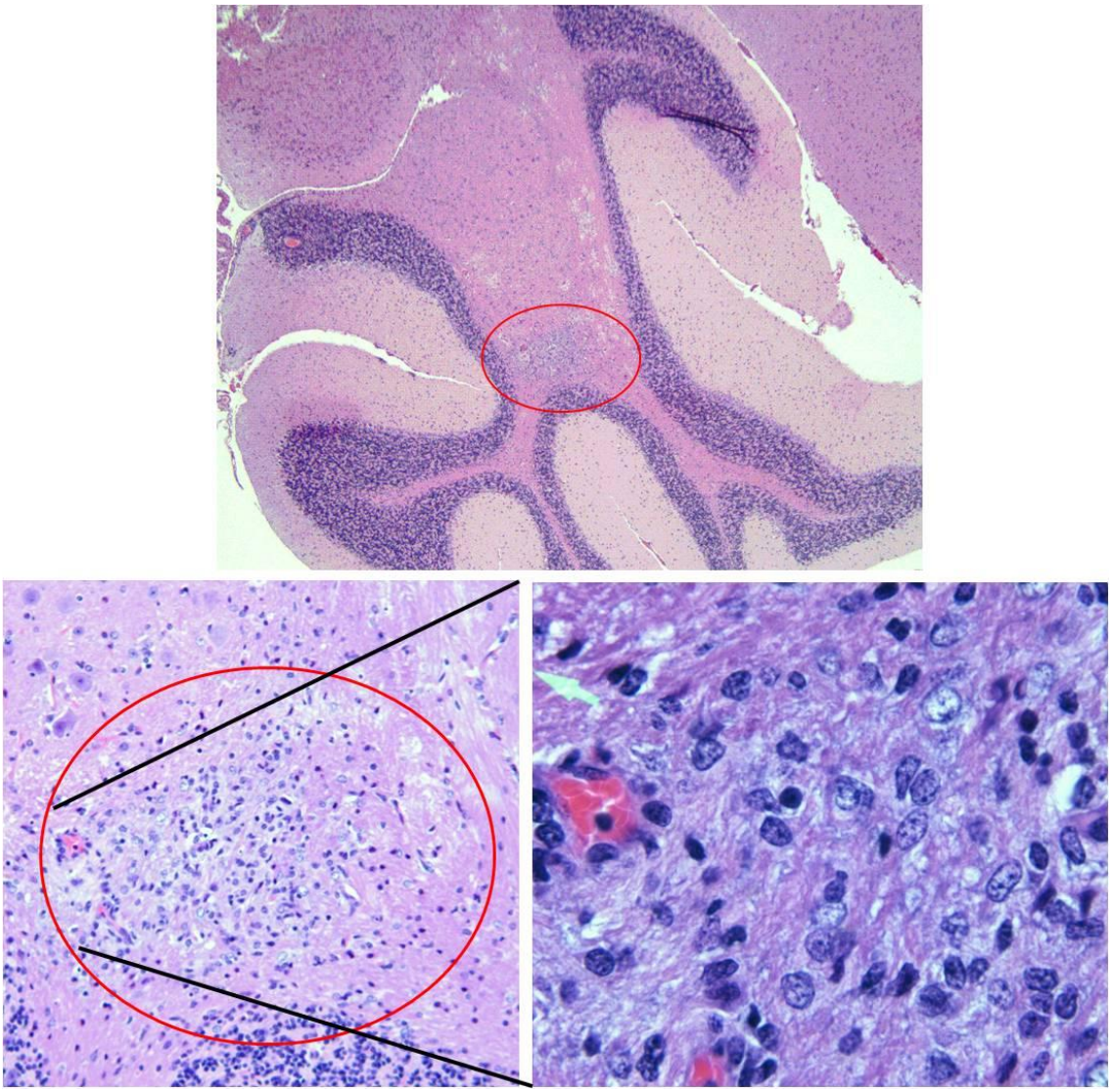

Figure 15: Lesions and infiltration in the cerebellum of a non-GA treated mouse. Wildtype mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55/CFA immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord sections were H&E and LFB stained revealing lesions (red circle), lymphocytic infiltration, and demyelination in a control mouse. Each tissue slide was analyzed by two blinded researchers. These experiments were done in collaboration with Dr. Christos Fotiadis, Neuropathology, UT-Southwestern Medical Center.

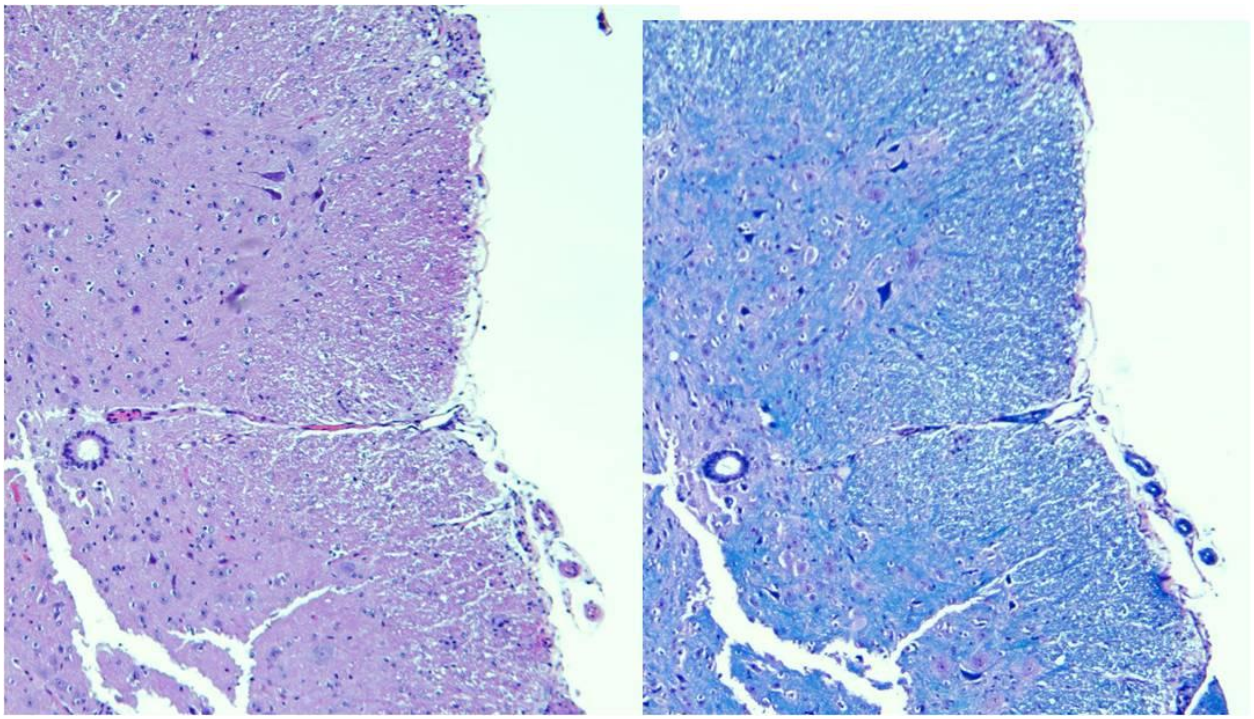

Figure 16: GA protects against demyelination in the lumbosacral spinal cord. Wildtype mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55/CFA immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord sections were H&E stained (left) or H&E and LFB stained (right) revealing little lymphocytic infiltration and demyelination in a GA-treated mouse. Each tissue slice was analyzed by two blinded researchers.

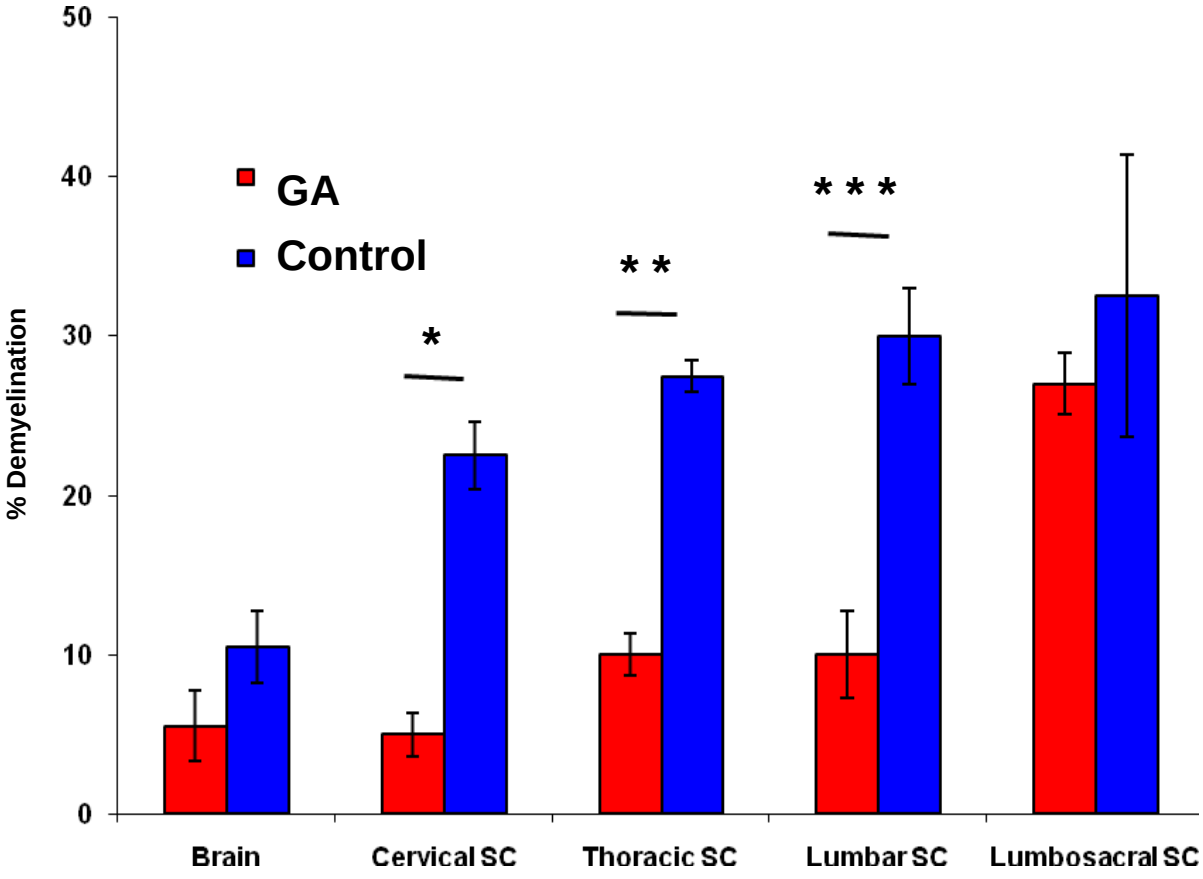

Figure 17: GA protects against demyelination in the spinal cord. Wildtype mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55 immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord sections were H&E stained or H&E and LFB stained revealing GA protected against demyelination in the spinal cord. Each tissue slide was analyzed by two blinded researchers. This figure is representative of 4 individually analyzed mice. * p= 0.006; ** p= 0.001; *** p= 0.017

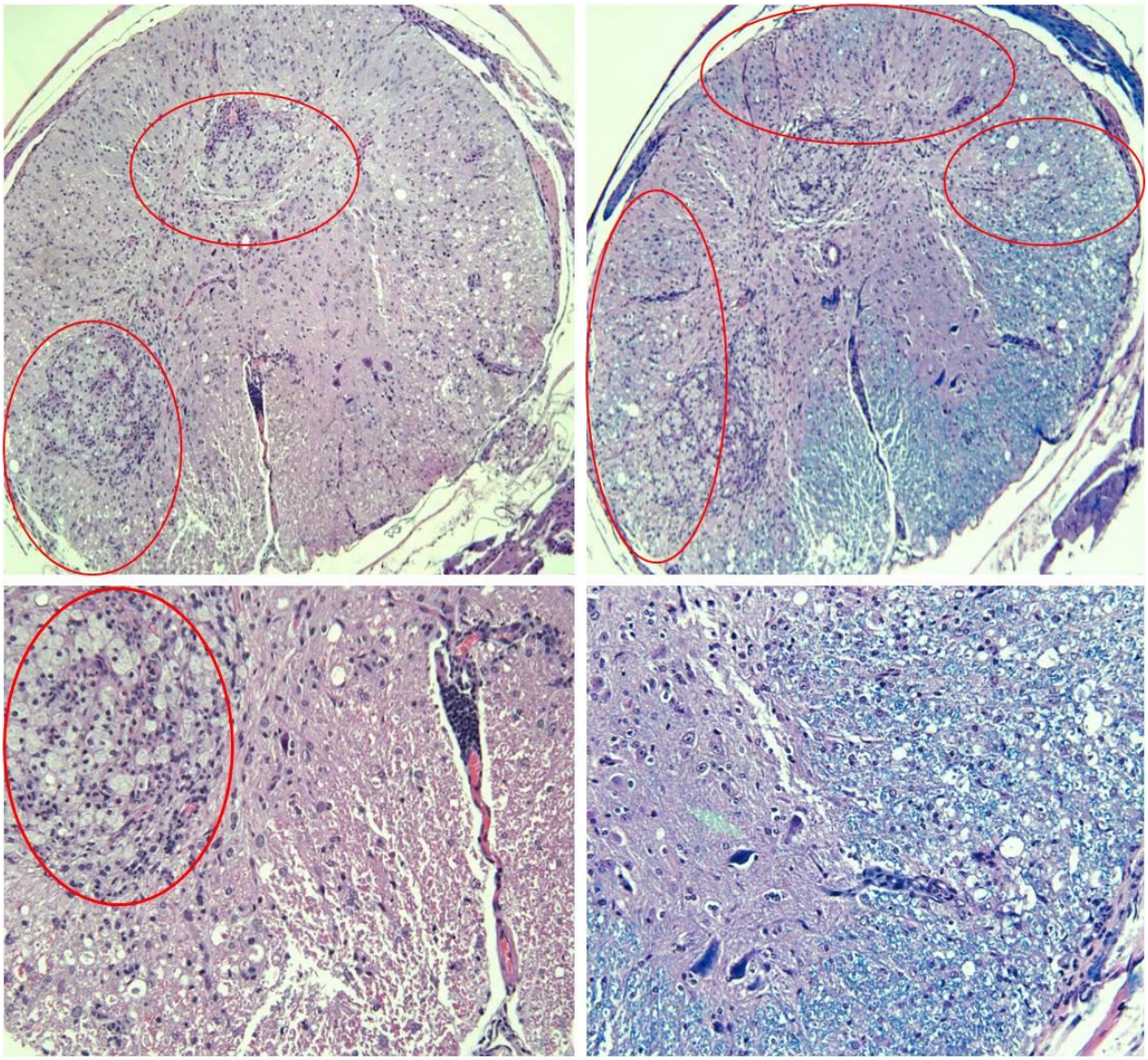

Figure 18: Lesions, infiltration, and demyelination in the cervical spinal cord of a non-GA treated CD8 (-/-) mouse. CD8 (-/-) mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55/CFA immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord sections were H&E (left) or H&E and LFB stained (right) revealing lesions (top left, red circles, bottom left, red circle), lymphocytic infiltration, and demyelination (right, red circles) in a control mouse. Each tissue slide was analyzed by two blinded researchers.

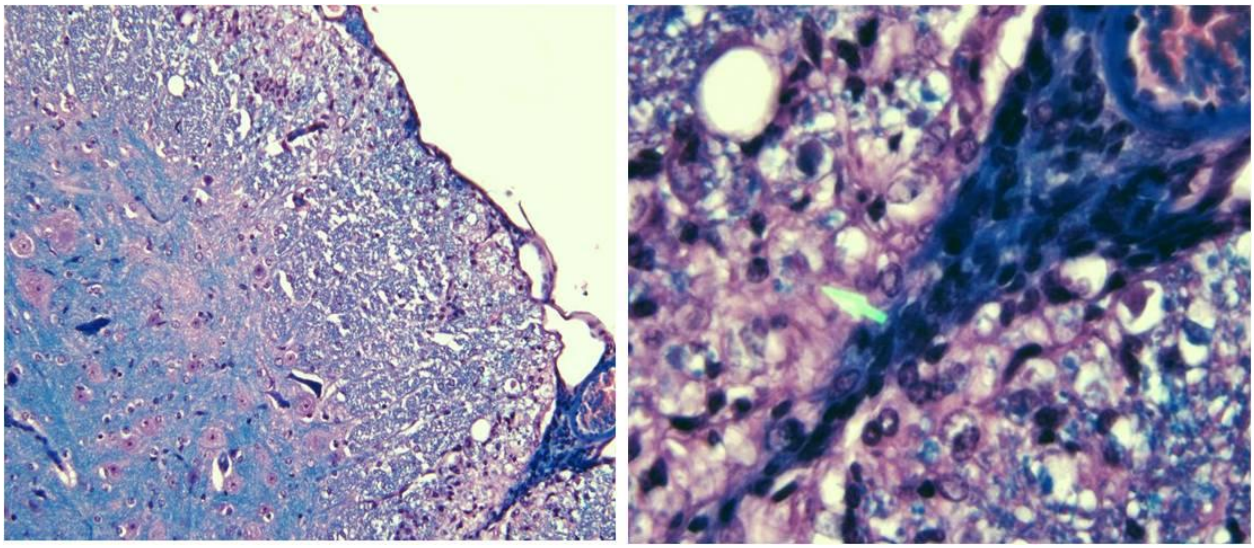

Figure 19: Infiltration and demyelination in the cervical spinal cord of a GA treated CD8 (-/-) mouse. CD8 (-/-) mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55/CFA immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord sections were H&E and LFB stained revealing lesions, lymphocytic infiltration, and demyelination in a GA-treated mouse. Each tissue slide was analyzed by two blinded researchers.

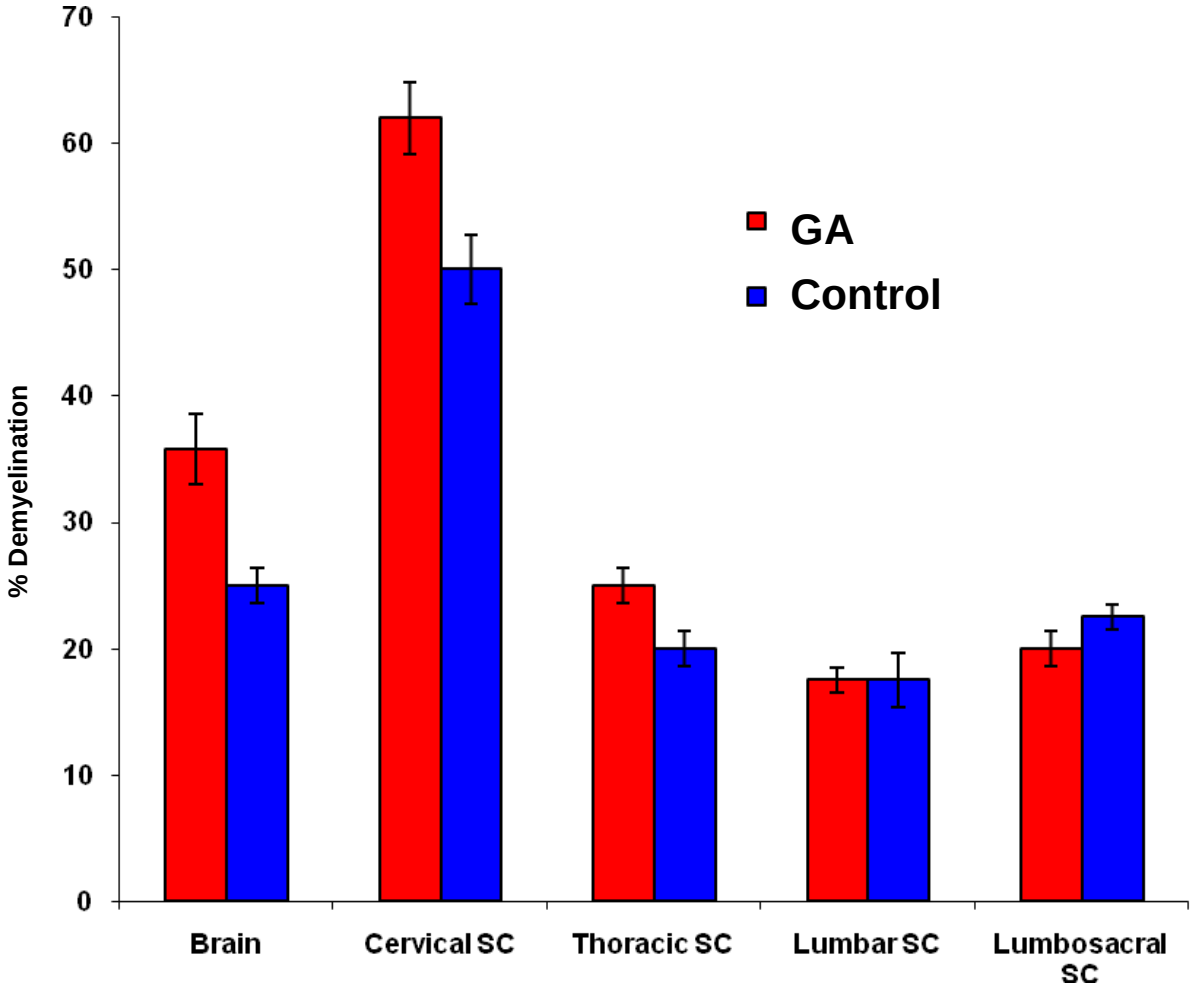

Figure 20: GA does not protect against demyelination in the absence of CD8$^+$ T cells. CD8 (-/-) mice were injected with 2 mg GA or control/IFA at day -10. EAE was induced by MOG 35-55/CFA immunization at day 0 and PT was injected at day 0 and day 2. At day 28, mice from both groups were euthanized and processed individually. Brain and spinal cord (SC) sections were H&E stained or H&E and LFB stained revealing GA did not protect against demyelination in the brain or spinal cord. Each tissue slide was analyzed by two blinded researchers. This figure is representative of 4 individually analyzed mice.

GA-induced CD8+ T cells confer protection from severe EAE in CD8 (-/-) mice

We have shown that $CD8^+$ T cells are required for GA protection through our CD8 (-/-) and CD8 depletion studies. To further investigate the *in vivo* relevance of GA reactive $CD8^+$ T cells, we sought to determine if GA protection from severe EAE could be conferred by adoptive transfer of these cells.

We immunized mice with either 1mg GA or OVA 323-339/IFA at day -25. At day -4 we euthanized these mice, harvested the splenocytes and put them in a bulk 72-hour culture with GA (20 μg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and $CD8^+$ T cells were purified by positive selection using a magnetic column. We intravenously (IV) transferred $2.5x10^6$ purified GA-induced $CD8^+$ T cells or OVA-induced $CD8^+$ T cells into naïve female CD8 (-/-) recipients. 24 hours later, on day 0 we immunized the 2 groups of CD8 (-/-) mice that received the $CD8^+$ T cell transfer with MOG 35-55/CFA and injected PT at day 0 and 2 as described above. In a blinded manner, we evaluated the mice for clinical symptoms of EAE until day 40.

