$CD8^+$ T CELLS ARE REQUIRED FOR THE THERAPEUTIC ACTION OF GLATIRAMER ACETATE IN AUTOIMMUNE DEMYELINATING DISEASE

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DEDICATION

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$CD8^+$ T CELLS ARE REQUIRED FOR THE ACTION OF GLATIRAMER ACETATE IN AUTOIMMUNE DEMYELINATING DISEASE

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Glatiramer acetate (GA, Copaxone®) is an FDA-approved immunomodulatory therapy for multiple sclerosis (MS), an immune-mediated demyelinating disease of the central nervous system. Our group has previously shown that GA therapy induces $CD8^+$ T cell responses responsible for suppression of $CD4^+$ T cell responses in MS patients. Using a murine model of MS, experimental autoimmune encephalomyelitis (EAE), we also demonstrated that $CD8^+$ T cells are necessary in mediating the therapeutic effects of GA, and adoptive transfer of GA-induced $CD8^+$ T cells resulted in amelioration of EAE, establishing a role as a viable

immunotherapy in demyelinating disease. Here, we show that GA treatment, as well as the suppressive function of GA $CD8^+$ T cells, requires IFNγ and perforin, but not IL-10, expression and activation by non-classical MHC class I molecules both *in vitro* and *in vivo*. GA-induced regulatory myeloid cells, previously shown to activate $CD4^+$ regulatory T cells in an antigen-independent manner, depend on $CD8^+$ T cells and MHC class I expression to suppress disease *in vivo*, an effect mediated by MHC class I-mediated induction of $CD8^+$, but not $CD4^+$, T cell responses. GA induces an anti-inflammatory "type 2" phenotype in monocytes *in vivo* that is unnecessary for the suppression of disease. The drug also inhibits the expression of surface markers of maturation such as CD11c and MHC class II. GA $CD8^+$ T cells reduce the proliferative potential of autoimmune, neuroantigen-specific $CD4^+$ T cells and induce $CD4^+CD25^+Foxp3^+$ regulatory T cells *in vivo*. Additionally, several MHC class I-binding peptide epitopes associated with GA treatment of dendritic cells were identified by LC/MS-MS and tested for disease suppression and ability to activate GA $CD8^+$ T cells. These studies demonstrate an essential role for $CD8^+$ T cells in GA therapy and identify their potential as an adoptive immunotherapeutic agent.

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LIST OF DEFINITIONS

μg – Microgram

μl – Microliter

μM – Micromolar

Ab – Antibody

APC – Antigen-presenting cell

APC – Allophycocyanin

APL – Altered peptide ligand

BSA – Bovine serum albumin

CD – Cluster of differentiation

CFA – Complete Freund's adjuvant

CFSE – 5,6-Carboxyfluorescein succinimidyl ester

CFDA-SE – 5,6-Carboxyfluorescein diacetate succinimidyl ester

CNS – Central nervous system

ConA – Concanavalin A

CPM – Counts per million

CTL – Cytotoxic lymphocyte

DC – Dendritic cell

EAE – Experimental autoimmune encephalomyelitis

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FBS – Fetal bovine serum

FITC – Fluorescein isothiocyanate

Foxp3 – Forkhead box P3

GA – Glatiramer acetate

IDO – Indoleamine-2,3-dioxygenase

IFA – Incomplete Freund's adjuvant

IFNβ – Interferon-beta

IFNγ – Interferon-gamma

IL-2 – Interleukin 2

IL-4 – Interleukin 4

IL-12 – Interleukin 12

IL-10 – Interleukin 10

i.p. – intraperitoneal

IU – International units

i.v. – intravenous

JC virus – John Cunningham virus

kDa – Kilodalton

LPS – Lipopolysaccharide

mAb – Monoclonal antibody

Mϕ - Macrophage

MBP – Myelin basic protein

MHC – Major histocompatibility complex

MHC I – Major histocompatibility complex class I

MHC II – Major histocompatibility complex class II

ml – Milliliter

MOG – Myelin oligodendrocyte glycoprotein

MOG_{35-55} – Myelin oligodendrocyte glycoprotein residues 35 to 55

mRNA – messenger ribonucleic acid

MS – Multiple sclerosis

MTb – *Mycobacterium tuberculosis*

MTD – 1-Methyltryptophan-D

NF-κB – Nuclear factor κB

NFAT – Nuclear factor of activated T cells

NK cell – Natural killer cell

OVA – Hen egg ovalbumin

OVAp – Hen egg ovalbumin residues 323-339

PBS – Phosphate-buffered saline

PE – Phycoerythein

PLP – Proteolipid protein

$PLP_{139-151}$ – Proteolipid protein residues 139 to 151

PML – Progressive multifocal leukoencephalopathy

PTx – Pertussis toxin

RAG – Recombinase activating gene

rtPCR – Reverse transcription polymerase chain reaction

SEM – Standard error of the mean

T-bet – T-box expressed in T cells

TGFβ – Transforming growth factor-beta

Th1 – T helper cell type 1

Th2 – T helper cell type 2

Th17 – T helper cell type 17

Treg – Regulatory T cell

TNFα – Tumor necrosis factor alpha

WT – Wild-type

CHAPTER ONE
Introduction

MULTIPLE SCLEROSIS

Demographics

Multiple sclerosis (MS) is a chronic, degenerative disease of the central nervous system (CNS) that affects over 400,000 Americans and 2.1 million people globally (1, 2). The disease is the most common neurological disorder of young adults. Like many autoimmune disorders, this disease more commonly affects women, at a rate of 2-3 times more frequently than men, and young people, with diagnosis most commonly occurring during the third decade of life (3). While MS affects most ethnic groups, people of northern European Caucasian background are the most commonly afflicted (4).

Symptoms and Diagnosis

Due to the neurological basis of MS, the range of symptoms varies widely, including bladder and bowel dysfunction, vertigo, pain, and cognitive issues (5-9). However, the most common presenting symptoms are fatigue, numbness, and blurry vision (10, 11). Gait disturbances are also very common and are the basis for determining the clinical severity of the disease (12). If left untreated, 50% of MS patients will need assistance walking by ten years; that number rises to 90% by 25 years (13). Diagnosis of the disease depends on finding evidence of central nervous symptom demyelination identified as gadolinium-enhancing lesions on magnetic resonance

imaging (MRI) (14). This CNS damage is correlated to clinical symptoms, giving rise to the characterization of MS as a "disease of space and time" (15). Other diagnostic criteria include the presence of oligoclonal banding of cerebrospinal fluid (CSF) and the use of evoked potentials to test for optic nerve and spinal cord dysfunction (16).

There are several forms of MS (17). The most prevalent is relapsing-remitting MS (rrMS), which affects 80% of patients. The form of the disease is characterized by evolving symptoms and signs of disease over days to weeks which stabilize and then improve, either spontaneously or in response to treatment. Twenty percent are found to have primary progressive MS (ppMS), which is characterized by the constant increase in disability without remission. While RRMS has a female predominance of 2:1, PPMS is nearly equal. Other forms of the disease include secondary progressive (spMS), defined as a decline in symptoms without periods of remission, occurring after an initial diagnosis of rrMS, as