The results indicate that GA-reactive $CD8^+$ T cells protected against severe EAE compared to the OVA $CD8^+$ T cell control. The CD8 (-/-) mice that received the GA-reactive $CD8^+$ T cells had a lower disease peak and recovered to a lower average clinical score during the effector phase of disease (Figure 21). These results were reproducible in all repeat experiments. Table 9 shows peak disease and disease onset for one representative experiment.

Prevention of severe EAE by GA-induced $CD8^+$ T cells is an interesting observation; however we wanted to know if GA-induced $CD8^+$ T cells could ameliorate established disease. We sought to adoptively transfer GA-induced $CD8^+$ T cells at peak disease as a therapeutic treatment in CD8 (-/-) mice.

Donor GA and OVA 323-339 induced $CD8^+$ T cells were prepared as described above. We estimated when disease onset would occur in our experimental design so that IV transfer would coincide with peak disease. This time, in addition to the 72-hour *in vitro* stimulation we attempted to transfer GA- and OVA-induced $CD8^+$ T cells without *in vitro* stimulation (*ex vivo*). From repeat experiments we found that $2.5x10^6$ up to $14x10^6$ GA-induced $CD8^+$ T cells IV transferred *ex vivo* did not alter the EAE disease course. However, we found that after the 72-hour *in vitro* stimulation, $5x10^6$ up to $10x10^6$ GA-induced $CD8^+$ T cells would ameliorate established EAE disease (Figure 22).

GA-induced $CD8^+$ T cells confer protection from severe EAE in wildtype mice

The next set of experiments was designed to determine if GA-induced $CD8^+$ T cells could confer GA protection in wildtype mice similar to our CD8 (-/-) model. GA- and OVA-induced $CD8^+$ T cells were acquired as described above for IV transfer *ex vivo* or after 72-hour *in vitro* stimulation. At day -1, $5x10^6$ and up $10x10^6$ GA- or OVA-induced $CD8^+$ T cells were IV transferred into female naïve wildtype mice. At day 0, all mice were immunized with 200 µg MOG 35-55/CFA and PT was injected at day 0 and day 2. We found that GA-induced $CD8^+$ T cells could not prevent severe EAE at dosages of $5x10^6$ to $10x10^6$ cells when injected *ex vivo*. By contrast, following a 72-hour *in vitro* stimulation, GA-induced $CD8^+$ T cells could prevent severe EAE at dosages of $5x10^6$ to $10x10^6$ cells ($p< 0.05$) (Figure 23). Table 10 depicts the peak disease and disease onset for both the *ex vivo* transfer and *in vitro* stimulation transfer conditions.

We next examined the effect of GA-induced $CD8^+$ T cells as therapy in wildtype mice. We acquired GA- and OVA-induced $CD8^+$ T cells as described earlier and IV injected $5x10^6$ and $10x10^6$ cells after a 72-hour *in vitro* stimulation into wildtype mice at peak disease (day 15). GA-induced $CD8^+$ T cells reduced established EAE in wildtype mice (Figure 24).

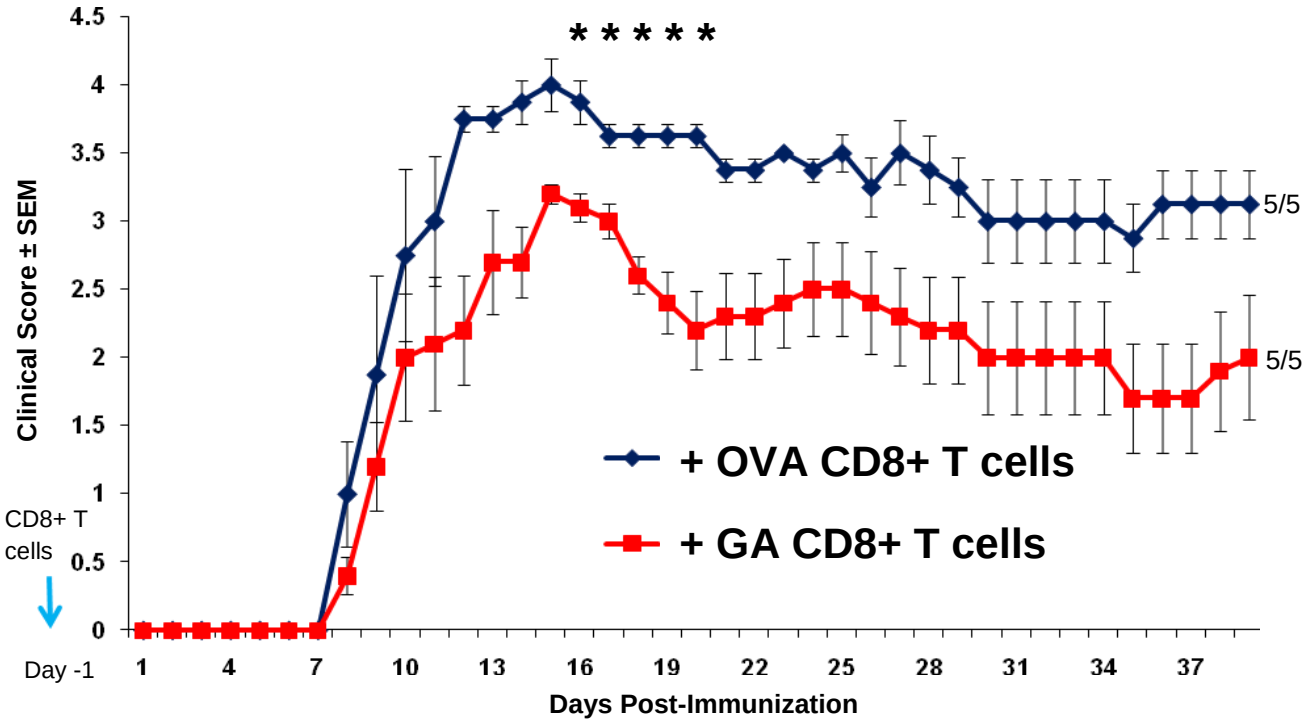

Figure 21: GA-Induced CD8$^+$ T cells confer protection against severe EAE in CD8 (-/-) mice. Female C57BL/6 wildtype donor mice were immunized with 1 mg GA or OVA/IFA at day -25. At day -4 mice were euthanized, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and CD8$^+$ T cells were purified by positive selection using a magnetic column. 2.5x10^6 purified GA-induced CD8$^+$ T cells or control CD8$^+$ T cells were IV transferred into naïve female CD8 (-/-) recipients. 24 hours later, all recipient mice were immunized with MOG/CFA. In a blinded manner, mice were evaluated for clinical symptoms of EAE until day 37. The graph shows mean clinical score ± SEM of 5 mice. This figure is representative of 3 independent experiments. * indicates $p < 0.05$

TABLE 9	Incidence	Peak Disease	Disease Onset
+ OVA CD8$^+$ T cells	5/5	3.9 +/- 0.08	9.3 +/- 0.50
+ GA CD8$^+$ T cells	5/5	3.4 +/- 0.10	10.2 +/- 0.67

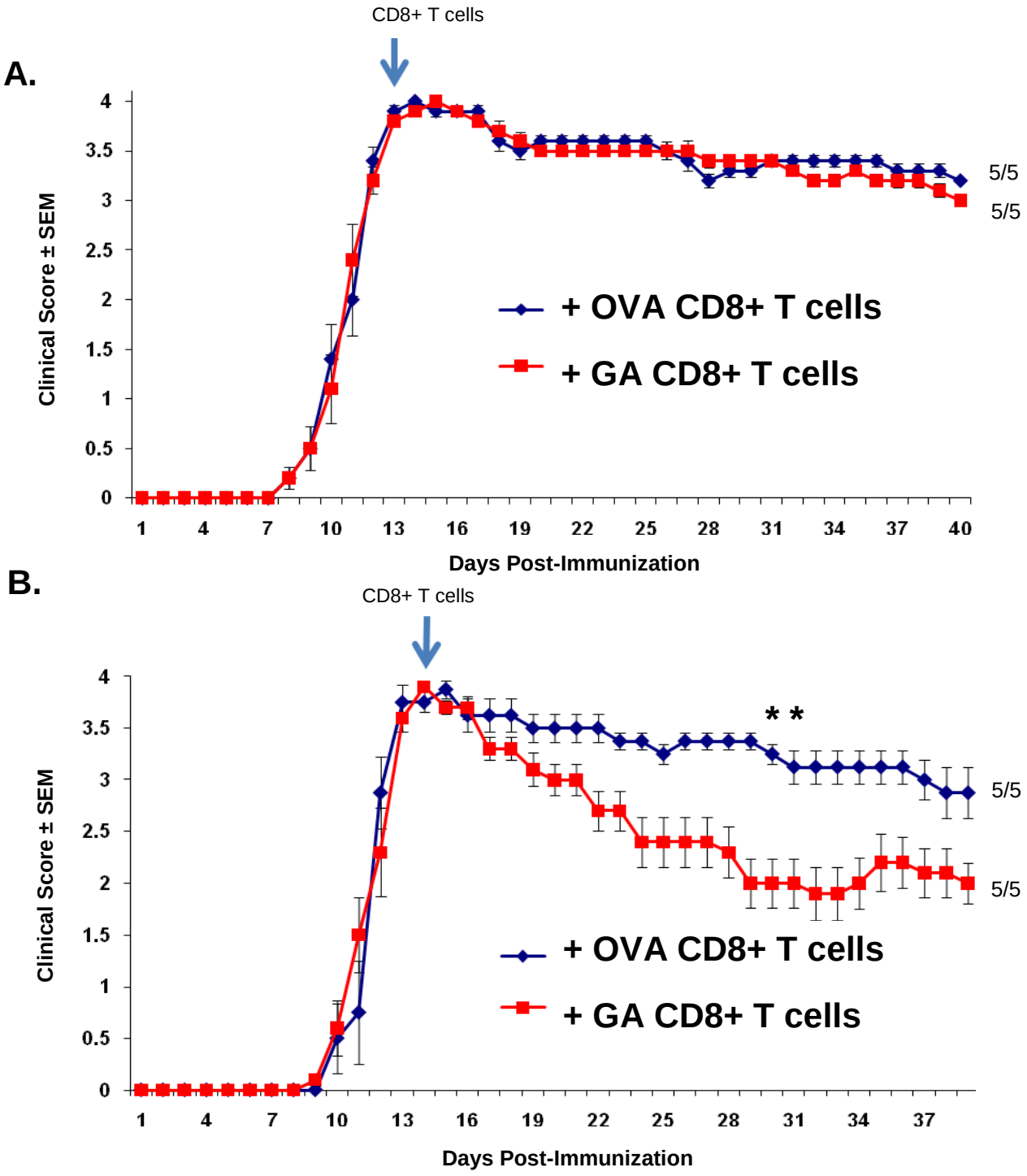

Figure 22: *In vitro*, but not *ex vivo* GA-Induced CD8$^+$ T cells reduce severe EAE in CD8 (-/-). Female C57BL/6 wildtype donor mice were immunized with 1 mg GA or OVA/IFA at day -10. On day 0, CD8 (-/-) recipient mice were immunized with MOG/CFA and PT injected at day

0 and 2. A. On the day of peak disease, donor GA and OVA mice were euthanized, splenocytes harvested, and GA and OVA CD8+ T cells purified by positive selection. $2.5x10^6$ up to $14x10^6$ GA- or OVA-induced $CD8^+$ T cells were IV transferred into diseased CD8 (-/-) mice. B. Donor wildtype mice were euthanized at day 10, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and $CD8^+$ T cells were purified by positive selection using a magnetic column. $5x10^6$ to $10x10^6$ purified GA-induced $CD8^+$ T cells or OVA $CD8^+$ T cells were IV transferred into peak diseased female CD8 (-/-) recipient mice that were randomized and normalized by average disease score. In a blinded manner, mice were evaluated for clinical symptoms of EAE until day 40. The graph shows mean clinical score ± SEM of 5 mice. This figure is representative of 3 independent experiments. * indicates $p< 0.05$

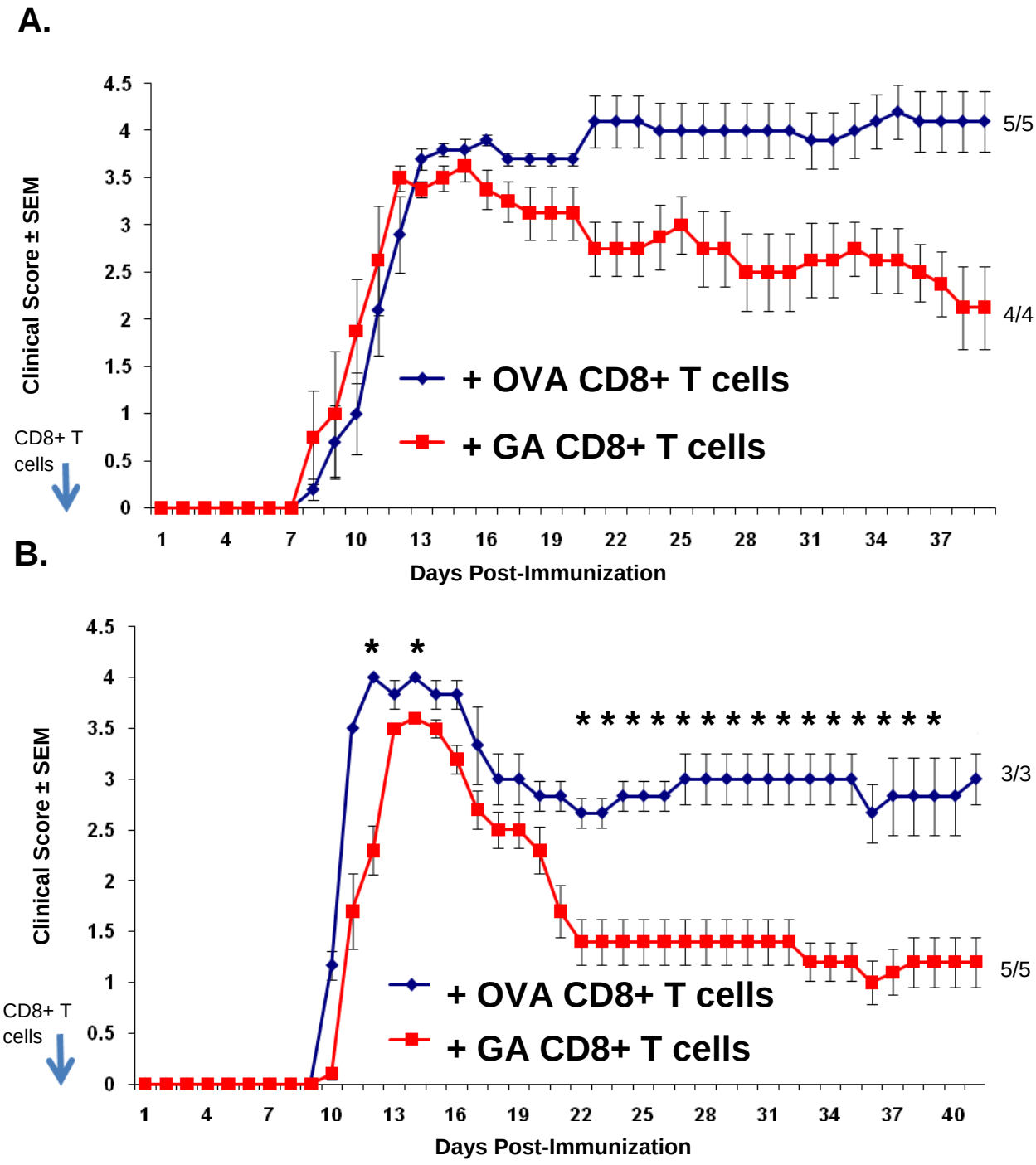

Figure 23: *In vitro stimulated* GA-induced CD8$^+$ T cells prevent severe EAE in wildtype mice. Female C57BL/6 wildtype donor mice were immunized with 1 mg GA or OVA/IFA at day -25. A. For *ex vivo*: at day -1 donor GA and OVA mice were euthanized, splenocytes harvested, and GA and OVA CD8+ T cells purified by positive selection. 5x10^6 up to 10x10^6 GA- or OVA-induced CD8$^+$ T cells were IV transferred into naïve wildtype mice. B. For *in vitro* stimulation:

GA and OVA donor wildtype mice were euthanized at day -4, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and $CD8^+$ T cells were purified by positive selection using a magnetic column. $5x10^6$ to $10x10^6$ purified GA-induced $CD8^+$ T cells or OVA $CD8^+$ T cells were IV transferred into female naïve wildtype recipient mice. 24 hours later, on day 0 all recipient mice were immunized with MOG 35-55/CFA and PT injected at day 0 and 2. In a blinded manner, mice were evaluated for clinical symptoms of EAE until day 40. The graph shows mean clinical score ± SEM of 3 mice (OVA) and 5 mice (GA). This figure is representative of 3 independent experiments. * indicates $p<0.05$

Table 10: CD8+ T cells into WT: Prevention

	Incidence	**Peak Disease** * p= 0.016	**Disease Onset** * p= 0.034
***Ex vivo* OVA**	**5/5**	**4.5 +/- 0.22**	**10.8 +/- 0.48**
***Ex vivo* GA**	**4/4**	**3.9 +/- 0.08**	**10.0 +/- 0.54**
***In vitro* Stim OVA**	**3/3**	**4.0 +/- 0.00 ***	**10.0 +/- 0.00 ****
***In vitro* Stim GA**	**5/5**	**3.6 +/- 0.06 ***	**11 +/- 0.18 ****

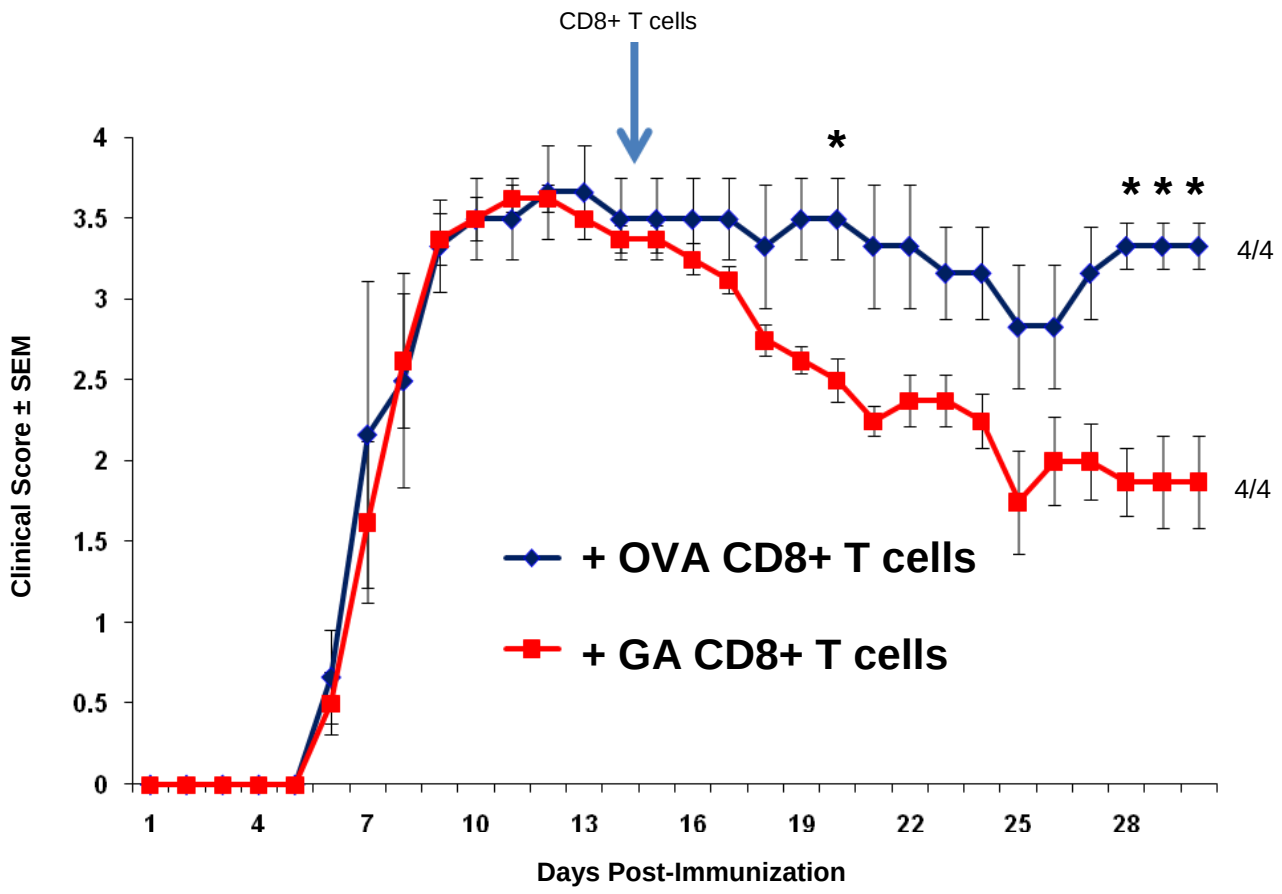

Figure 24: GA-induced CD8$^+$ T cells reduce severe EAE in wildtype mice. Female C57BL/6 wildtype donor mice were immunized with 1 mg GA or OVA/IFA at day -10. On day 0, CD8 (-/-) recipient mice were immunized with MOG 35-55/CFA and PT injected at day 0 and 2. Donor wildtype GA or OVA mice were euthanized at day 10, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and CD8$^+$ T cells were purified by positive selection using a magnetic column. 5x10^6 and 10x10^6 purified GA-induced CD8$^+$ T cells or OVA CD8$^+$ T cells were IV transferred into peak diseased female wildtype recipient mice that were randomized and normalized by average disease score. In a blinded manner, mice were evaluated for clinical symptoms of EAE until day 30. The graph shows mean clinical score ± SEM of 4 mice. This figure is representative of two independent experiments. * indicates $p < 0.05$

Co-transfer of GA-induced $CD8^+$ T cells with MOG $CD4^+$ T cells reduces passive adoptive disease

We have now shown that GA-induced $CD8^+$ T cells have a regulatory role on active disease as a prevention (at day -1) and as a therapeutic modality (at peak disease). In a preliminary experiment, we now sought to determine if GA-induced $CD8^+$ T cells also exert a regulatory effect on adoptively transferred disease. Wildtype mice were immunized with MOG 35-55/CFA and injected with PT at day 0 and day 2. At day 10, these diseased mice were euthanized and lymph node cells harvested as per our standard passive disease protocol. These bulk cell cultures were stimulated *in vitro* for 72-hours with MOG (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, the dead cells were removed by ficoll gradient and live cells enriched for $CD4^+$ T cells by positive selection with a magnetic column. We acquired GA- and OVA-induced $CD8^+$ T cells as described above. Naïve mice were then injected with purified MOG $CD4^+$ T cells alone, MOG $CD4^+$ T cells + GA-induced $CD8^+$ T cells, or MOG $CD4^+$ T cells + OVA-induced $CD8^+$ T cells. In this preliminary experiment, results showed that GA-induced $CD8^+$ T cells could prevent severe EAE in adoptive transfer disease, suggesting that GA-induced $CD8^+$ T cells have a regulatory effect on encephalitogenic MOG $CD4^+$ T cells (Figure 25). This experiment should be repeated to verify these intriguing results.

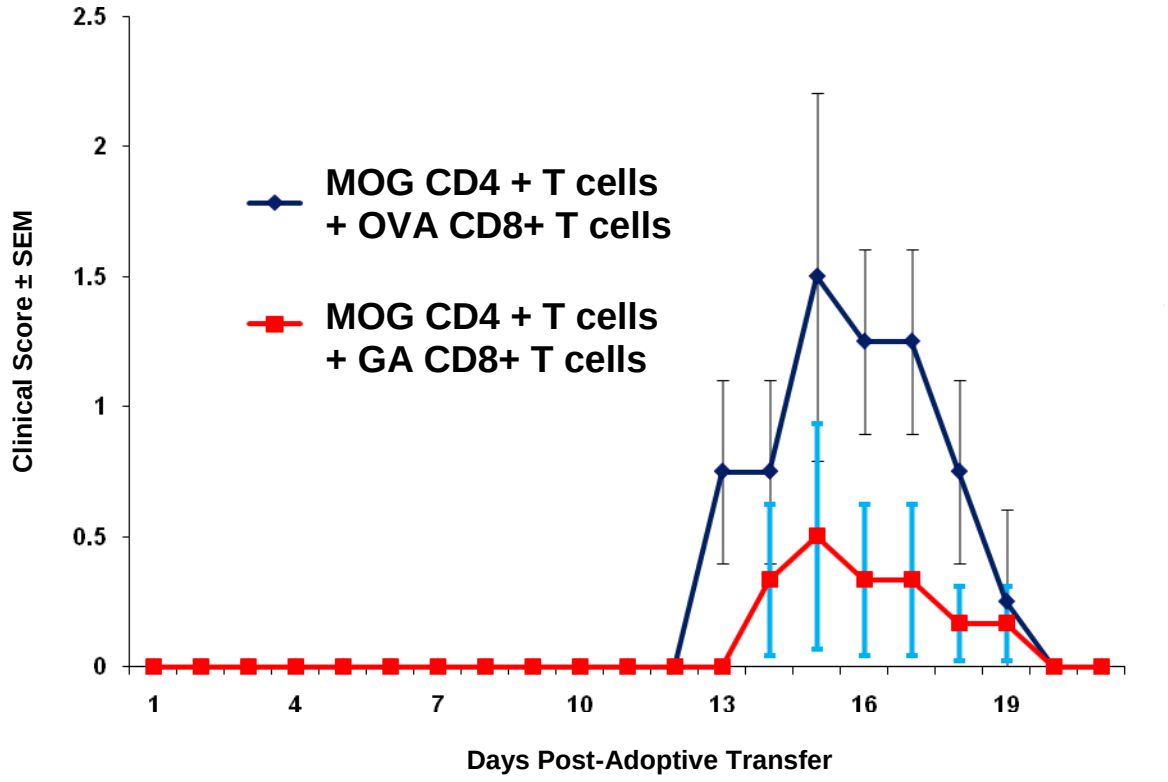

Figure 25: Effect of GA-induced CD8$^+$ T cells in severe passively transferred EAE. Wildtype mice were immunized with MOG 35-55/CFA on day -13 and injected with PT on day 0 and day 2. Diseased mice were euthanized at day -3 and lymph node cells were harvested and stimulated with MOG (20 µg/mL) and IL-2 (10 pg/mL) for 72 hours. Separately, wildtype mice were immunized with 1 mg GA or OVA/IFA at day -23 and euthanized at day -3. Harvested cells were stimulated with either GA or OVA (20 µg/mL) and IL-2 (10 pg/mL) for 72 hours. MOG CD4$^+$ T cells and GA- and OVA-induced CD8$^+$ T cells were purified by positive selection with a magnetic column. MOG CD4$^+$ T cells + GA or OVA-induced CD8$^+$ T cells were injected into naïve wildtype recipient mice at day 0. Mice were evaluated for clinical symptoms until day 22 in a blinded manner.

Chapter VI: Mechanism of Glatiramer Acetate $CD8^+$ T cell Regulation

In vitro Cytotoxicity: GA-induced $CD8^+$ T cells kill GA-loaded target cells

Now that we have shown that $CD8^+$ T cells are required for the *in vivo* efficacy of GA, the next logical step is to elucidate the mechanism of action. Many possibilities exist, but we started our investigation using the human MS studies in our lab as a cornerstone. We have shown that GA-induced cytotoxic $CD8^+$ T cells directly kill $CD4^+$ T cells in a GA specific manner [133]. Therefore, first we asked if GA-specific $CD8^+$ T cells were cytotoxic in nature. For our murine studies we adapted a flow cytometry-based cytotoxicity assay. This assay measures the ability of GA-induced CD8+ T cells to kill GA-loaded targets cells. Briefly, naïve wildtype mice were injected with 200 μg GA/CFA at day 0. On day 12, mice were euthanized and harvested splenocytes are stimulated in culture with GA (20 μg/mL) for 7 days. On day 19, $CD8^+$ T cells were purified by negative selection. These purified GA-induced $CD8^+$ T cells were the "Effectors". In parallel, on day 18, naïve mice were euthanized and the splenocytes harvested. These cells are then stimulated overnight with GA (20 μg/mL) and ConA (0.5 μg/mL). After the overnight culture these cells were CFSE-stained and used as "Targets". The "Effectors" and "Targets" were combined at different ratios, 0:1, 1:1, up to 64:1 and incubated for 24 hours. At the end of the 24 hours, APC compensation beads were added to each sample to serve an internal number control that is utilized when calculating % killing. Samples were run on a flow cytometer and % killing was calculated:

$$\%\ \text{killing: } 1 - \frac{(\text{Live CFSE target cell per 1000 beads for a particular effector ratio})}{(\text{Live CFSE target cell count for no effector cell well})} \times 100$$

The positive control for this assay was the P815 tumor cell line, which was used as the target cell for a redirected anti-CD3 lysis. The results from our *in vitro* cytotoxicity assay showed that GA-induced $CD8^+$ T cells are killing GA-loaded target cells (Figure 26). This cytotoxic property of GA-induced $CD8^+$ T cells may be partially responsible for our *in vivo* observations.

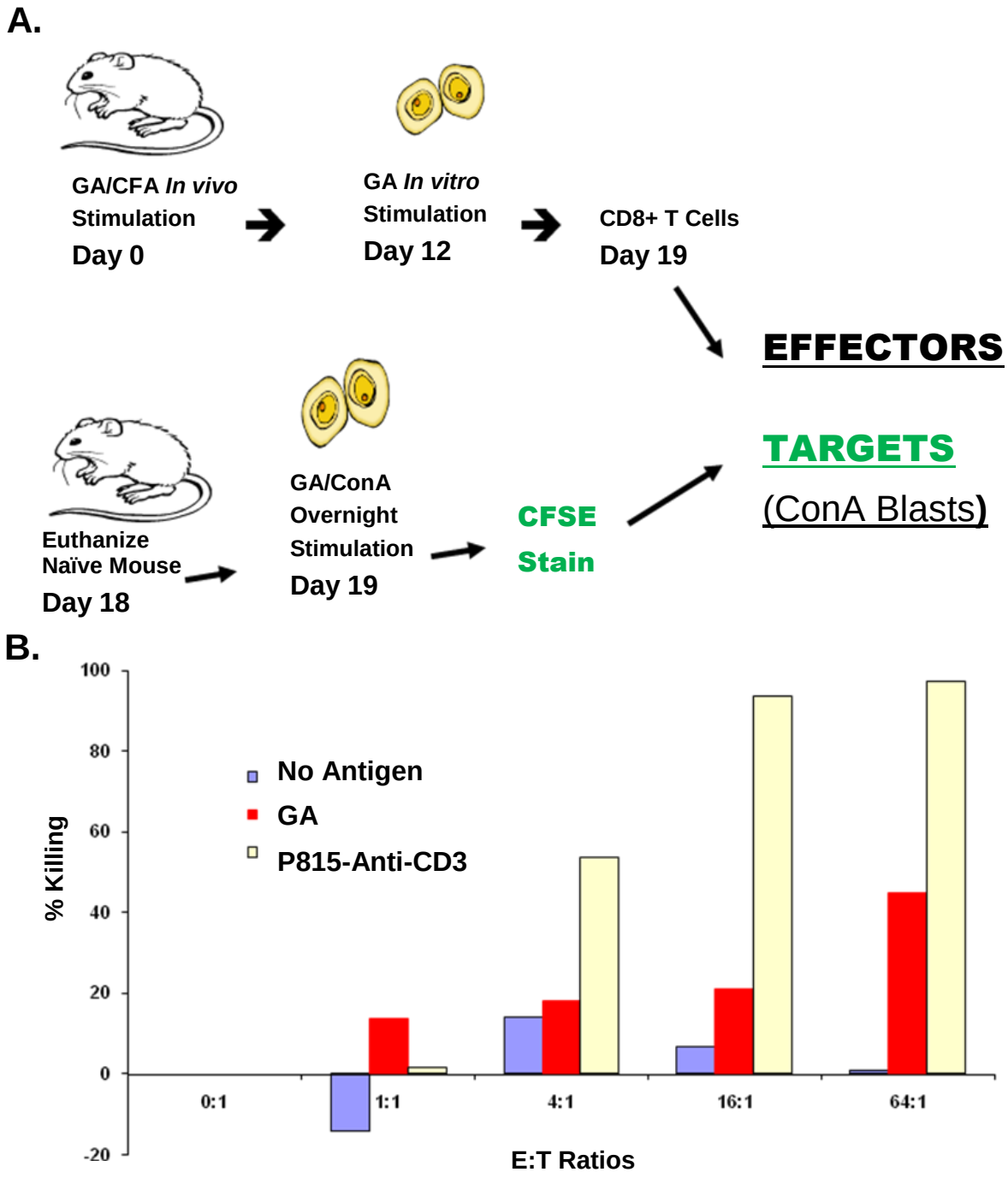

Figure 26: GA-specific CD8$^+$ T cells kill antigen loaded targets in an antigen-specific manner. A. Wildtype mice were injected with 200 µg GA/CFA at day 0. At day 12, mice were euthanized and splenocytes were stimulated *in vitro* with GA (20 µg/mL) for 7 days. At day 19, CD8+ T cells were purified by negative selection (Effectors). In parallel, at day 18, naïve mice

were euthanized and splenocytes were incubated overnight in GA (20 µg/mL) and ConA (0.5 µg/mL). After culture, cells were CFSE stained (Targets). Effector and Target populations were incubated together at different ratios. B. APC beads were added as internal number control and samples are run on flow cytometer. % Killing was calculated as 1- (Live CFSE target cells per 1000 beads for effector target ratio)/ (Live CFSE target cell per 1000 beads for no effector cell well) x 100.

GA-induced $CD8^+$ T cell suppression: mice protected by GA from severe EAE have less MOG-specific proliferation

Another potential mechanism of action of GA-induced $CD8^+$ T cells is suppression of $CD4^+$ T cell proliferative responses. Since the encephalitogenic MOG $CD4^+$ T cell from diseased mice proliferate when confronted with MOG 33-55 *in vitro*, this provides a readout for measuring potential *in vivo* suppression. Naïve wildtype mice were injected with either 2 mg GA or control/IFA at day -10. At day 0, all mice were immunized with MOG 35-55/CFA and injected with PT on day 0 and day 2 as described earlier. Mice were observed for clinical signs of EAE and then euthanized at day 25. Splenocytes were harvested and used in thymidine incorporation assays. Cells were incubated in 96-well plates for 72-hours with no antigen, increasing dosages of MOG, or ConA. Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. Our results indicate that mice protected by GA from severe EAE had less MOG 35-55-specific proliferation at day 25 of disease (Figure 27). On day 25 of disease, protected WT mice were on average at least one full clinical grade lower than unprotected mice (Figures 7, 8, 9, 10, and 23).

A decrease in MOG-specific proliferation could mean several things, most notably either a decrease in the absolute number of encephalitogenic $CD4^+$ T cells that are responding to MOG 33-55 or alternatively a decrease in response by the encephalitogenic $CD4^+$ T cells. It is possible that these effects are mediated by GA-induced $CD8^+$ T cells, although we cannot be certain at this time. Alternatively, GA-induced $CD8^+$ T cells may have an effect on a different cell type and therefore indirectly decrease MOG-specific proliferation of the $CD4^+$ T cells. These possibilities will be discussed later in more detail.

Since GA treatment decreases MOG-specific proliferation and $CD8^+$ T cells are required for the *in vivo* efficacy of GA treatment, we sought to determine if depleting $CD8^+$ T cells during treatment *increases* MOG-specific proliferation. As discussed earlier, $CD8^+$ T cell depletion during GA treatment blocks the beneficial clinical protection against severe EAE (Figure 14). We injected mice with anti-CD8 or injection control on days -11, -8, and -5. 2 mg GA/IFA was injected into all mice at day -10 and EAE was induced at day 0 by immunization with MOG 35-55/CFA and PT was injected on day 0 and day 2. Mice were evaluated for clinical disease until day 30 and then euthanized. The spleens were harvested and the cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of MOG, or ConA. Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. The results indicate that $CD8^+$ T cell depletion of GA-treated mice increases MOG-specific proliferation compared to non-CD8-depleted GA treated mice (Figure 28). Due to the problems we were having with the anti-CD8 antibody killing mice that were injected after disease induction and the troubleshooting that resulted, we did not perform this experiment in non-GA treated diseased mice.

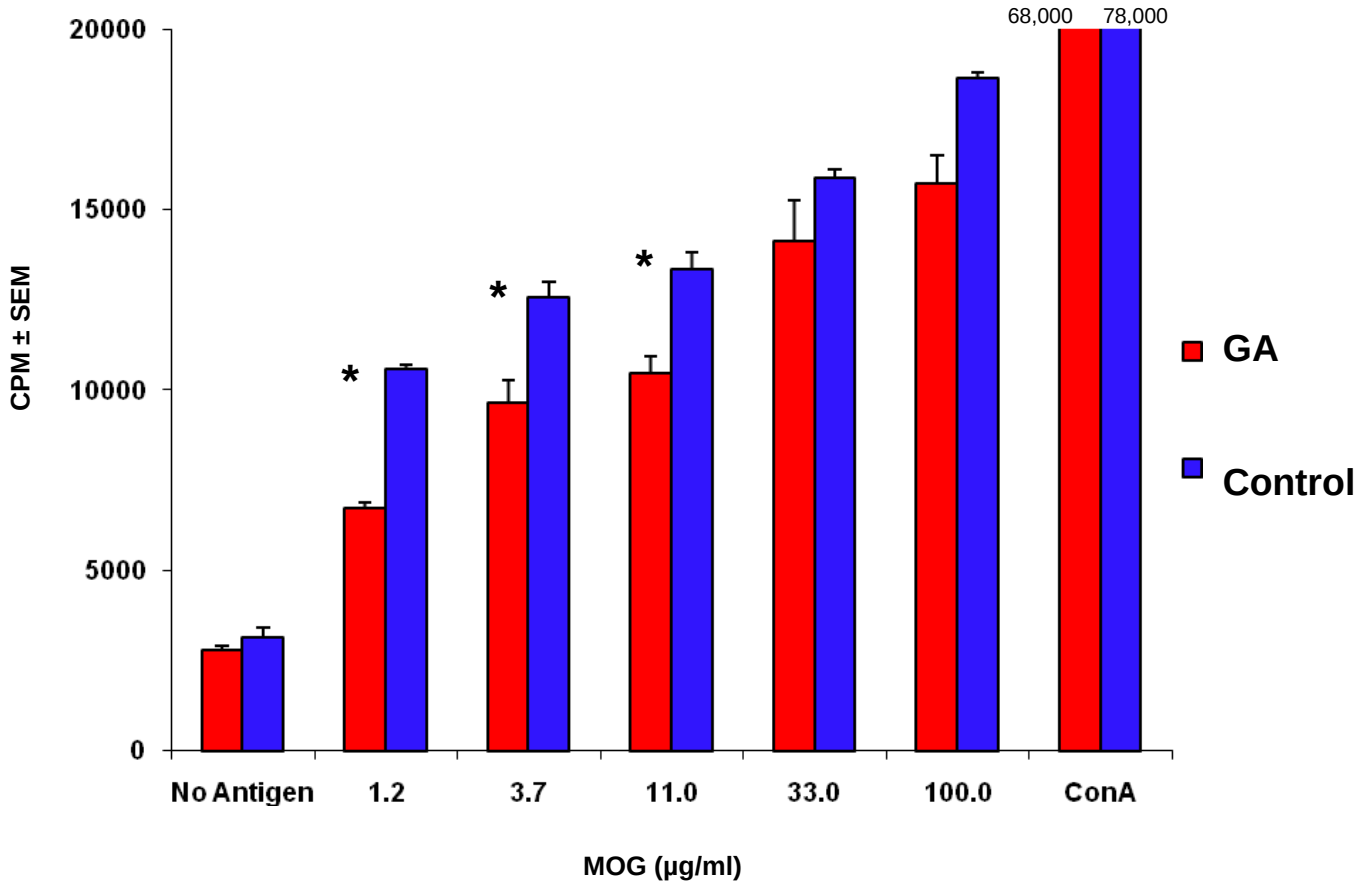

Figure 27: Mice protected from severe EAE by GA have less MOG specific proliferation. Wildtype naïve mice were injected with either 2 mg GA or Control/IFA at day -10. At day 0, all mice were immunized with MOG 35-55/CFA and injected with PT on day 0 and day 2. Mice were observed for clinical signs of EAE and then euthanized at day 25. Splenocytes were harvested and used in thymidine incorporation assays. Cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of GA, or ConA. Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. This figure is representative of 4 independent experiments. * indicates $p < 0.05$

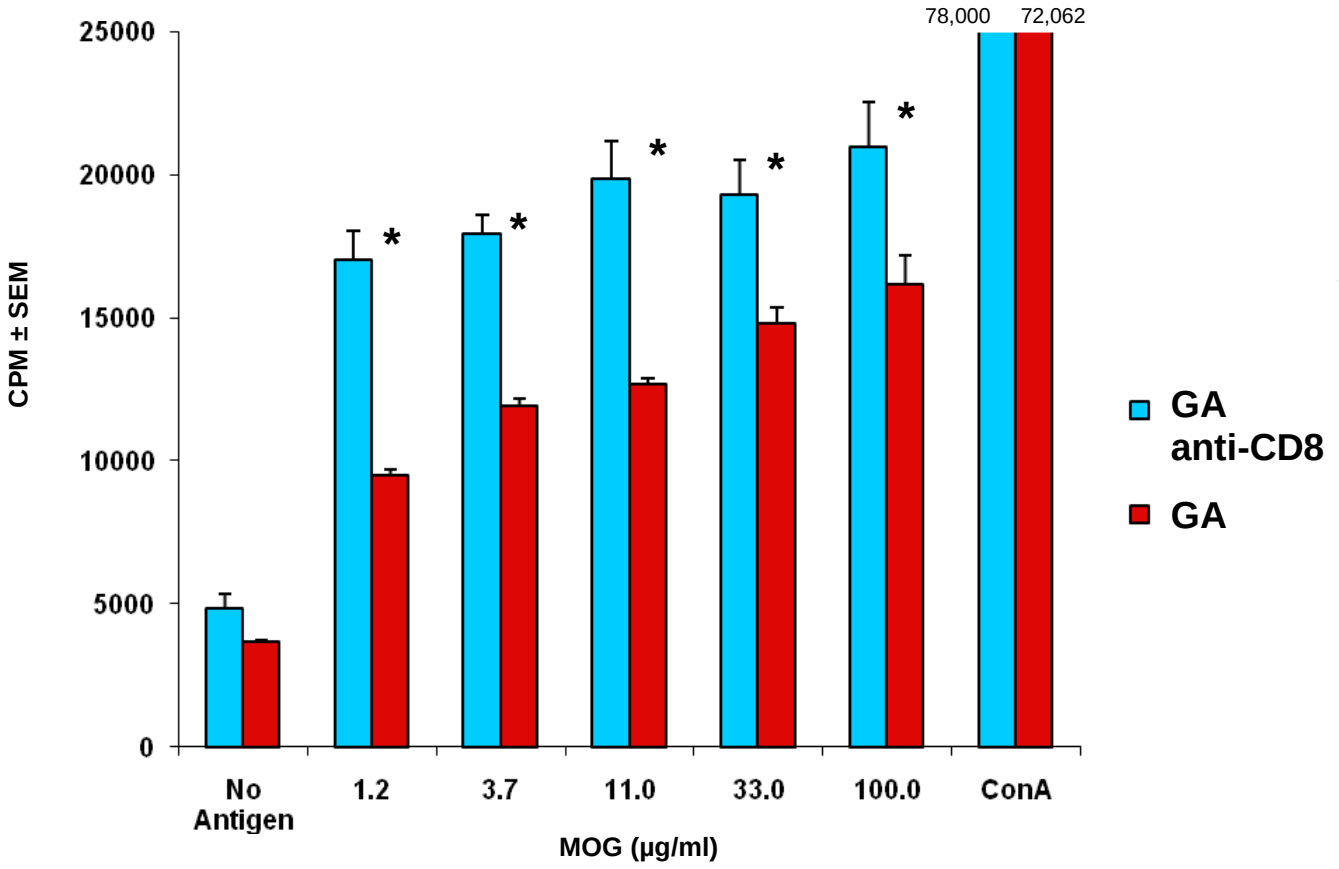

Figure 28: CD8$^+$ T cell depletion increases MOG specific proliferation in GA-treated diseased mice. Anti-CD8 was injected at day -11, -8, and -5. 2 mg GA/IFA was injected at day -10 and EAE was induced at day 0 with MOG 35-55/CFA + PT immunization. Mice were evaluated for clinical disease until day 30. Mice were euthanized and splenocytes harvested. Cells were incubated in 96-well plates for 72 hours with no antigen, increasing dosages of GA, or ConA. Cells were then pulsed with tritiated thymidine for 18 hours and analyzed for incorporation. * indicates $p < 0.05$

GA-induced $CD8^+$ T cells do not suppress OVA-specific responses

We have shown that GA-induced $CD8^+$ T cells have a preventative and therapeutic effect on EAE and that depleting $CD8^+$ T cells increases MOG-specific $CD4^+$ T cell proliferation (Figure 28). We wanted to determine if GA-induced $CD8^+$ T cells or GA treatment could suppress OVA-specific responses or if the observed suppressive effect was specific to MOG responses. We injected female, C57BL/6 wildtpe donor mice with 1 mg GA or OVA/IFA at day -25. At day -4 mice were euthanized, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and $CD8^+$ T cells were purified by positive selection using a magnetic column. $9x10^6$ purified GA-induced, OVA-induced, or naïve $CD8^+$ T cells, were intravenously (IV) transferred into naïve female wildtype recipients. 24 hours later, on day 0 all recipient mice were immunized with OVA 323-339/CFA and PT injected at day 0 and 2. At day 10, proliferation to no antigen, increasing dosages of OVA 323-339, and ConA was checked by a 5-day CFSE proliferation assay. We observed that OVA, GA, or naïve $CD8^+$ T cells do not inhibit an OVA-specific response (Figure 29). In each group that received $CD8^+$ T cells IV, the $CD4^+$ T cells from the OVA immunized mice were still able to respond to OVA *in vitro*, Stimulation Index (SI)>2.

Daily GA/PBS treatment does suppress the $CD4^+$ T cell OVA-specific response.

We also examined the OVA-specific response following GA/PBS daily therapy. We immunized female wildtype C57BL/6 mice with 200 µg OVA 323-339/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. From day 2 to day 10 half the mice were treated subcutaneously with 20 µg GA/PBS daily and the other half with an injection control. At day 10, proliferation with no stimulation, increasing dosages of OVA 323-

339, and ConA was checked by a 5-day CFSE proliferation assay. We found that daily GA/PBS therapy was able to suppress the OVA-specific $CD4^+$ T cell proliferation (Figure 30). The control group had SI's of 3 to 5 with OVA antigen, but the daily GA/PBS treated group did not have a positive proliferative response to OVA (SI<2). This result is not surprising since one of the known mechanisms of action of GA is MHC class II competitive binding [118].

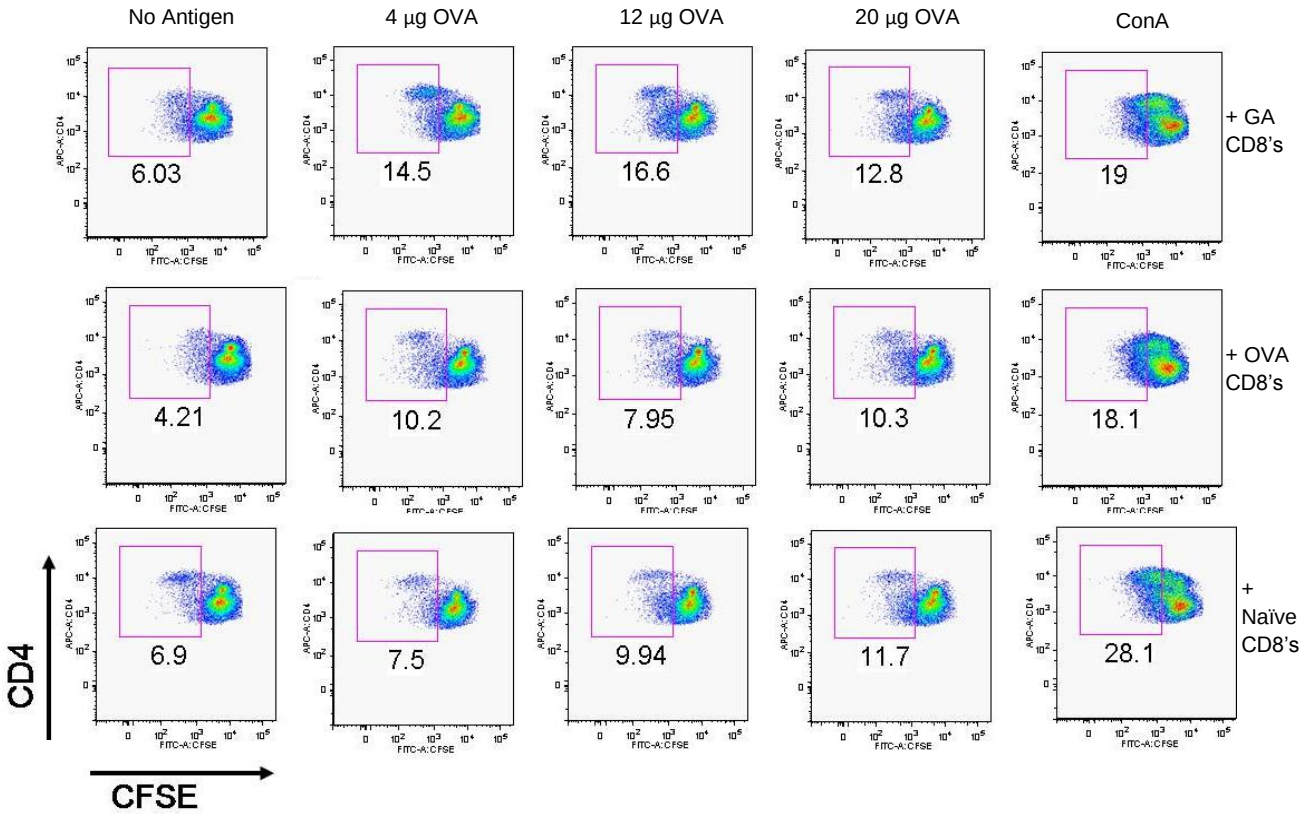

Figure 29: GA, OVA, or Naïve CD8+ T cells do not inhibit OVA specific proliferation. Female C57BL/6 wildtype donor mice were immunized with 1 mg GA or OVA/IFA at day -25. At day -4 mice were euthanized, splenocytes harvested and cells incubated in a bulk 72-hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour incubation, dead cells were removed by ficoll gradient and $CD8^+$ T cells were purified by positive selection using a magnetic column. $9x10^6$ purified GA-induced, OVA-induced, or naïve $CD8^+$ T cells, were intravenously (IV) transferred into naïve female wildtype recipients. 24 hours later, on day 0 all recipient mice were immunized with OVA 323-339/CFA and PT injected at day 0 and 2. At day 10, proliferation to no antigen, increasing dosages of OVA 323-339, and ConA was checked by a 5-day CFSE proliferation assay.

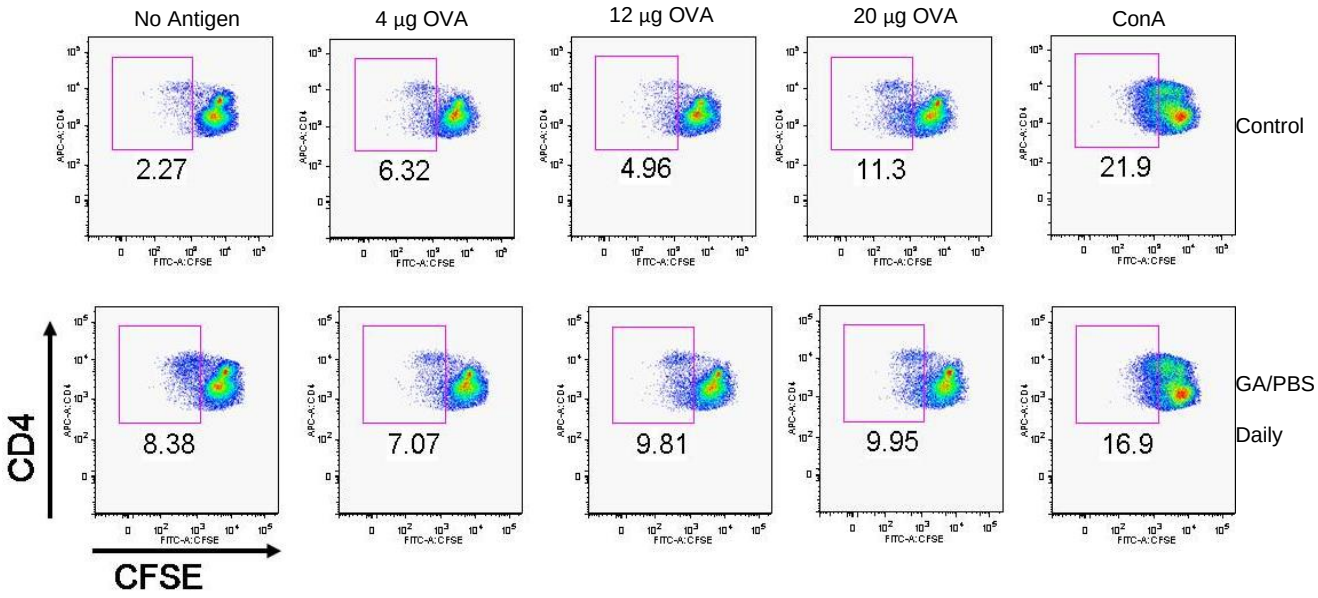

Figure 30: Daily GA/PBS treatment inhibits OVA 323-339 specific proliferation. Female wildtype C57BL/6 mice were immunized with 200 µg OVA 323-339/CFA supplemented with *M. tuberculosis* (4 mg/mL). 250 ng PT was injected at day 0 and day 2. From day 2 to day 10 half the mice were treated subcutaneously with 20 µg GA/PBS daily or injection control. At day 10, proliferation to no antigen, increasing dosages of OVA 323-339, and ConA was checked by a 5-day CFSE proliferation assay.

GA-induced $CD8^+$ T cells suppress MOG $CD4^+$ T cell Proliferation

Another method of accessing GA-induced $CD8^+$ T cell suppression of $CD4^+$ T cell responses is to set up an *in vitro* flow based suppression assay. We induced EAE in wildtype naïve mice at day 0 with MOG 35-55/CFA and PT immunization. One group of diseased mice was treated with 20 μg GA/PBS daily from day 11 until day 24 and protection from severe EAE was observed. After following the clinical symptoms until day 35 we euthanized these mice for use in our suppression assay. Cells from the non-GA treated diseased mice were CD8-depleted and CFSE-stained. This group of CD8-depleted cells was the "responders". The "suppressors" cells came from GA-protected mice. $CD8^+$ T cells from this group were purified by positive selection. Responders and suppressors were set-up at increasing ratios (1:0, 1:0.1, 1:0.2, 1:0.5), incubated for 5 days with either no stimulus, OVA, GA, GA + MOG, or ConA and then analyzed by flow cytometry. Our results show that no suppression occurred in the no stimulus, OVA, or MOG groups as $CD8^+$ T cells are added in increasing ratios. In the group incubated with GA, baseline proliferation went from 30% (1:0 ratio) down to 17.9% in the 1:0.5 ratio. However, proliferation in the no antigen 1:0 tube was 30%, therefore, percentages cannot be considered positive responses. Visual inspection also indicated that a significant amount of cells were no longer present with increasing $CD8^+$ T cells from GA-treated mice. The MOG only and GA + MOG groups had positive proliferation responses (SI>2) versus the no antigen condition, but the proliferation did not decrease with increasing $CD8^+$ T cells. The ConA condition did show suppression with increasing numbers of $CD8^+$ T cells from GA-treated mice and an obvious decrease in the $CD4^+$ T cell population (Figure 31).

In a second attempt, responder cells came from two sources: wildtype or CD8 (-/-) diseased mice at day 17. Bulk cultures from the WT mice were first CD8-depleted before they

were used as the responder cells. Suppressor $CD8^+$ T cells came from wildtype mice immunized with GA or OVA/IFA 20 days prior.

% suppression was calculated by:

$$\frac{\text{(No Ag\% Proliferation – New Ratio \% Proliferation)}}{\text{(No Ag\% Proliferation)}} \times 100$$

The percent suppression of WT CD8-depleted responders by GA or OVA-induced $CD8^+$ T cells for each antigen is charted in Figure 32. In the no stimulus condition, the responder cells proliferated despite the absence of stimulating antigen. Addition of GA-induced $CD8^+$ T cells suppressed this proliferation by up to 60% (Figure 32.A). In the presence of OVA or MOG antigen, there were no positive proliferative responses in the 1:0 conditions (data not shown). When GA- or OVA-induced $CD8^+$ T cells were added, proliferation went up slightly, which resulted in a calculated negative % suppression. It is odd that there was no positive proliferative response to MOG in the 1:0 conditions since these responders cells are from day 17 diseased mice. In Figure 32B, in the presence of GA as the antigen, GA-induced $CD8^+$ T cells suppressed proliferation of MOG $CD4^+$ T cells by up to 85%. In Figure 32C, ConA, both groups of $CD8^+$ T cells suppressed, but the GA-induced $CD8^+$ T cells suppressed up to 85%. Based on these data, GA-induced $CD8^+$ T cells can effectively suppress $CD4^+$ T cells from diseased CD8 (-/-) mice *in vitro*.

In Figure 33, part A depicts the % suppression of MOG $CD4^+$ T cells from CD8 (-/-) mice by GA-induced $CD8^+$ T cells. GA-induced $CD8^+$ T cells highly suppress the MOG $CD4^+$ T cells even without an antigenic stimulus. Neither GA- nor OVA-induced $CD8^+$ T cells were able to suppress MOG $CD4^+$ T cells in the presence of OVA antigen. This is probably due to the fact that the responding MOG $CD4^+$ T cells weren't stimulated to proliferate by OVA (data not shown). The negative % suppression indicates an increase in proliferation as $CD8^+$ T cells were

added. In the presence of GA, GA-induced $CD8^+$ T cells suppressed a weak proliferative response by the MOG $CD4^+$ T cells (Figure 33.B). This is not surprising since the MOG $CD4^+$ T cells did not have their own cognate antigen to respond to, but the suppressor cells were activated by GA. In part C, in the presence of MOG antigen, the MOG $CD4^+$ T cells again did not elicit a strong proliferative response. The meager response that was present was highly suppressed by the GA-induced $CD8^+$ T cells (up to 55%). In part D, in the presence of ConA, both $CD8^+$ T cell groups highly suppressed the MOG $CD4^+$ T cell response by up to 55%.

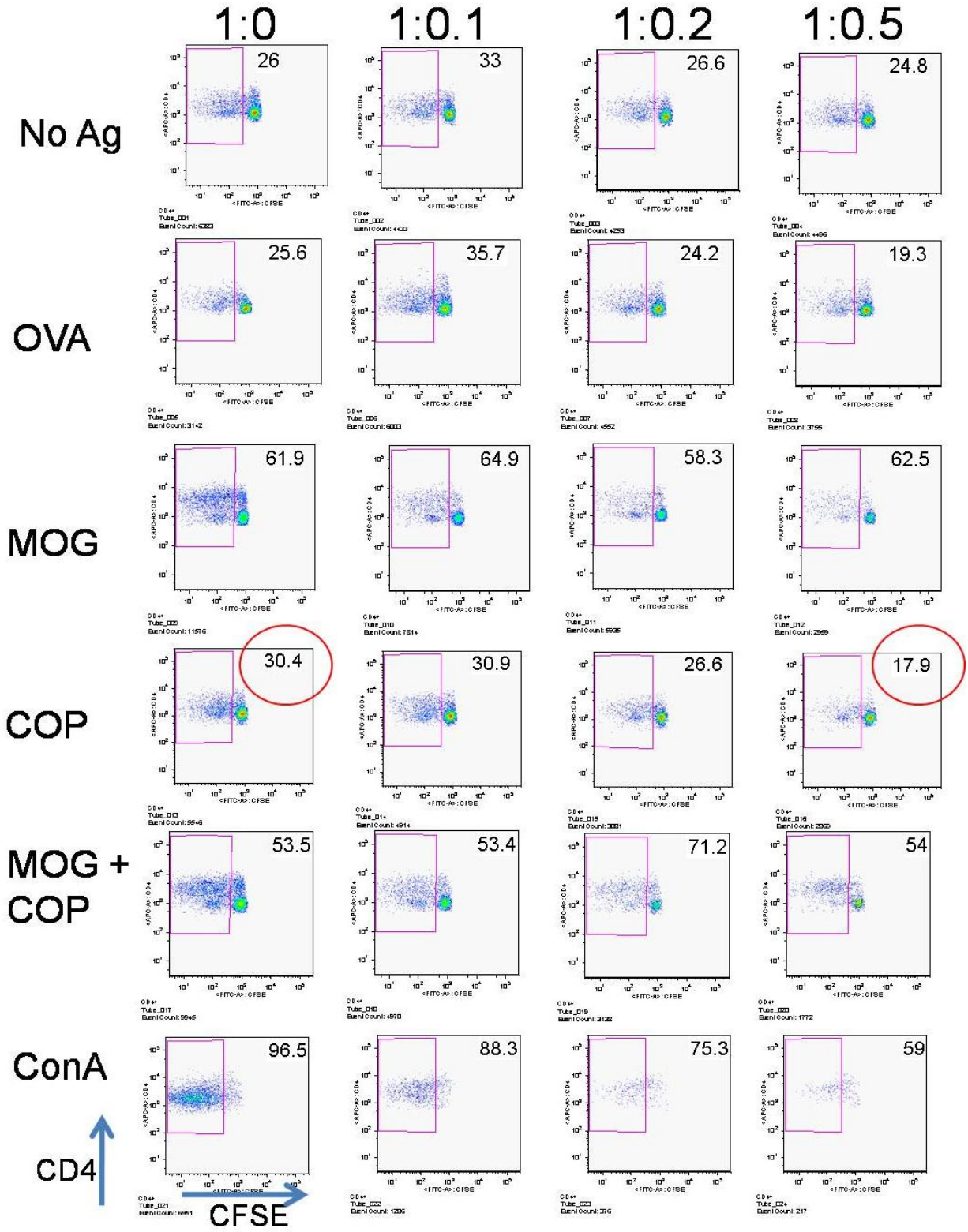

Figure 31: CD8$^+$ T cells from GA treated mice suppress only with ConA. CFSE stained, CD8$^+$ T cell-depleted "responder" cells from diseased mice are incubated with purified CD8$^+$ T

"suppressor" cells from GA treated mice at increasing ratios (1:0, 1:0.1, 1:0.2, 1:0.5). Cells were incubated with no antigen, OVA, MOG, GA, MOG + GA, or ConA for 5 days and then analyzed by flow cytometry.

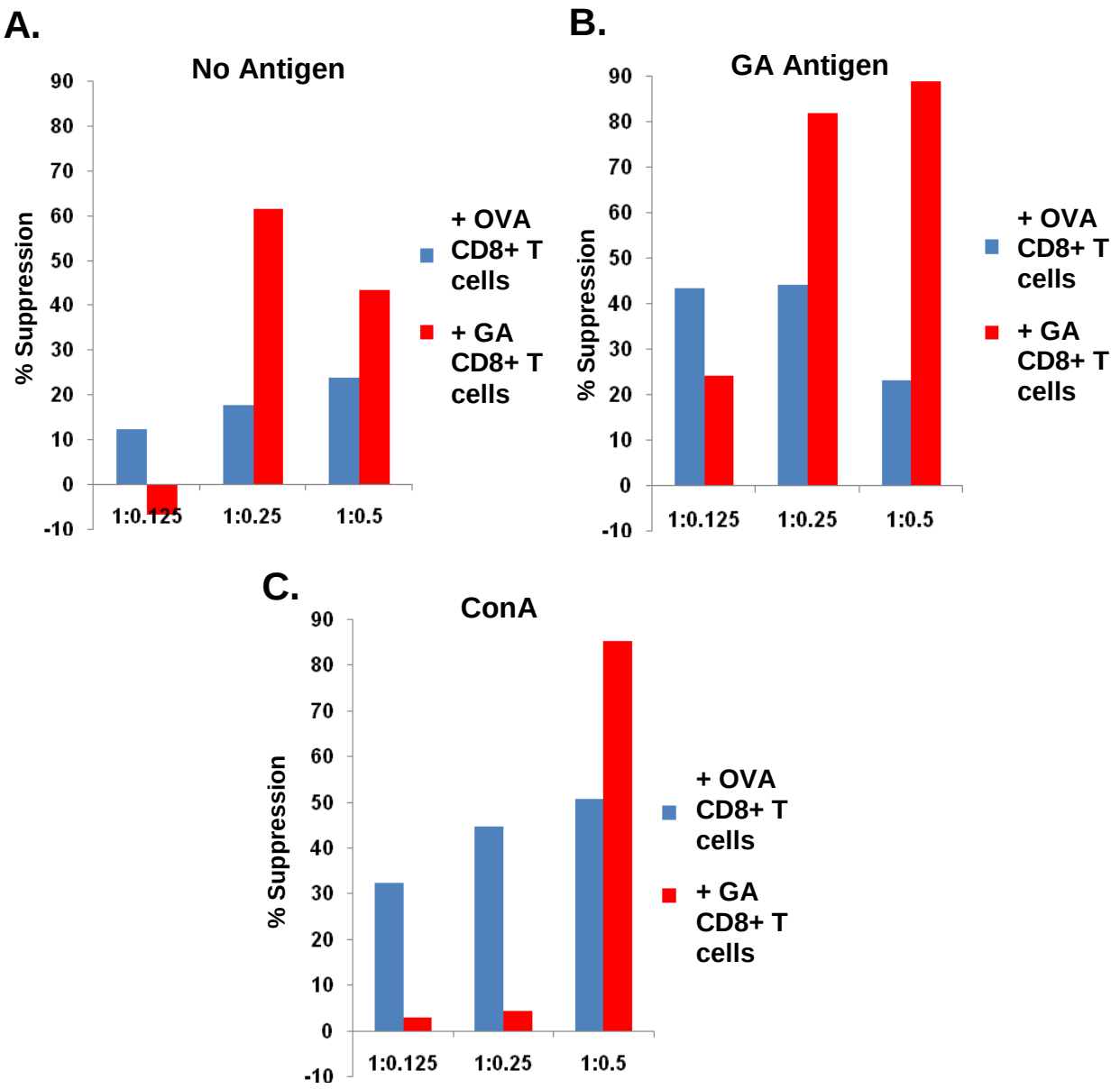

Figure 32. GA-induced CD8$^+$ T cells suppress MOG CD4$^+$ T cells in the presence of no antigen, GA, or ConA. Wildtype CD8-depleted "responder" cells were incubated with increasing number of OVA- or GA-induced CD8$^+$ T cells in the presence of A. No antigen, B. GA, or C. ConA for 5 days. % suppression is calculated: (No Ag% Proliferation – New Ratio % Proliferation)/ (No Ag% Proliferation) x 100.

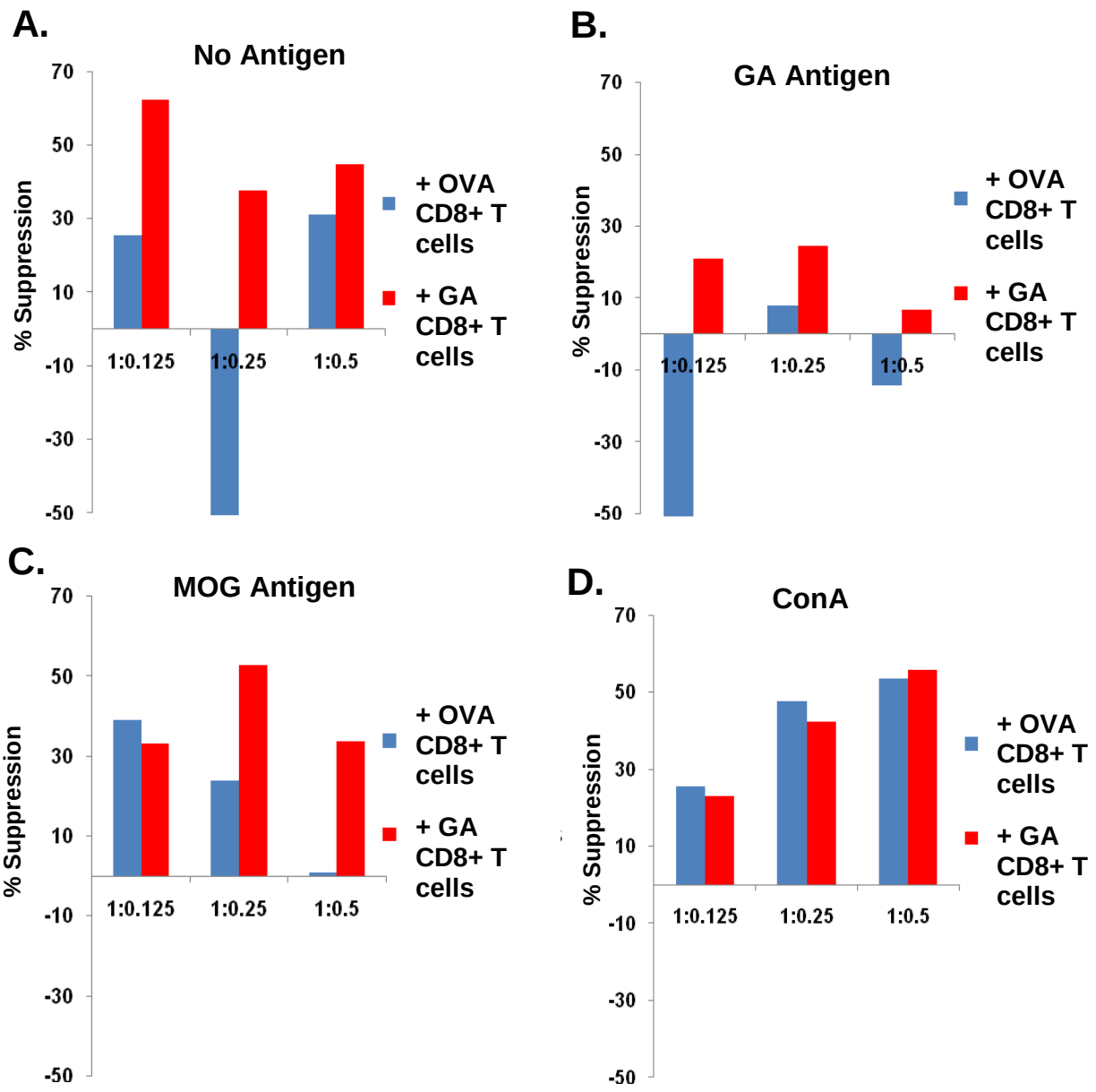

Figure 33. GA-induced CD8$^+$ T cells suppress MOG CD4$^+$ T cells from CD8 (-/-) mice in the presence of no antigen, GA, or ConA. CD8 (-/-) "responder" cells were incubated with increasing number of OVA- or GA-induced CD8$^+$ T cells in the presence of A. No antigen, B. GA, C. MOG 35-55, or D. ConA for 5 days. % suppression is calculated: (No Ag% Proliferation – New Ratio % Proliferation)/ (No Ag% Proliferation) x 100.

GA-induced $CD8^+$ T cells secrete IFN-γ, IL-10, and TNF-α

Another potential mechanism of GA-induced $CD8^+$ T cells may be cytokine secretion. To analyze this possibility we selected for GA-induced $CD8^+$ T cells in different conditions to replicate situations in which we see *in vivo* efficacy. Female wildtype naïve mice were injected with 1mg GA or OVA/IFA at day 0. At day 10, we euthanized half the mice from each group, harvested the cells, and purified $CD8^+$ T cells by positive selection. These $CD8^+$ T cells were incubated with naïve APCs at a 1:5 ratio with no antigen, GA, or ConA. At 72 hours supernatants were removed and frozen. We repeated this process at day 20 and saved the supernatants. IFN-γ and IL-10 ELISAs were performed on day 10 and day 20 samples in side by side experiments. IFN-γ production was below the level of detection in day 10, but was increased in the GA *in vitro* condition at day 20 post GA immunization (Figure 34.A). IL-10 production from GA-induced $CD8^+$ T cells increased from day 10 to day 20 as shown in Figure 34.B. IL-10 production increased by day 20 at both low and high dosages of GA *in vitro*. These results indicate that following 20 days of *in vivo* GA stimulation, GA-induced $CD8^+$ T cells are secreting IFN-γ and IL-10. This observation is relevant to our *in vivo* data since all of our adoptive transfer experiments were performed after 20 days of GA *in vivo* stimulation and then 72 hour *in vitro* GA stimulation. These results were reproducible in 3 independent experiments for IFN-γ and 2 independent experiments for IL-10. These experiments were done in collaboration with Maycie Garibay, Pathology, UT-Southwestern Medical Center.

In a separate experiment we injected naïve wildtype mice with 1 mg GA or OVA/IFA. 20 days later we euthanized those mice, harvested the cells, and stimulated them in a bulk 72-hour culture with GA or OVA (20 μg/mL) and IL-2 (10 pg/mL). After the 72-hour stimulation, we positively selected $CD8^+$ T cells and isolated the RNA for real-time PCR. We used primers for

IFN-γ, TNF-α, IL-10, IL-6, and TGF-β. We found that GA-induced $CD8^+$ T cells produce more IFN-γ, TNF-α, and IL-10 than OVA-induced $CD8^+$ T cells (Figure 35). GA-induced $CD8^+$ T cells produced more than 3-fold as much IFN-γ and 7-fold more IL-10 than OVA-induced $CD8^+$ T cells. These experiments were done in collaboration with Sterling Ortega, Pathology, UT-Southwestern Medical Center.

$CD8^+$ T cells from GA protected mice secrete IFN-γ and IL-10

Thus far, we have shown that $CD8^+$ T cells are required for GA efficacy *in vivo* and we have determined the cytokine profile of the adoptively transferred GA-induced $CD8^+$ T cells that confer protection. We now wanted to know the cytokine profile of $CD8^+$ T cells during *in vivo* protection from severe EAE. To examine the properties of $CD8^+$ T cells during EAE protection we immunized female wildtype naïve mice with MOG 35-55/CFA at day 0 and PT at day 0 and day 2. We followed our standard protocol for 20 μg GA/PBS daily treatment from day 2 until day 15. At day 10, which is prior to disease onset, we euthanized half the mice from each group. Cells were harvested and $CD8^+$ T cells were purified by positive selection. These $CD8^+$ T cells were incubated with naïve APCs at a 1:5 ratio with no antigen, MOG 35-55 (11, 33 and 100 μg/mL), GA (11 and 33 μg/mL), or ConA. At 72 hours, supernatants were removed and frozen. We repeated this process at day 20 and saved the supernatants. As previously seen in our daily GA/PBS treatments (Figure 10), GA protection was observed up until the mice were euthanized at day 20 day. IFN-γ and IL-10 ELISAs were performed on day 10 and day 20 samples in side by side experiments. The results showed that at day 10, more IFN-γ was secreted by $CD8^+$ T cells from non-GA treated mice versus $CD8^+$ T cells from GA-treated mice (Figure 36.A). Since we know the IFN-γ phenotype of GA-induced $CD8^+$ T cells at day 10 (undetectable), this IFN-γ secretion can be attributed to the disease course. Disease onset did not occur in parallel mice

until day 12 suggesting we were likely observing the early stages of disease induction at day 10. These observations will be discussed in more detail later. At day 20, $CD8^+$ T cells from protected mice secreted very high amounts of IFN-γ in response to both MOG antigen and GA antigen compared to $CD8^+$ T cells from unprotected mice with severe EAE (Figure 36.B). Taken together, these data indicate IFN-γ secreted by $CD8^+$ T cells may play a role in the protective *in vivo* efficacy of GA.

The results from the IL-10 data revealed that at day 10, IL-10 secretion was below the level of detection in both GA-treated and non-GA-treated mice. However, at day 20 during GA protection from severe EAE, $CD8^+$ T cells from both the GA-protected and unprotected mice secrete IL-10. As expected, only the CD8+ T cells from GA-protected mice secrete IL-10 in response to GA in vitro, 5-fold higher than unprotected mice and 2- to 3- fold higher than MOG-induced IL-10 secretion (Figure 37). Taken together, this indicates that GA induced IL-10 secretion may play a role in the concurrent observed protection from severe EAE.

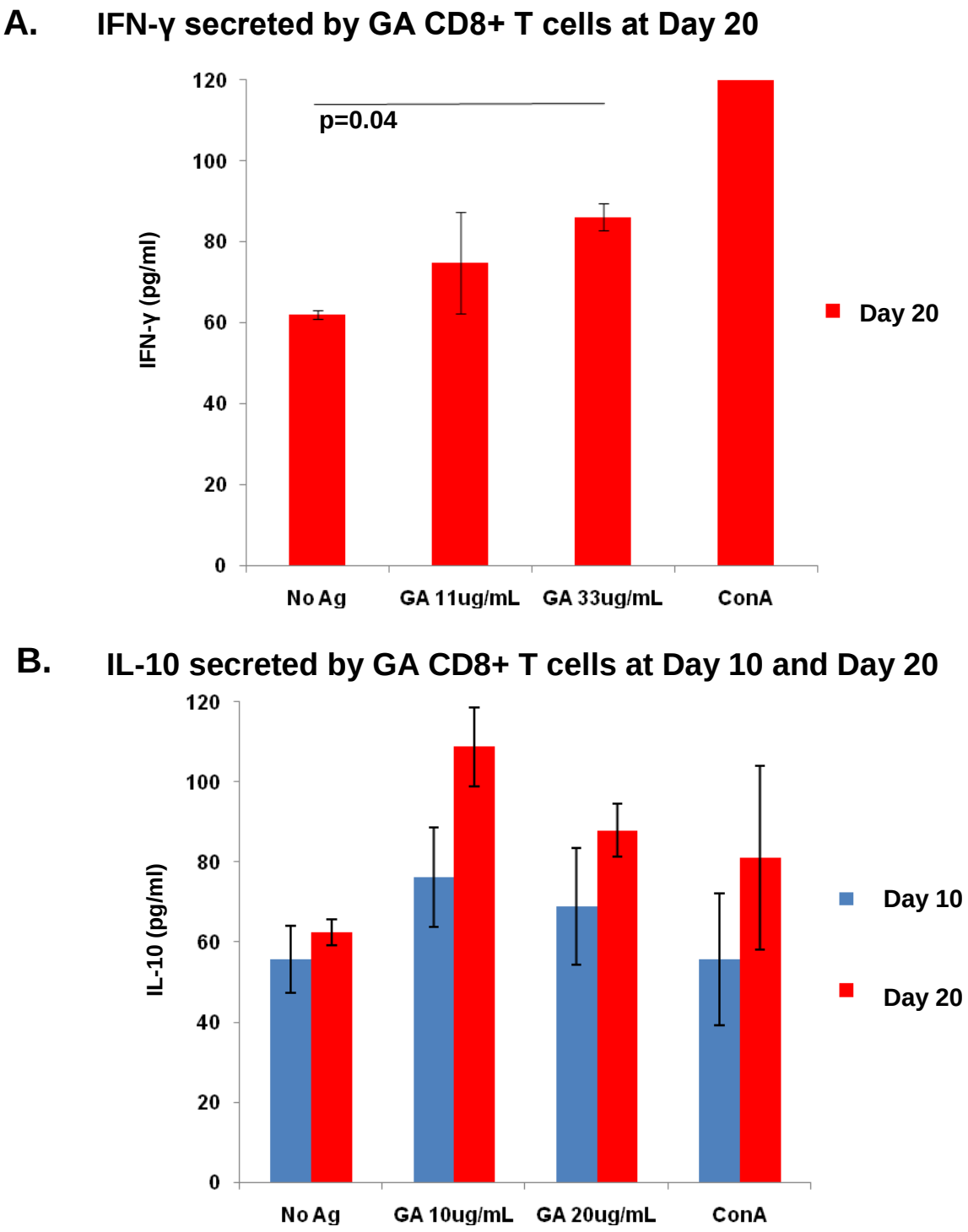

Figure 34: GA-induced CD8$^+$ T cells secrete IFN-γ and IL-10. Female wildtype naïve mice were injected with 1 mg GA on day 0. At day 10, half the mice from each group were

euthanized, cells harvested, and the $CD8^+$ T cells were purified by positive selection. $CD8^+$ T cells were incubated with naïve APCs at a 1:5 ratio with no antigen, GA, or ConA. At 72 hours supernatants were removed and frozen. We repeated this process at day 20 and saved the supernatants. A. IFN-γ and B. IL-10 ELISAs were performed on day 10 and day 20 samples. These results were reproducible in 3 independent experiments for IFN-γ and 2 independent experiments for IL-10. $p=0.04$. These experiments were done in collaboration with Maycie Garibay, Pathology, UT-Southwestern Medical Center.

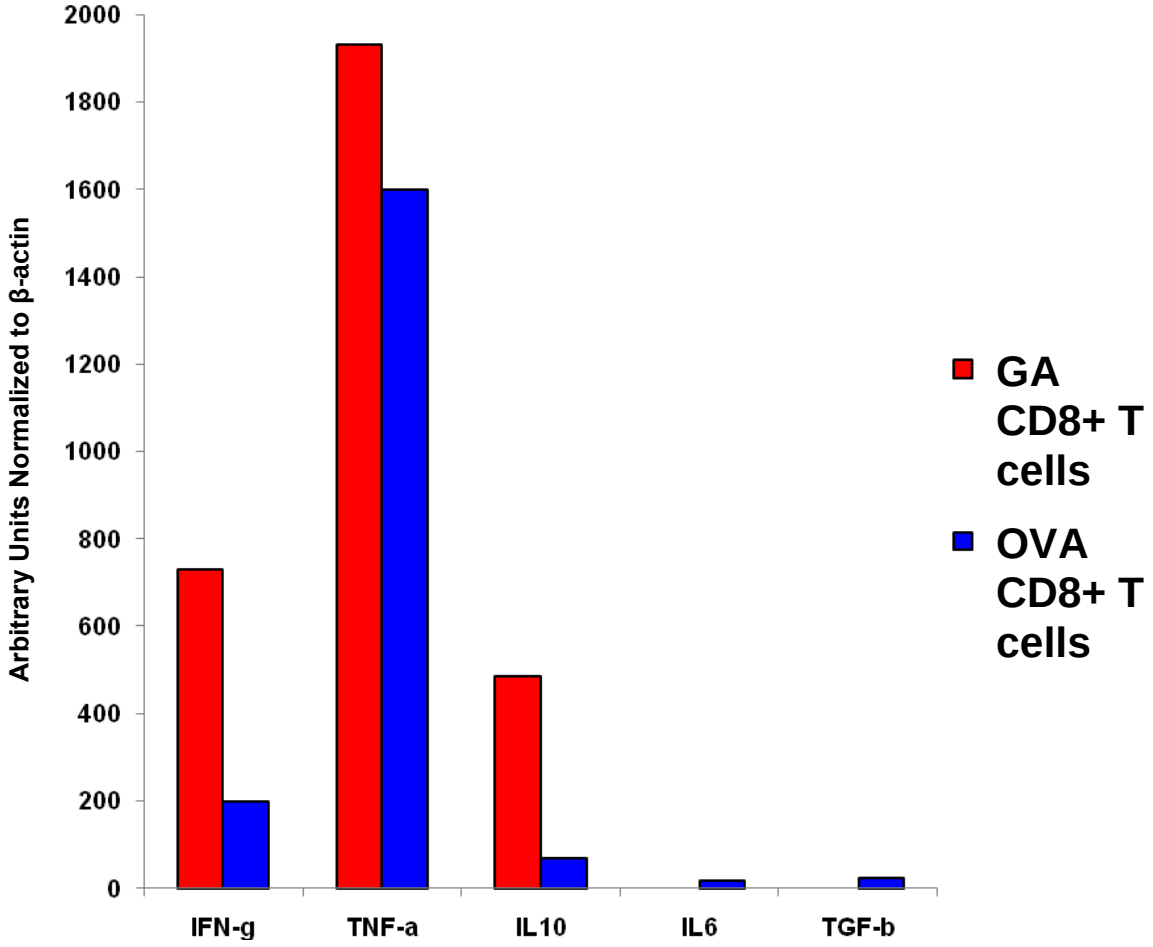

Figure 35: GA-induced CD8+ T cells express IFN-γ, TNF-α, and IL-10. Naïve wildtype mice were injected with 1mg GA or OVA/IFA. 20 days later mice were euthanized, cells harvested, and stimulated in a 72 hour culture with GA or OVA (20 µg/mL) and IL-2 (10 pg/mL). After the 72 hour stimulation, $CD8^+$ T cells were purified by positive selection and RNA isolated for reverse transcriptase PCR. Primers for IFN-γ, TNF-α, IL-10, IL-6, and TGF-β were used to determine dCT. Cytokine levels are expressed as arbitrary units normalized to β-actin expression levels. These experiments were done in collaboration with Sterling Ortega, Pathology, UT-Southwestern Medical Center.

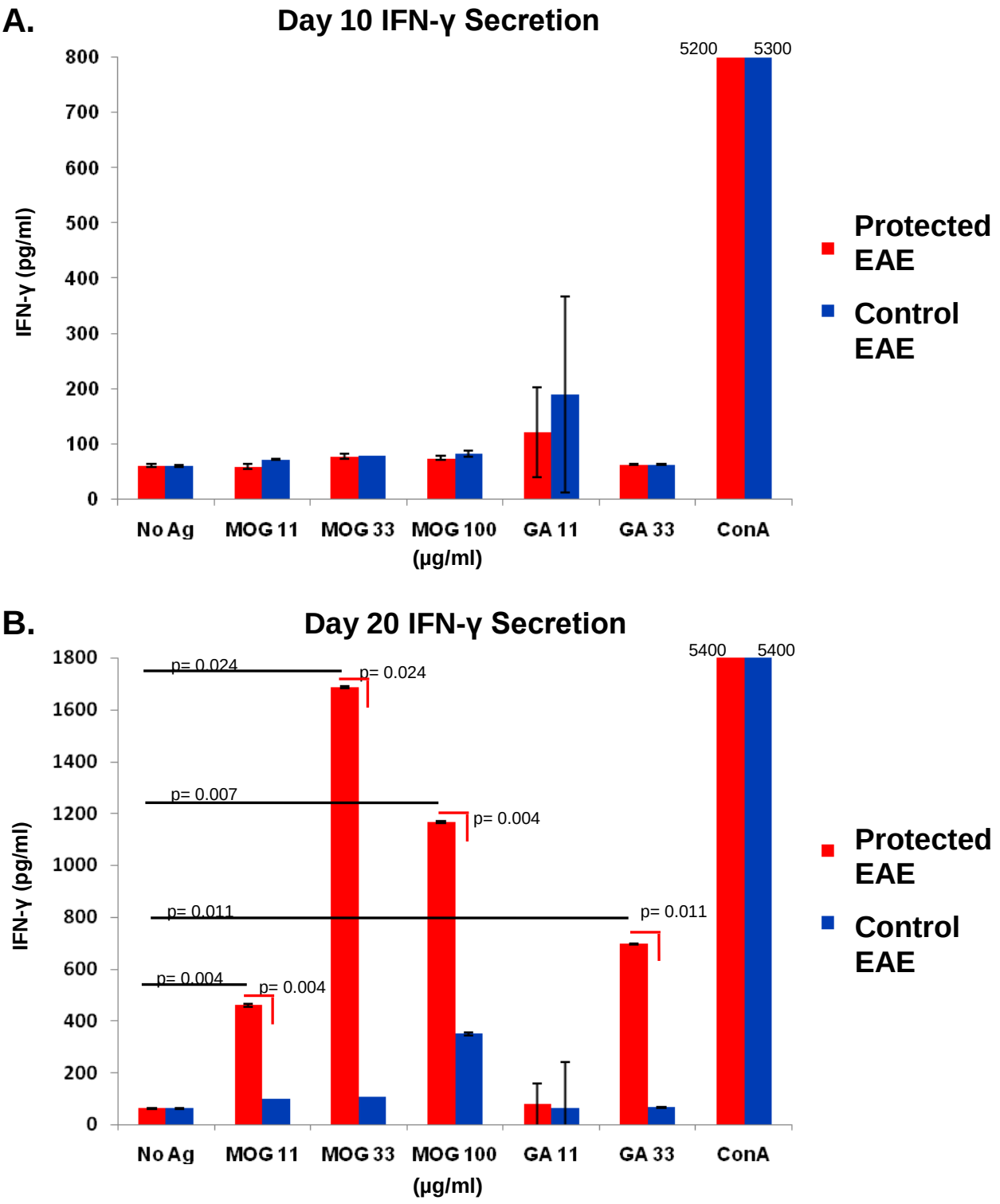

Figure 36: IFN-γ secretion by CD8+ T Cells from GA-treated or non-treated mice. Female wildtype naïve mice were immunized with MOG 35-55/CFA at day 0 and PT at day 0 and day 2. Mice were treated with 20 µg GA/PBS daily treatment or injection control from day 2 until day

15. At day 10, prior to disease onset, half the mice were euthanized from each group. Cells were harvested and $CD8^+$ T cells were purified by positive selection. These $CD8^+$ T cells were incubated with naïve APCs at a 1:5 ratio with no antigen, MOG 35-55 (11, 33 and 100 μg/mL), GA (11 and 33 μg/mL), or ConA. At 72 hours, supernatants were removed and frozen. This process was repeated at day 20. IFN-γ ELISAs were performed on A. day 10 and B. day 20 samples. These results were reproducible in 2 independent experiments. These experiments were done in collaboration with Maycie Garibay, Pathology, UT-Southwestern Medical Center.

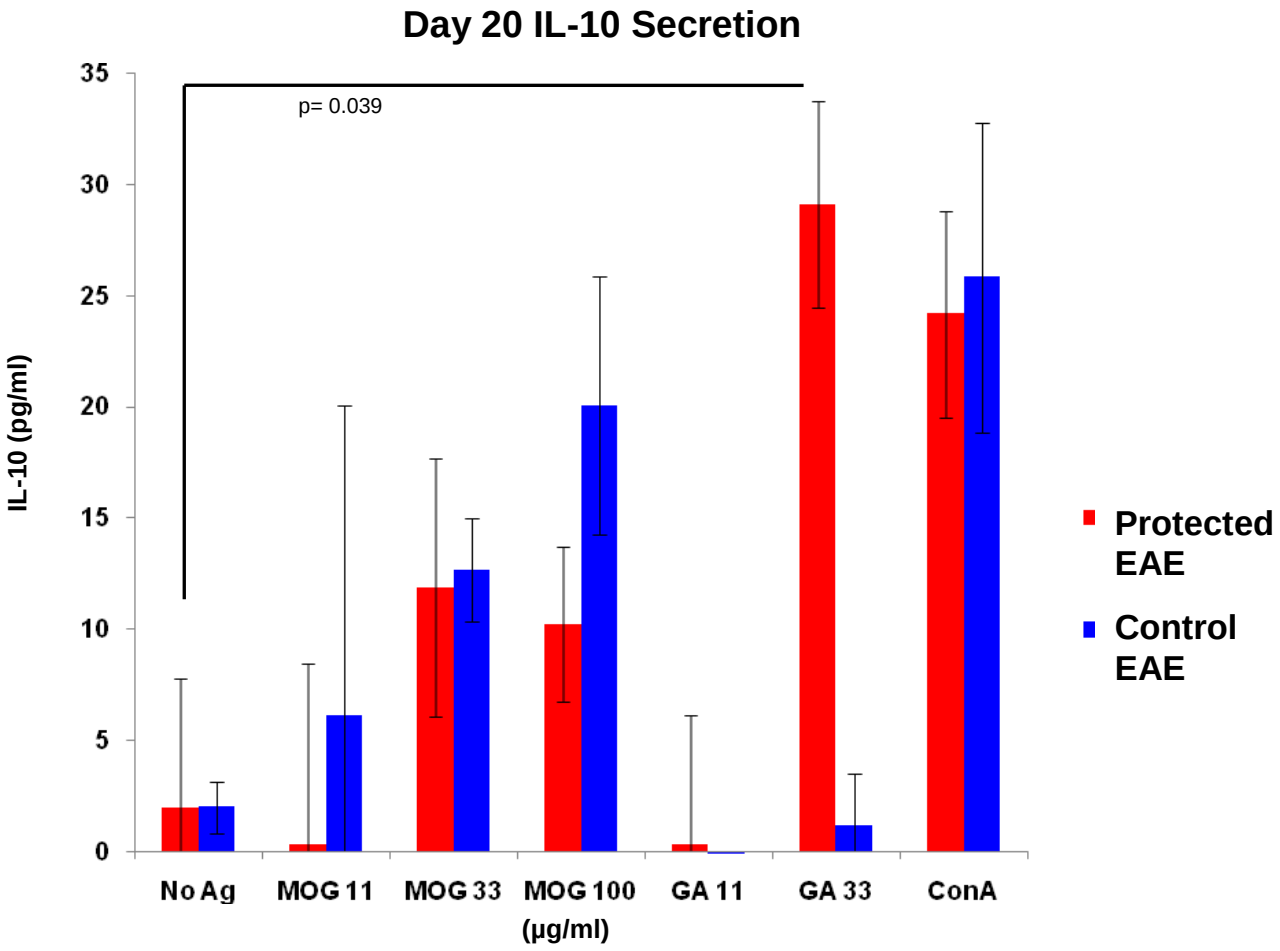

Figure 37: IL-10 secretion by CD8+ T Cells from GA treated or non-treated mice. Female wildtype naïve mice were immunized with MOG 35-55/CFA at day 0 and PT at day 0 and day 2. Mice were treated with 20 µg GA/PBS daily treatment or injection control from day 2 until day 15. At day 10, prior to disease onset, half the mice were euthanized from each group. Cells were harvested and $CD8^+$ T cells were purified by positive selection. These $CD8^+$ T cells were incubated with naïve APCs at a 1:5 ratio with no antigen, MOG 35-55 (11, 33 and 100 µg/mL), GA (11 and 33 µg/mL), or ConA. At 72 hours, supernatants were removed and frozen. This process was repeated at day 20. IFN-γ ELISAs were performed on day 10 and day 20 samples. Day 10 samples were below the level of detection. These results were reproducible in 2 independent experiments. These experiments were done in collaboration with Maycie Garibay, Pathology, UT-Southwestern Medical Center.

Chapter VII: Mechanism: Glatiramer Acetate Influence on other Cell Types

GA may alter MOG-specific $CD4^+$ T cells

As discussed earlier, mice protected by GA from severe EAE have less MOG-specific proliferation. We were interested to see if $CD4^+$ T cells from GA-protected mice have different encephalitogenic properties than non-protected mice. Utilizing our adoptive transfer protocol to induce passive disease we can determine if MOG $CD4^+$ T cells from GA-treated mice induce less severe EAE. Female wildtype naïve mice were immunized with MOG 35-55/CFA on day 0 and injected with PT on day 0 and day 2. We treated the mice with either 20 µg GA/PBS or injection control from day 2 until day 15. At day 20 of disease we euthanized both groups of mice and harvested lymph nodes and spleens. Cells were stimulated for 72 hours *in vitro* with MOG (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour stimulation, $CD4^+$ T cells were purified by positive selection and IV transferred into naïve mice to induce passive disease. Our preliminary results showed that on a small scale, $CD4^+$ T cells from GA-protected mice may be less encephalitogenic than $CD4^+$ T cells from unprotected mice (Figure 38). Because of the small numbers involved in this experiment, repeat experiments are necessary to conclude that GA alters MOG-specific $CD4^+$ T cells.

GA alters type II monocytes

It has been reported that GA has an effect on the formation of type II monocytes. Type II monocytes are defined by their cytokine profile of secreting less TNF-α, and IL-12, but more IL-10 and TGF-β. This report states that type II monocytes are responsible for the immunomodulatory effect of GA [123]. Although type II monocytes play a role in GA immune modulation, we hypothesize that CD8+ T cells are required for their therapeutic effects. Therefore, we investigated whether or not type II monocytes develop in the CD8 (-/-) mouse.

While type II monocytes clearly play a role in GA immune modulation, they may require CD8+ T cells for their therapeutic effects.

We injected 150 µg GA/PBS daily for 7 days as previously described as a method of inducing anti-inflammatory type II monocytes in wildtype mice as well as CD8 (-/-) mice. We purified monocytes by $CD11b^+$ positive selection and set up the *in vitro* cultures with either no antigen, 10U or 100U IFN-γ. Supernatants were collected at 48, 72, and 120 hours. We then performed cytokine analysis by ELISAs for TNF-α and IL-10. Our results showed that we could replicate the published data from wildtype mice showing increased IL-10 production, as well as decreased TNF-α production following GA therapy. Unlike our wildtype results, our CD8 (-/-) data showed that in the absence of $CD8^+$ T cells, TNF-α secretion increased (Figure 39). The IL-10 CD8 (-/-) data was not conclusive. One trial showed that in the absence of $CD8^+$ T cells, IL-10 production decreased after GA therapy. However, repeat experiments showed no difference, following the same trend as wildtype (Figure 40). Taken together, the absence of $CD8^+$ T cells does not clearly affect type II monocytes production, but may alter one or two of the anti-inflammatory cytokines. Since Type II monocytes are defined by their cytokine profile, it is not clear what effect an increase in TNF-α would have on their immunomodulatory properties. It is possible that in the CD8 (-/-) a different kind of monocyte as defined by its cytokine profile is formed, but it's not clear what effect this would have on monocytes' GA therapeutic effect. One possibility is that alteration in TNF-α does not change the clinical benefit of the type II monocytes and that it's decrease in wildtype mice is simply a phenotypic description in this setting. Additional experiments should be done to elucidate these possibilities and examine the production of TGF-β by monocytes in the CD8 (-/-) mouse.

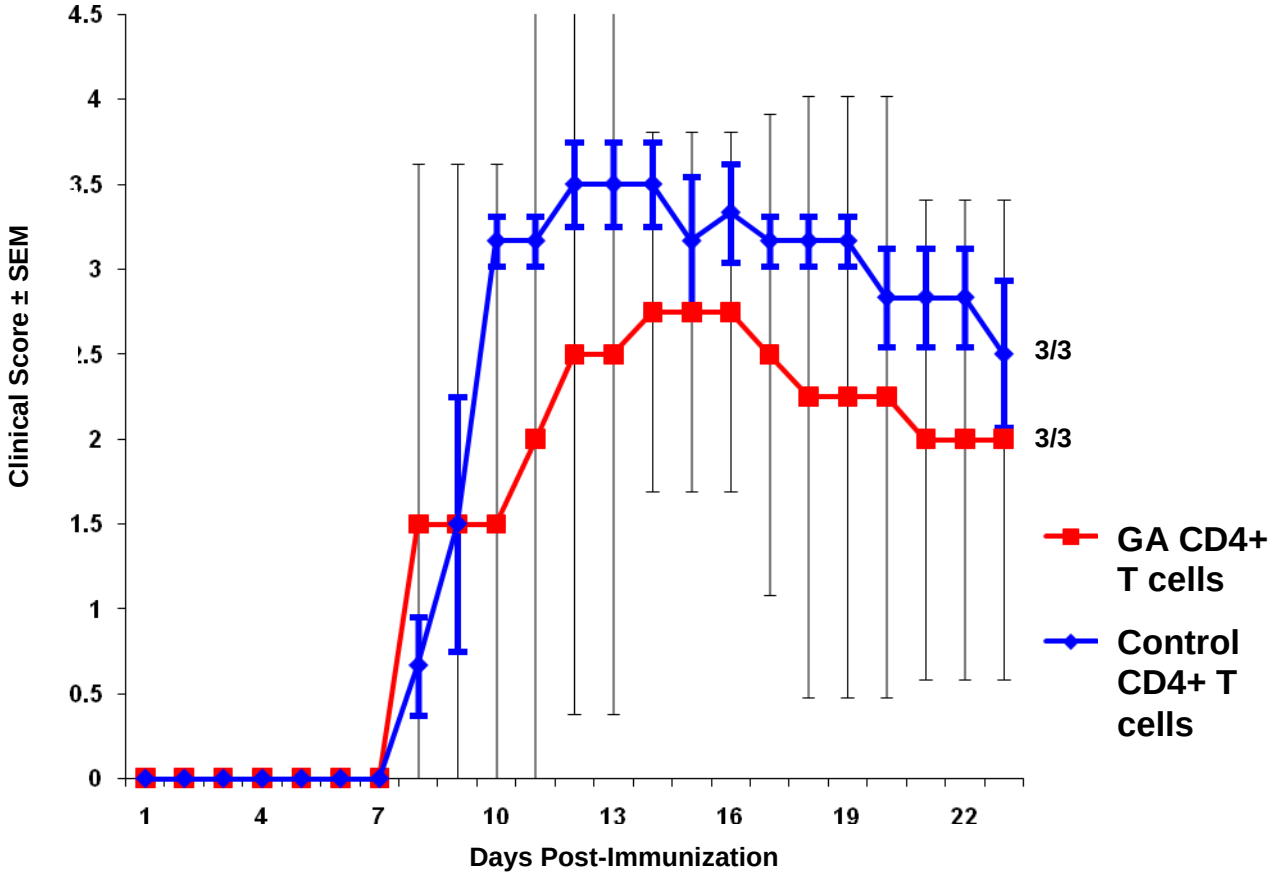

Figure 38: CD4$^+$ T cells from GA-protected mice may have less encephalitogenic properties than CD4$^+$ T cells from unprotected diseased mice (preliminary data). Female wildtype naïve mice were immunized with MOG 35-55/CFA on day 0 and injected with PT on day 0 and day 2. We treated the mice with either 20 µg GA/PBS or injection control from day 2 until day 15. At day 20 of disease we euthanized both groups of mice and harvested lymph nodes and spleens. Cells were stimulated for 72 hours *in vitro* with MOG (20 µg/mL) and IL-2 (10 pg/mL). After the 72-hour stimulation, CD4$^+$ T cells were purified by positive selection and 4x10^6 MOG CD4$^+$ T cells were IV transferred into naïve mice to induce passive disease. Mice were evaluated for disease until day 24. The graph shows mean clinical score ± SEM of three mice.

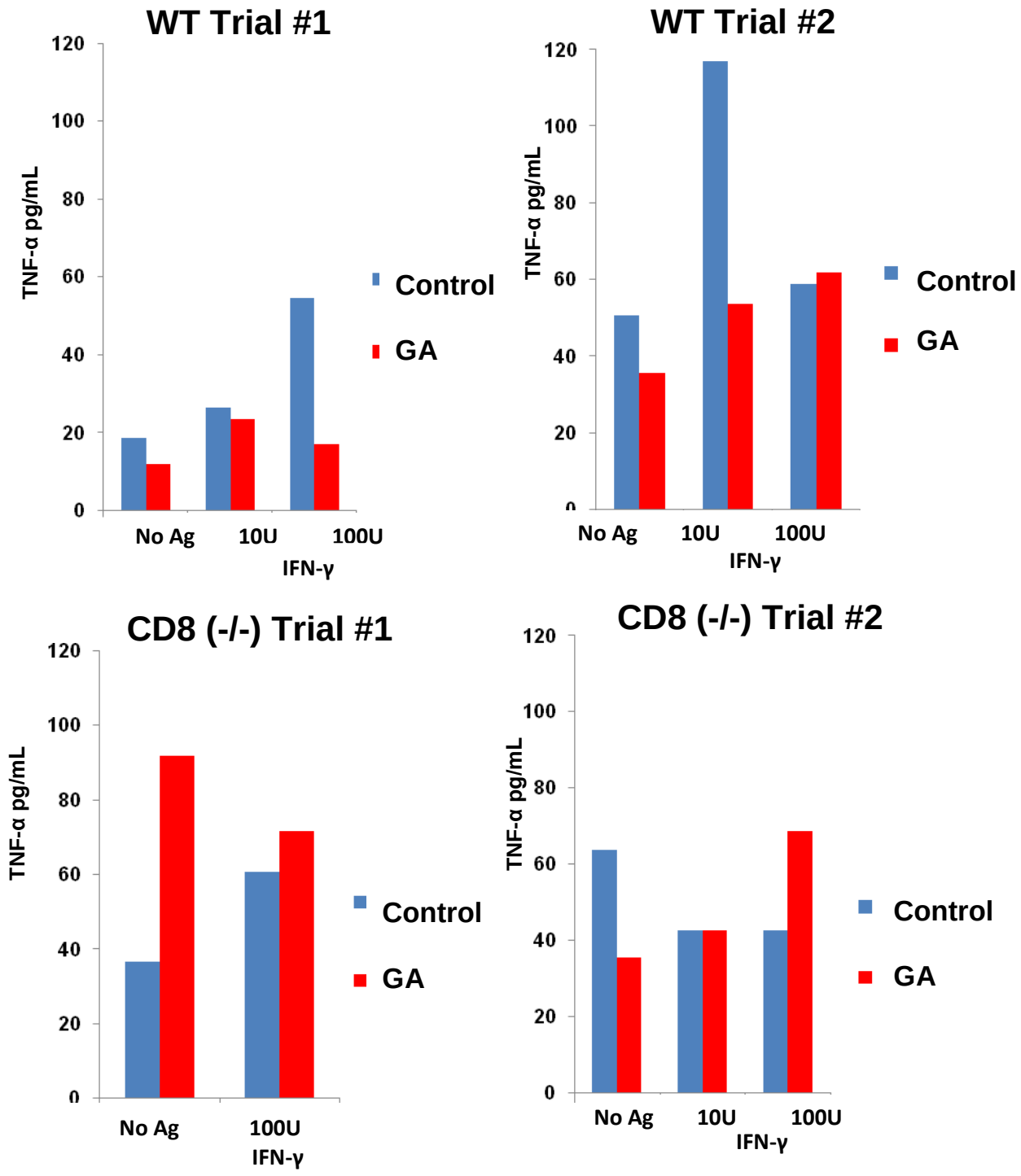

Figure 39: TNF-α secretion is decreased in WT mice following GA therapy, but increased in CD8 (-/-) mice. 150 µg GA/PBS daily was injected for 7 days in wildtype and CD8 (-/-) mice. We purified monocytes by $CD11b^+$ positive selection and set up the *in vitro* cultures with either

no stimulation, 10U or 100U IFN-γ. Supernatants were collected at 48, 72, and 120 hours. Cytokine analysis was performed by ELISA for TNF-α. These figures are representative of 3 independent experiments.

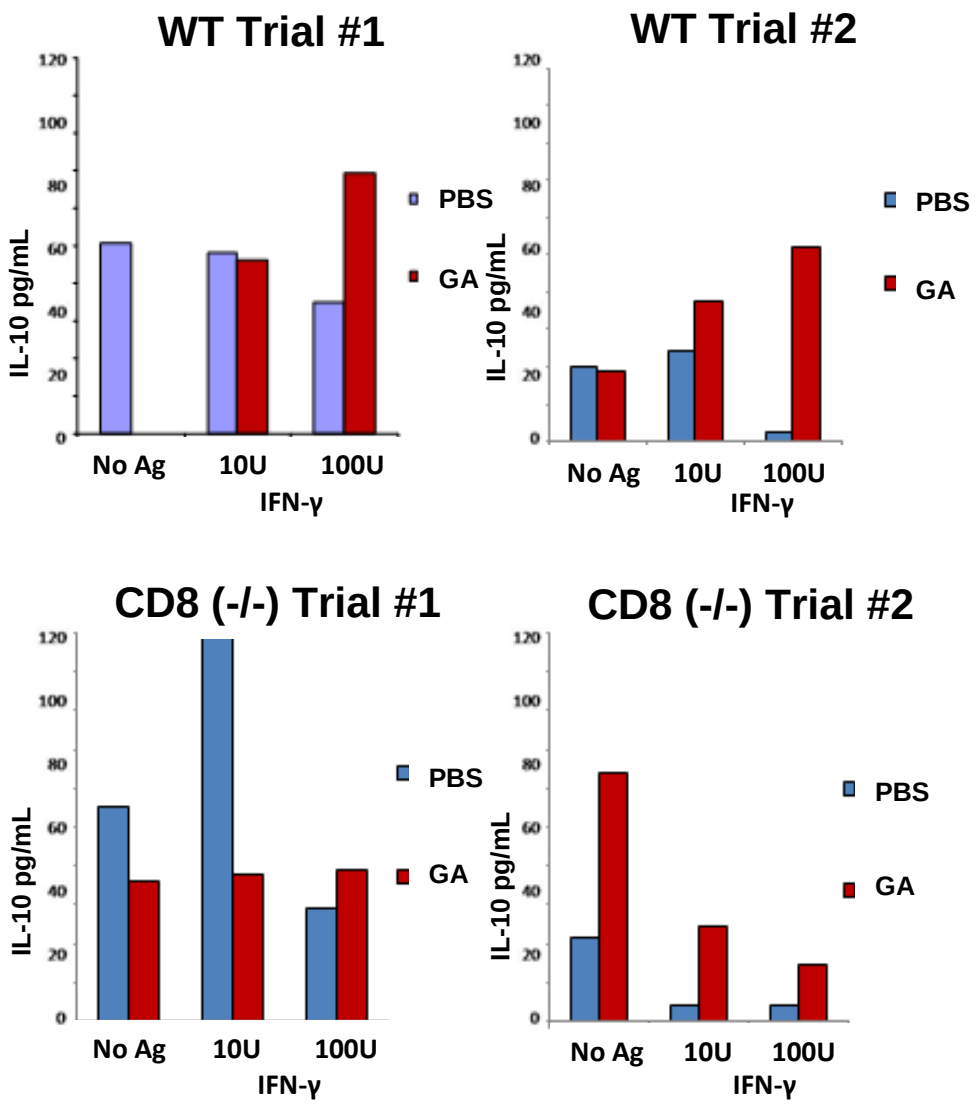

Figure 40: IL-10 secretion is increased in WT mice following GA therapy, but decreased in CD8 (-/-) mice. 150µg GA/PBS daily was injected for 7 days in wildtype and CD8 (-/-) mice. We purified monocytes by $CD11b^{+}$ positive selection and set up the *in vitro* cultures with either no stimulation, 10U or 100U IFN-γ. Supernatants were collected at 48, 72, and 120 hours. Cytokine analysis was performed by ELISA for IL-10. These figures are representative of 4 independent experiments.

Chapter VIII: $CD8^+$ T cells and EAE

In the absence of $CD8^+$ T cells, mice develop more severe EAE

We wanted to determine the influence $CD8^+$ T cells have during EAE. We reasoned that if $CD8^+$ T cells have a regulatory role in EAE, as some groups have reported [82, 84, 136-138], then we should observe a noticeable difference between the disease curves of WT and CD8 (-/-) mice if we tritrated the MOG concentrations we use in the immunizations. We injected both WT and CD8 (-/-) mice with 200 µg, 100 µg, or 50 µg MOG 35-55/CFA to induce EAE. As expected, in wildtype mice, we found that as we decreased the MOG concentration from 200 µg to 100 µg to 50 µg we observed a decline in both disease incidence and severity (Figure 41A). Disease incidence dropped from 100% in the normal 200 µg MOG concentration to 80% and 60% for the 100 µg and 500 µg MOG concentrations respectively. Very interestingly, we observed 100% incidence in CD8 (-/-) mice regardless of the decreasing MOG concentrations (Figure 41B). Also, peak disease severity and disease onset remained identical in the absence of $CD8^+$ T cells.

We next looked at EAE in CD8 (-/-), (+/-), and (+/+) littermates. This is the only accurate way to compare WT and CD8 (-/-) disease curves. Age-matched female mice from each group were immunized with 200 µg MOG 35-55 emulsified in CFA supplemented with *M. tuberculosis* (4 mg/mL) at day 0. 250 ng pertussis toxin was injected at day 0 and day 2. Mice were evaluated for clinical symptoms until day 30 in a blinded manner. We found that CD8 (-/-) mice got more severe EAE than their CD8 (+/+) progeny (Figure 42). The CD8 (+/-) mice had a disease curve that mimicked the CD8 (-/-) mice indicating there may be threshold of CD8 receptor required to observe the regulatory effects during EAE. These data reaffirmed our hypothesis that $CD8^+$ T

cells have a regulatory role in EAE and that this cell type plays a role in the therapeutic modulation of disease.

$CD8^+$ T cell depletion at day -1 results in more severe EAE

As shown in Figure 14, when we deplete $CD8^+$ T cells prior to disease induction during the time of GA/IFA treatment, the therapeutic effect of GA is removed. We next sought to deplete $CD8^+$ T cells after EAE disease induction to examine the kinetics of GA protection. Determining the importance of $CD8^+$ T cells at each phase of EAE disease may be important to fully elucidating the mechanism of GA protection. First it was necessary to determine the EAE disease course when $CD8^+$ T cells were depleted at different time points of disease. Three groups were designed to receive initial injections of 200 µg anti-CD8 at either day -1, day 6, or day 13 of disease. Each group would receive 100 µg anti-CD8 boosters 7 days later and a 50 µg booster after an additional 7 days (Figure 43). Unfortunately, two mice died during the first 100 µg booster injections at day 6 (from the day -1 group). These mice died within an hour of receiving the anti-CD8 prompting us to redesign the CD8 depletion protocol. We immediately stopped additional booster anti-CD8 injections and continued with the experiment. Each group only received the initial 200 µg anti-CD8 injection. We evaluated the mice for clinical EAE symptoms until day 36 in a blinded manner.

The day -1 CD8-depleted group had more severe EAE versus the other depletion time points and no depletion group (Figure 44A). Figure 44B depicts only the day -1 group and the no depletion group. CD8+ T cells may have an effect during both the induction and effector phases of EAE. This day -1 depletion time point is important for understanding our success with the day -1 prevention adoptive transfer studies that will be discussed later.

Since multiple mice died during the CD8-depletion kinetic experiment we needed to first determine the cause of their death before we could set up a GA protection/CD8-depletion experiment. We suspected antibody-immune complexes/serum sickness may have rapidly developed after the second anti-CD8 injection. To determine if the death was due to the CFA in the MOG injection or the PT injected at day 0 and day 2, we injected 20 mice with 200 µg anti-CD8 and then divided those mice up into the following groups: CFA/PBS + PT, IFA/PBS + PT, PT only, and CFA/PBS only. All 4 groups received their injection regimens at day 0 and/or day 2 and then all groups were given a 100 µg anti-CD8 booster at day 6. Two out of the five mice in the CFA/PBS + PT group died within 2 hours of receiving the 100 µg anti-CD8 booster while all mice in the other groups survived without complications. We administered a 50 µg anti-CD8 booster at day 13 to all 4 groups, but another mouse died in the CFA/PBS + PT group within an hour of the injection. The CFA + PT combination highly activates the immune system and results in 60% mortality when anti-CD8 is administered. Since EAE disease is induced using these conditions we abandoned any further post day 0 studies with the CD8-depleting antibody.

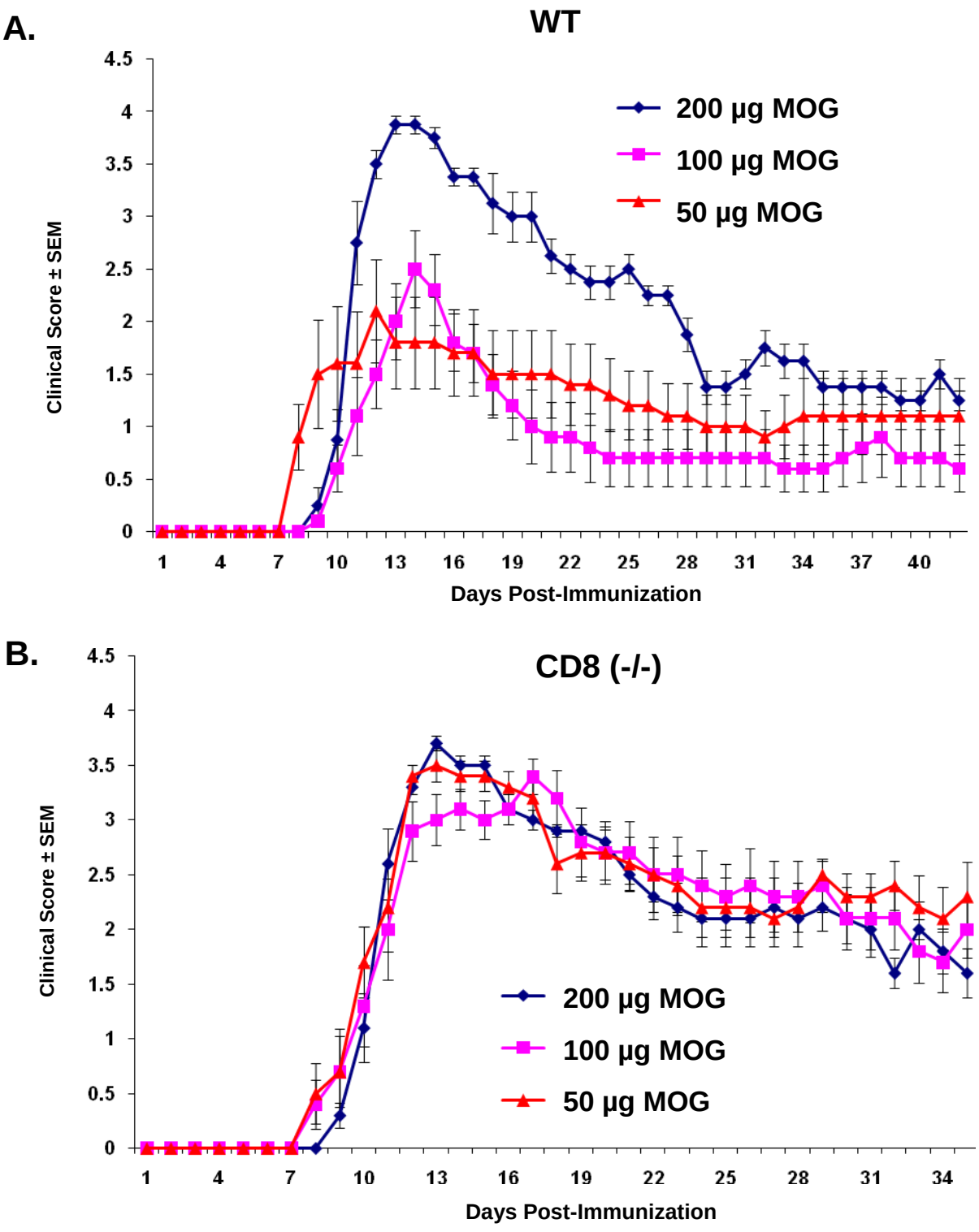

Figure 41: Different Concentrations of MOG 33-55 induce different disease severity in wildtype mice, but the same severity of EAE disease in the absence of CD8$^+$ T cells. Female WT or CD8 (-/-) mice were immunized with either 200 µg, 100 µg, or 50 µg MOG 35-55

emulsified in CFA supplemented with *M. tuberculosis* (4 mg/mL) at day 0. 250 ng pertussis toxin was injected at day 0 and day 2. Mice were evaluated for clinical symptoms until day 35 or 41. A. WT disease. B. CD8 (-/-) disease. The graph depicts the mean clinical score ± of 5 mice.

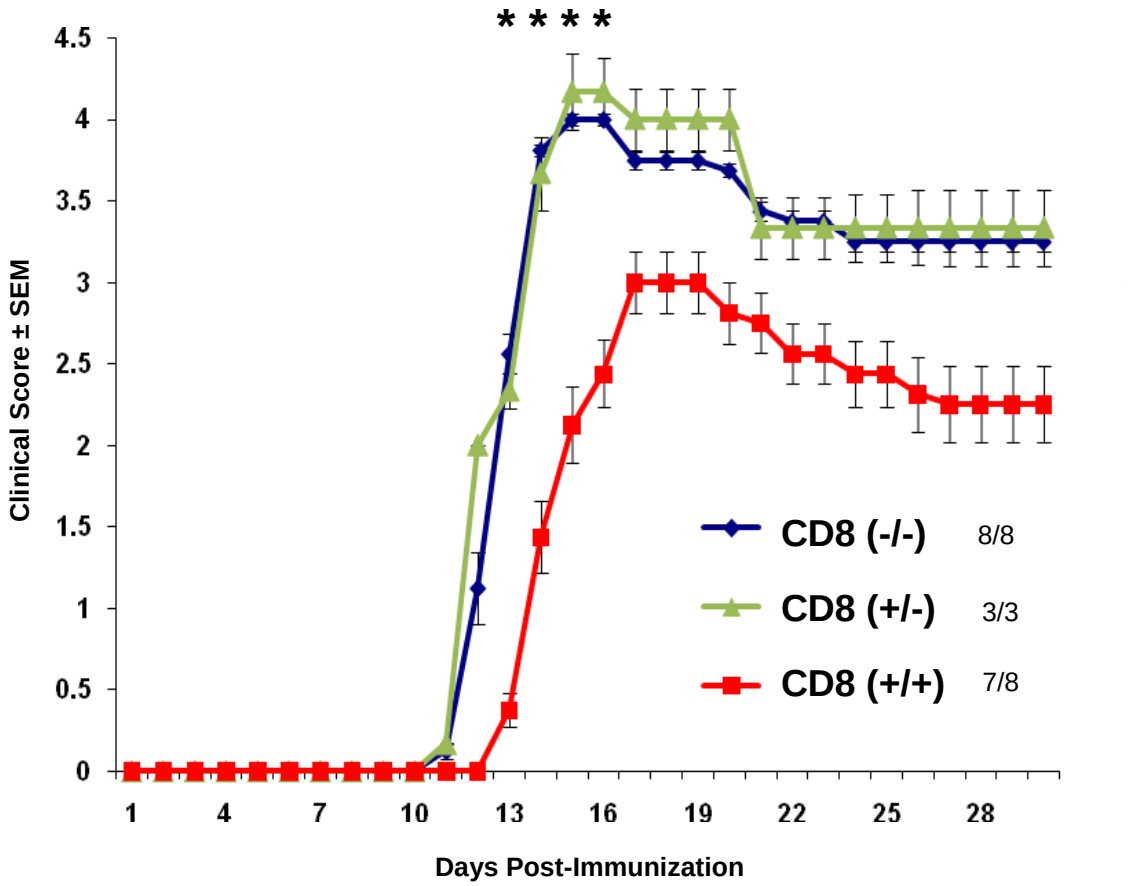

Figure 42: EAE is more severe in the absence of CD8$^+$ T cells. Female CD8 (-/-), (+/-), and (+/+) littermates were bred and then age matched. Each group was immunized with 200 µg MOG 35-55 emulsified in CFA supplemented with *M. tuberculosis* (4 mg/mL) at day 0. 250 ng pertussis toxin was injected at day 0 and day 2. Mice were evaluated for clinical symptoms until day 30 in a blinded manner. The graph depicts mean clinical score ± SEM of 8 or 3 mice. * indicates p=0.05

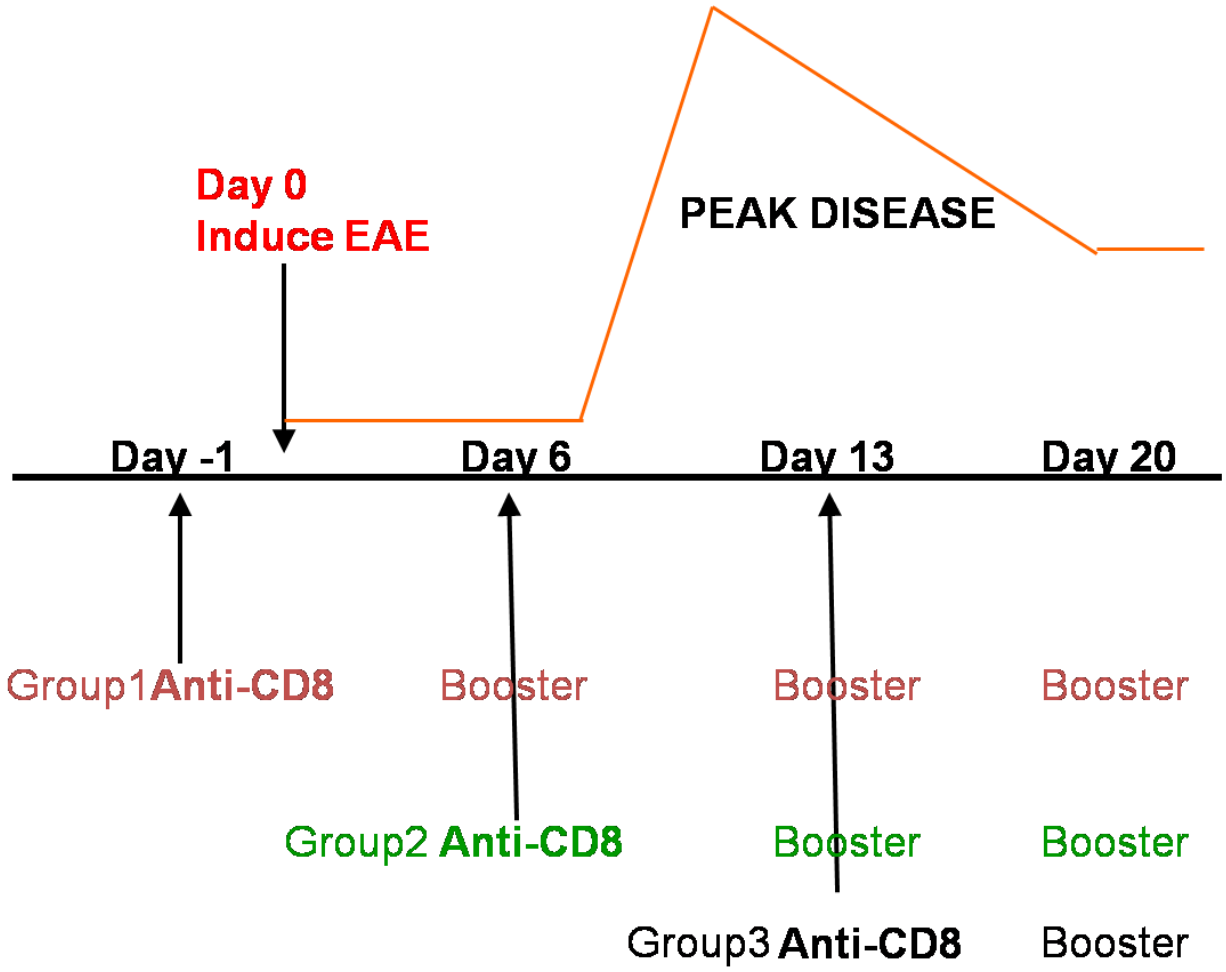

Figure 43: Experimental Design for CD8+ T cell depletion during EAE disease. Three groups of mice received initial injections of 200 µg anti-CD8 at either Day -1, 6, or 13. Each group would subsequently receive 2 or 3 booster injections of 100 µg anti-CD8 7, 14, and 21 (group 1 only) days later. These experiments were done in collaboration with Dr. Mihail Firan, Pathology, UT-Southwestern Medical Center.

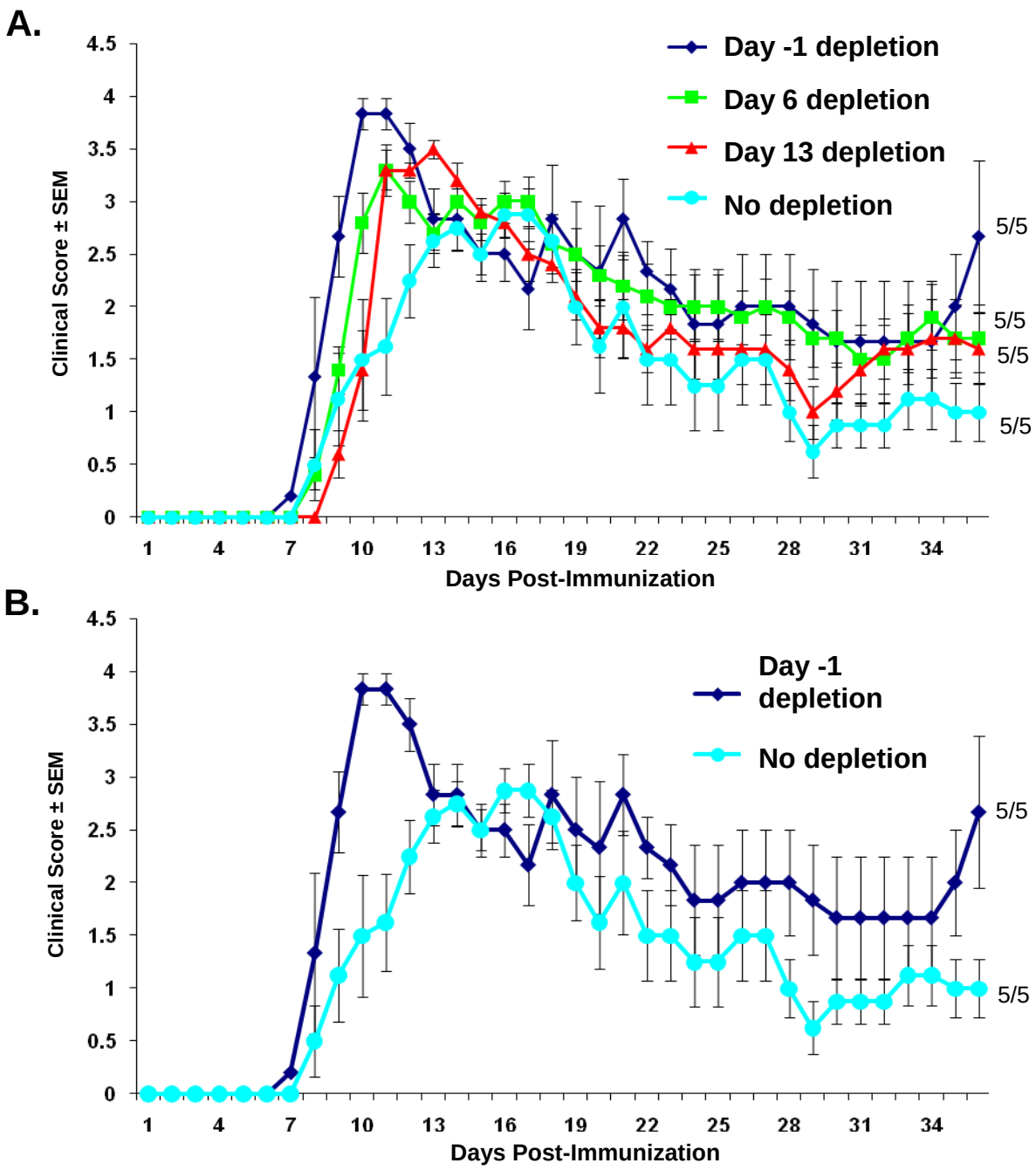

Figure 44: CD8$^+$ T cells depleted at Day -1, 6, or 13 of EAE disease course. Three groups of mice received initial injections of 200 µg anti-CD8 at either Day -1, 6, or 13. EAE was induced with MOG 35-55/CFA immunization at day 0 and PT injections at day 0 and 2. The day -1 group received a booster of 100 µg anti-CD8 at day 6 which resulted in the immediate death of two mice suspending further boosters. Mice were evaluated for EAE clinical symptoms until day 36. Graph depicts mean clinical scores ± SEM. * indicates $p<0.05$

Chapter IX: Discussion and Future Directions

The exact mechanism of action of GA remains elusive and very complicated. Many different groups have reported various cell types involved in the therapeutic modulation of disease [122, 123, 128, 133, 139-142]. Previously, accepted dogma was that GA worked by skewing from a Th1 to a Th2 immune response in the $CD4^+$ T cell subset and thereby working by bystander suppression [115-117]. GA has also been reported to compete with myelin related antigens for MHC binding [118]. Other groups have reported the involvement of T regulatory cells, $CD4^+$ $CD25^+Foxp3^+$, as being responsible for some immune modulation and tolerance mechanisms [125, 139]. GA has also been shown to affect type II monocytes and dendritic cells [123, 126]. Our lab has shown that in MS patients, following daily GA therapy, the deficient $CD8^+$ T cell response is restored to levels comparable to healthy adults [128]. These initial observations indicated that $CD8^+$ T cells play a role in the therapeutic modulation of MS and inspired us to pursue this research project in the murine system. By using the EAE mouse model, we are able to explore the role of $CD8^+$ T cells mechanistically.

The role of $CD8^+$ T cells in EAE has been somewhat controversial. There is literature that supports both a pathogenic role and a regulatory role for $CD8^+$ T cells. It has been shown that CD8 (-/-) mice and CD8-depleted mice have worse EAE or more relapses of the disease [82, 84, 136]. In β2-microglobulin (-/-) mice, which have no $CD8^+$ T cells due to an MHC I deficiency, a regulatory role is also shown for CD8+ T cells [137]. While most literature has focused on the $CD4^+$ regulatory T cell subset, $CD8^+$ T cells may have an important subset of regulatory cells as well. Najafian et al. reported the existence of $CD8^+$ $CD28^-$ T suppressor cells in mice [138]. Another study has reported that $CD8^+$ $CD122^+$ T cells are regulatory in the EAE model and produce IL-10, suppress IFN-γ production and proliferation of $CD8^+$ T cells [143].

Despite this evidence for a regulatory role for $CD8^+$ T cells or a regulatory subset of $CD8^+$ T cells, it was also reported that $CD8^+$ T cells had a pathogenic role by inducing disease. One group reported that in C3H mice a CD8 infiltrating disease could be induced by injecting with myelin basic protein. It was also reported that passive disease could be induced by transfer of pathogenic $CD8^+$ T cells and peptide-specific $CD8^+$ T cells could be indentified using MHC I tetramers [79-81].

Our studies show a regulatory role for $CD8^+$ T cells in EAE. MOG-induced EAE is more severe in CD8 (-/-) mice compared to WT mice at the same concentrations. A decrease in the MOG concentration results in a decrease in both incidence and disease severity in WT mice, but in CD8 (-/-) mice, 100% incidence and severe EAE is observed even at ¼ of the normal MOG concentration (Figure 41). $CD8^+$ T cells seem to have a threshold for a tolerance mechanism that is overwhelmed or "broken" by the immune cascade induced by high concentrations of MOG. Our day -1 CD8 depletion data depicting worse disease in the absence of $CD8^+$ T cells indicates that $CD8^+$ T cells are playing a regulatory role during the induction of disease which matches other published depletion studies by Jiang et al. and Montero et al [84, 85].

We hypothesized that similar to our human observations, following GA treatment, a GA-specific $CD8^+$ T cell response was present in mice. The main goals of this work were to first verify the existence of GA-specific $CD8^+$ T cell responses, prove their *in vivo* relevance during therapeutic modulation of autoimmune demyelination, and then elucidate its regulatory mechanism.

The phenotype of our GA-induced $CD8^+$ T cells is not fully characterized. Thus far, in our adoptive transfer studies we utilize the bulk $CD8^+$ T cell population. Because these $CD8^+$ T cells are stimulated *in vivo* and then *in vitro* one might assume it is the antigen responsive

population that is suppressive. However, we don't have direct evidence of this. One possibility is that the suppressive GA-induced $CD8^+$ T cells are actually the non-proliferating faction of cells. This subset would be the CFSE high population in our CFSE proliferation assays. One way to resolve this issue is to antigen stimulate a bulk population as we have described, but flow sort the CFSE high (non-dividing) and CFSE low (dividing) cells. We would then IV transfer these cells in our prevention or therapeutic assay to determine their suppressive capabilities. If the suppressive population is actually the non-proliferating population then low numbers from a flow sort should not be an issue. We reproducibly observed prevention from severe EAE when adoptively transferred at day -1 with cell numbers as low as $2.5x10^6$ GA-induced $CD8^+$ T cells. This implies that if the majority of those cells are proliferating then 100,000 to 500,000 non-dividing $CD8^+$ T cells may be enough to suppress severe EAE if the suppressive population is the non-dividing cells. If both the 100,000 to 500,000 non-dividing $CD8^+$ T cells and $2.5x10^6$ bulk GA-induced $CD8^+$ T cells prevent severe EAE then we know the non-dividing faction is responsible for the suppression.

As previously mentioned, our day -1 CD8 depletion data suggests that depleting $CD8^+$ T cells removes a tolerance mechanism. Our $CD8^+$ T cell-depleted mice developed more severe disease than our non-depleted mice (Figure 46). The observation that depleting $CD8^+$ T cells at day -1 makes EAE worse complements our adoptive transfer data. By transferring GA-induced $CD8^+$ T cells during this same time frame we are able to prevent severe EAE- the exact opposite result of the CD8 depletion studies. It should be noted that we have attempted to IV transfer OVA $CD8^+$ T cells or naïve $CD8^+$ T cells at day -1, but neither could prevent severe clinical symptoms. GA-induced $CD8^+$ T cells have regulatory properties that naïve and other activated $CD8^+$ T cells do not possess.

Potential Mechanisms of Action

As mentioned earlier, GA is able to suppress MOG-specific proliferation. One potential explanation is that GA-induced $CD8^+$ T cells are specifically activated by GA, but globally suppress thereafter. To address this hypothesis, we set up two different experimental approaches. In the first approach, naïve, OVA, or GA-induced $CD8^+$ T cells were IV transferred into naïve WT recipient mice at the same cell numbers we used to observe prevention of EAE. Analysis of OVA-specific proliferative responses showed that there was no global suppression by IV transfer of GA-induced $CD8^+$ T cells (Figure 29).

In the second approach, we immunized with OVA and started treating with daily GA/PBS. The daily GA regimen is one method by which we observe protection from severe EAE and preliminary data showed a decrease in the encephalitogenic properties of MOG $CD4^+$ T cells from these protected mice. OVA-specific proliferative responses were inhibited by GA daily treatment, which is not surprising since the literature provides evidence that GA competitively inhibits T cell activation [118]. Thus, it appears GA $CD8^+$ T cells suppress specifically, but aqueous GA treatment is able to suppress proliferative responses in a non-specific manner

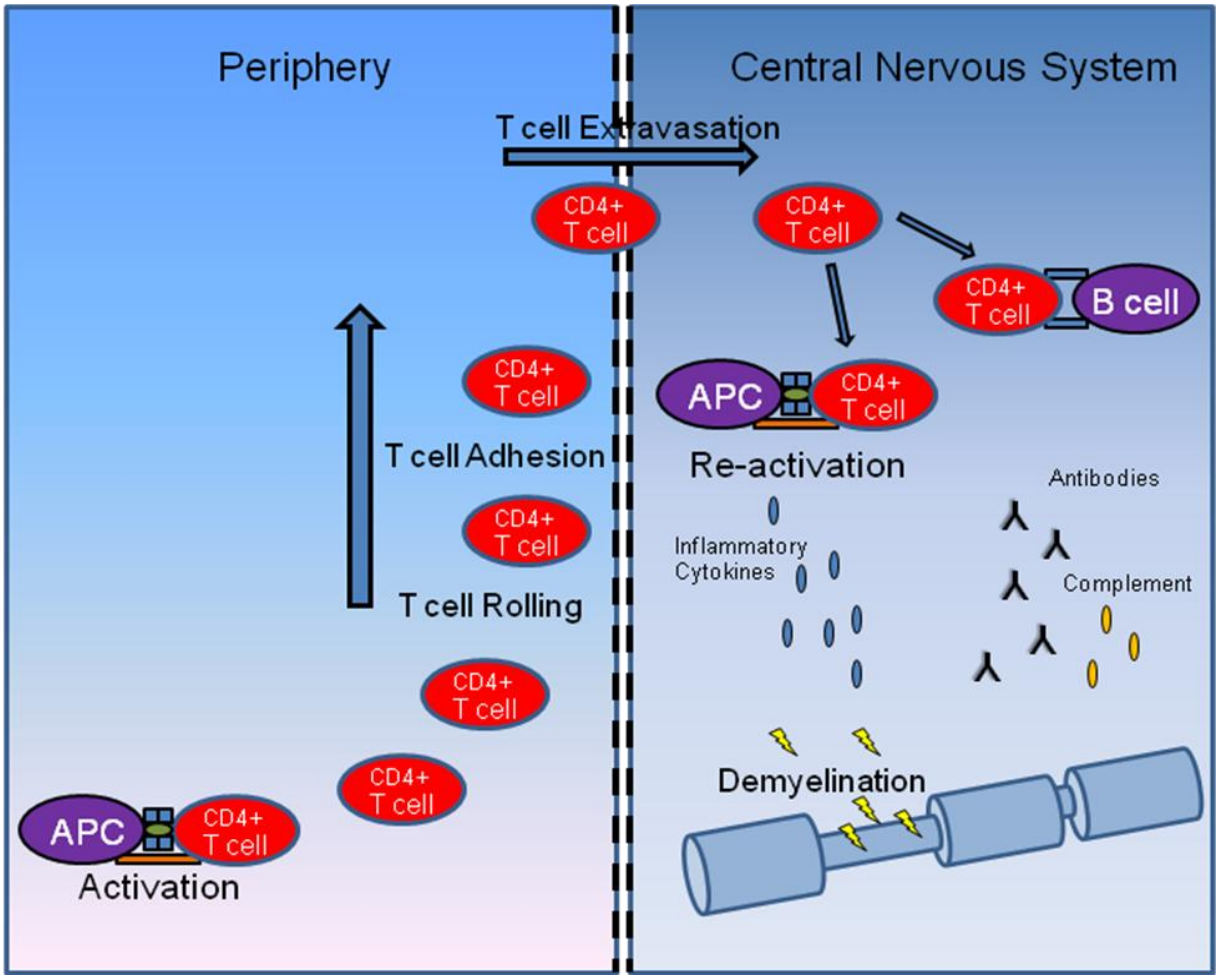

Figure 45: Typical immune cascade during disease

The typical immune cascade in EAE is shown in Figure 45. What remains to be answered is exactly how our preventative and therapeutic observations of GA-induced $CD8^+$ T cells prevent severe EAE. There are two mechanistic possibilities regarding the adoptive transfer of GA-induced $CD8^+$ T cells. The first possibility is that the GA-induced $CD8^+$ T cells have the same mechanistic effect in the CNS at day -1 as they do at day 13 during peak disease (Figure 46). For this hypothesis to be true, these GA-induced $CD8^+$ T cells would have to traffic to the CNS immediately following IV transfer. This is not an unreasonable assumption as activated $CD8^+$ T cells are known to traffic across the blood-brain barrier. This has been shown with

activated OVA $CD8^+$ T cells. The important detail is whether or not these trafficking GA-induced $CD8^+$ T cells see their cognate antigen in the brain. While they do not see GA in the brain, it is possible that they cross-react with myelin related antigens such as myelin basic protein (MBP) since GA was designed on the MBP backbone. Even if the GA-induced $CD8^+$ T cells do not encounter stimulating antigen, if they simply do not traffic out of the CNS immediately, that may be enough time for the EAE immunological cascade to start 24 hours later at day 0. Once the pathogenic $CD4^+$ T cells have trafficked to the CNS, they will recognize myelin related components presented by APCs. We do not know exactly what the mechanism of GA efficacy actually is in the CNS, but there are several possibilities. GA-induced $CD8^+$ T cells could be killing by perforin/granzymeB, Fas/FasL, or IFN-γ [144-146]. We have shown that these adoptively transferred cells are secreting high levels of IFN-γ (Figures 39 and 40). IFN-γ up-regulates APCs which could have downstream effects including the release of IL-2 and IL-12 to activate NK cells and T cells [147-149]. Additionally, IFN-γ may be responsible for killing of pathogenic $CD4^+$ T cells or killing of APCs. Killing APCs would have an indirect effect on the pathogenic $CD4^+$ T cells. Alternatively, GA-induced $CD8^+$ T cells may be affecting APCs in a non-killing mechanism by down regulating co-stimulatory molecules and altering antigen presenting capabilities. The large amounts of IFNγ during protective disease may also be inducing the suppressor molecule, indoleamine 2,3-dioxygenase (IDO). IDO is an inhibitor of tryptophan metabolism and tryptophan depletion can suppress T cell proliferation [150]. Our lab has started to investigate the role of this molecule in EAE.

If GA-induced $CD8^+$ T cells have the same mechanism at day -1 as they do a day 13 then at day 13 $CD8^+$ T cells would also traffic to of the CNS. Since this transfer of GA-induced $CD8^+$ T cells would be during peak disease, the GA-induced $CD8^+$ T cells in the CNS could engage in

one of their potential killing mechanisms directly on pathogenic $CD4^+$ T cells or on APCs (Figure 46).

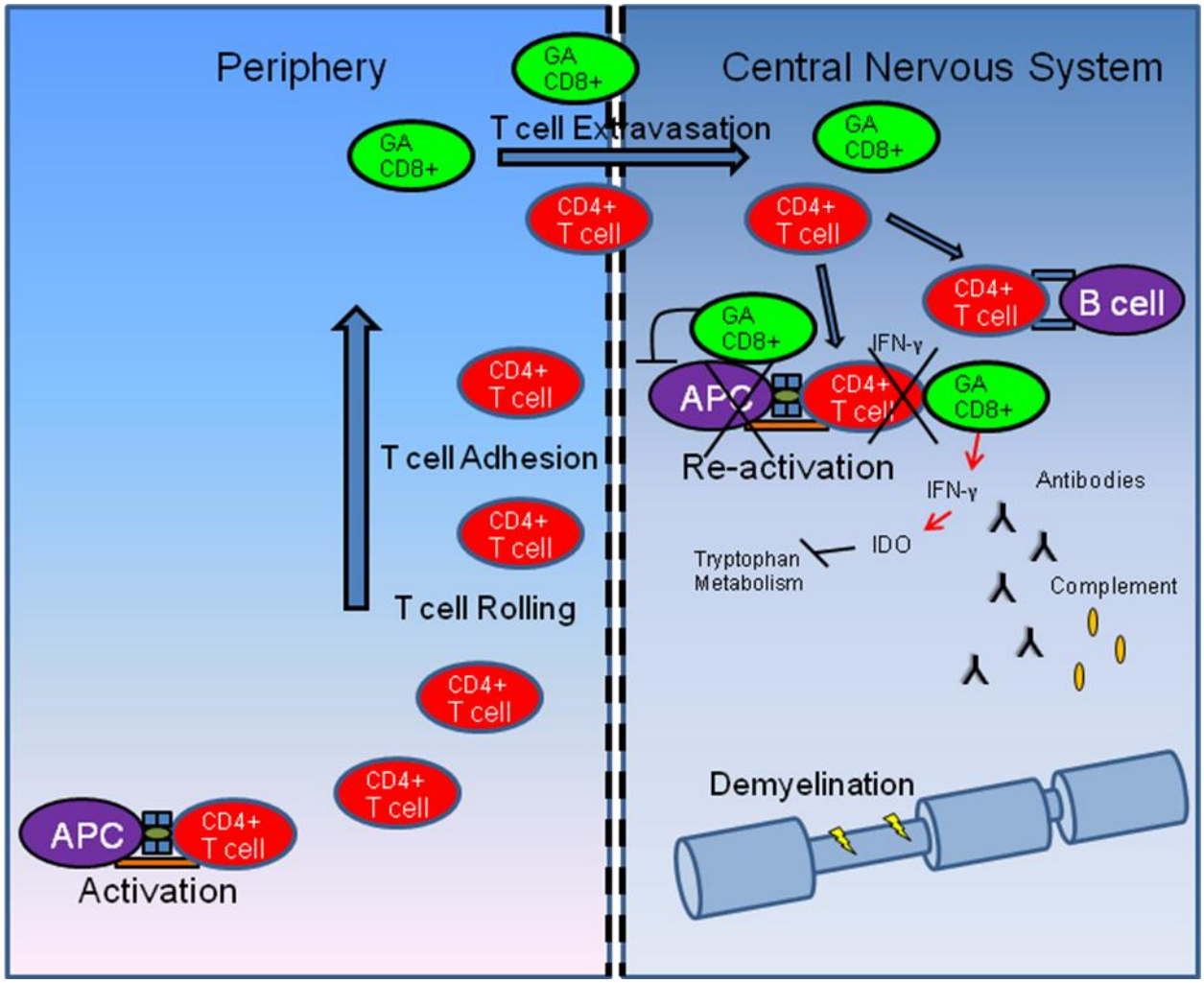

Figure 46: Day -1 and Day 13 have the same mechanism in the CNS: GA-induced $CD8^+$ T cells may exert their immunomodulatory effects in the CNS at Day -1 (Prevention) and Day 13 (Therapy).

Alternatively, the effect of GA-induced $CD8^+$ T cells may have two different mechanisms depending on the time point they are transferred. The preventative effect at day -1 may occur in the periphery while the therapeutic effect may all occur in the CNS. For this to be correct, GA-induced $CD8^+$ T cells would traffic to the lymph nodes where they would engage with pathogenic $CD4^+$ T cells. It's possible that a direct killing or suppressive mechanism of $CD4^+$ T cells could occur in the lymph node thereby preventing the same yield of encephalitogenic $CD4^+$ T cells from trafficking to the CNS. Alternatively, GA-induced $CD8^+$ T cells may affect the APCs in the lymph node. One possibility is that APCs do traffic to the brain and GA-induced $CD8^+$ T cells are actually preventing them from trafficking. This idea of APC trafficking to the brain is not commonly accepted, but when pathogenic $CD4^+$ T cells reach the brain, they must see presented antigen. This could actually be an APC that trafficked to the brain, not just brain APCs that present myelin antigen (Figure 47).

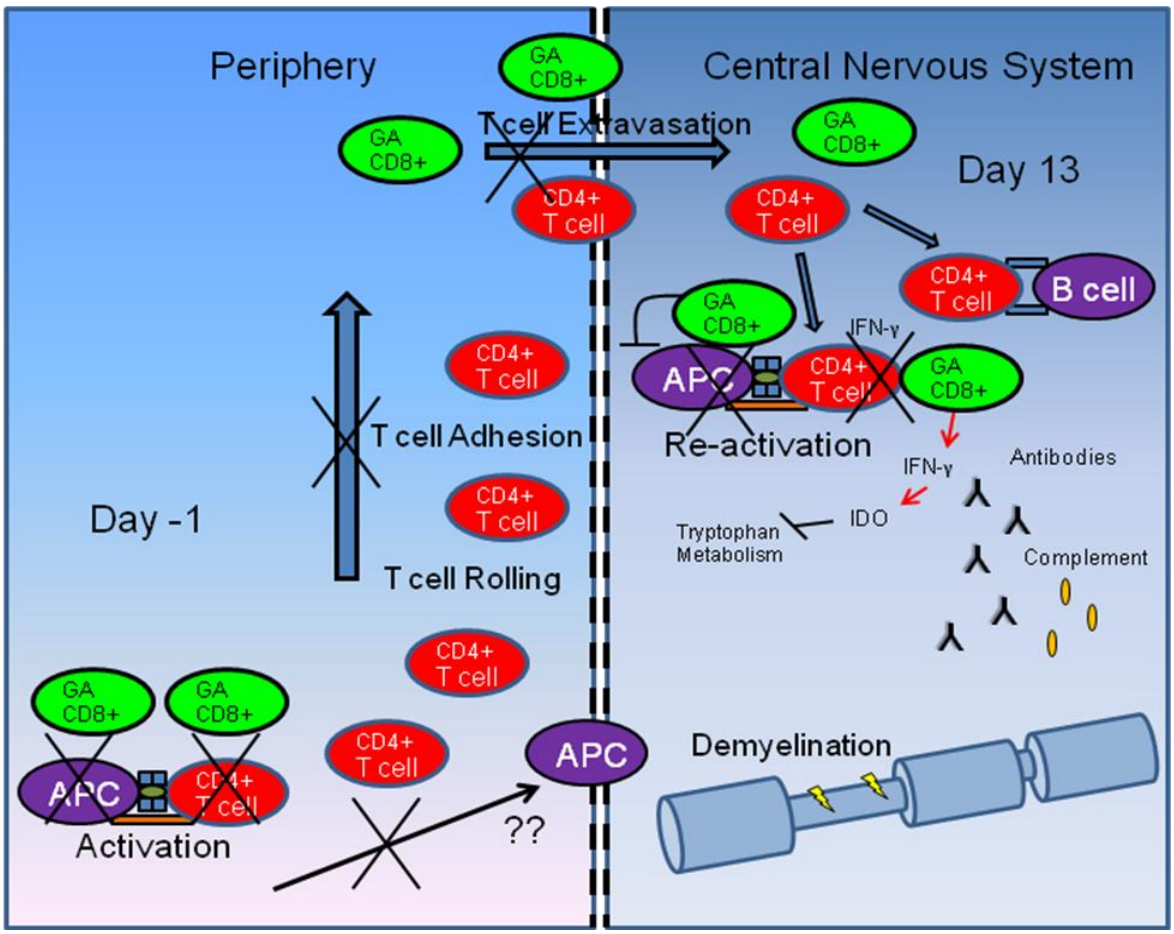

Figure 47: Two mechanisms: GA-induced $CD8^+$ T cells transferred at Day -1 (Prevention) have their effect in the periphery, but GA-induced $CD8^+$ T cells transferred at Day 13 (Therapy) have their effect in the CNS.

Cytokines

The role of cytokines in GA-induced $CD8^+$ T cell protection is important. Our data show that IFN-γ secretion is up-regulated from day 10 to day 20 in GA immunized animals. Since all of our adoptive transfer studies use GA-induced $CD8^+$ T cells with 20 days of stimulation, this is a critical observation to the therapeutic mechanism. Our RT-PCR data shows that IFN-γ is specific to GA stimulation, as mice immunized with OVA had 3-4 fold less IFN-γ secretion. Our

data from protected mice from daily GA/PBS treatment is even more intriguing. At day 10, just prior to disease onset, $CD8^+$ T cells from non-GA treated animals secreted twice as much IFN-γ as GA-treated mice (Figure 38). GA protection was observed in this experiment starting with onset at day 12 until all remaining mice were euthanized at day 20. At day 20, we observed striking differences in the IFN-γ secretion in response to MOG antigen. At one MOG dosage (33μg/mL), GA-induced $CD8^+$ T cells secreted 17 times as much IFN-γ as non-GA treated mice. The question remains, why is IFN-γ so important during clinical protection and what is actually occurring *in vivo*? As previously mentioned, one possibility is there is direct IFN-γ mediated killing of pathogenic $CD4^+$ T cells or APCs as a potential mechanism for the adoptive transfer protection. Pathogenic $CD4^+$ T cells may be releasing more IL-2, activating $CD8^+$ T cells to release more IFN-γ. If GA-induced $CD8^+$ T cells are already secreting IFN-γ then this effect may be amplified during the effector phase of EAE. The use of IFN-γ (-/-) mice would provide an excellent method of determining the extent of IFN-γ's influence. Other studies have shown that IFN-γ (-/-) mice get worse EAE, however we would utilize the IFN-γ (-/-) mice for donor $CD8^+$ T cells. In addition, IFN-γ (-/-) mice could be used as recipient mice for an *in vivo* killing assay if killing is observed in the wildtype mice. Alternatively, the large amounts of IFN-γ observed at day 20 during protective disease may actually be inducing the suppressor molecule, IDO as previously mentioned.

Cytotoxic Ability

The cytotoxic abilities of GA-induced $CD8^+$ T cells provide another logical explanation for the *in vivo* protection. Our next step would be to perform an *in vivo* killing assay which has been optimized in our lab in the murine system. With the *in vivo* killing assay, the readout is

disappearance of CFSE target cells in the spleen. The disappearance of a population could also mean a difference in trafficking of the antigen and ConA stimulated targets cells. In addition, the killing assay would need to be performed with an irrelevant antigen control such as OVA for both the killer cells with an OVA/ConA target population to ensure that the disappearance of a population is actually specific to our antigen- GA. A positive result here would have to be coupled with our *in vitro* killing data to prove its value. An important question yet to be addressed is whether or not GA-induced $CD8^+$ T cells have direct cytotoxic effects on oligodendrocytes. This question would not be easy to answer, but is important to understanding the overall cell-cell killing interactions that could occur in the brain. Alternatively, MOG $CD4^+$ T cells could also be added *in vitro* to the oligodendrocyte killing assay since this situation occurs *in vivo*.

GA-induced $CD8^+$ T Cell Trafficking

The most important issue that remains is trafficking of GA-induced $CD8^+$ T cells. We can only hypothesize that the GA-induced $CD8^+$ T cells effect occurs in certain regions, but T cells from congenic mice or CFSE-labeled GA-induced $CD8^+$ T cells should be tracked throughout the mouse during disease. Using congenic mice would be easiest approach since you would adoptively transfer GA-induced $CD8^+$ T cells and search throughout the mouse at different time points. Using a percoll gradient, lymphocytes can be recovered from the CNS. By using a duel stain such as high and medium CFSE or CMTPX you could track co-injected OVA and GA-induced $CD8^+$ T cells. This would address where the GA-induced $CD8^+$ T cells are exerting their effect and at what timeframes. In addition, because we are co-injecting with activated OVA $CD8^+$ T cells, kinetic differences in trafficking between the two groups could be measured.

Qa-1 and HLA-E

Previously, our lab has shown through our human studies that GA-induced $CD8^+$ T cells directly kill GA-loaded targets, specifically $CD4^+$ T cells. This killing was contact-dependant and HLA-E-restricted [133]. One possible mechanism for our murine GA-induced $CD8^+$ T cells is that they are Qa-1 restricted. The MHC I-like Qa-1 locus is equivalent to HLA-E in humans and restricts peptide recognition by $CD8^+$ T suppressor cells [86]. Activated $CD4^+$ T cells and APCs express Qa-1, therefore there is a possibility pathogenic $CD4^+$ T cells and APCs may present myelin related antigens such as MOG through Qa-1. The GA-induced $CD8^+$ T cells could kill pathogenic $CD4^+$ T cells or APCs through the Qa-1 mechanism in that situation [151-154]. If this did occur, EAE disease would curtailed from two sides, the killing of pathogenic $CD4^+$ T cells and by the killing of APCs that would normally be presenting to autoreactive $CD4^+$ T cells.

Application to Human MS

The possibility exists that in the future, autologous cell therapy treatments may be developed for MS. Dendritic cell vaccines are currently being developed for individual cancer patients, but the cost is estimated to be over $50,000 per person [155-157]. For now, we will utilize our murine studies to clarify the roles of different cell types and their mechanisms of action in the therapeutic modulation of the EAE disease course.

The EAE mouse model has many advantages when it comes to studying the immunological and pathological mechanisms of autoimmune demyelination. The availability of knockout mice, depletion antibodies, and genetically identical subjects make murine studies very effective for researching a particular disease such as EAE with a human parallel- MS. Of course there are drawbacks, such as the artificially induced disease and differences in clinical symptoms. EAE tends to be a disease of ascending paralysis which is not how multiple sclerosis presents in patients. There has also been contradicting information regarding IFN-γ. IFN-γ (-/-) mice get worse EAE and recently in human MS, antibody against IFN-γ receptor reduced disease progression [60]; however, older studies report that MS patients who received IFN-γ got exacerbated disease [59]. Nevertheless, much can be learned from studies in the EAE model of demyelination.

GA was first discovered when working with animal models so certainly defining its mechanism of action in the EAE model is fitting. Due to the complex nature of the immune system, many more studies need to be done before we can begin to fully elucidate which cell types are truly the most important for therapeutic modulation of disease. Based on our current *in vivo* observations we can confidently state that $CD8^+$ T cells are required for the therapeutic effects of GA therapy on autoimmune modulation.

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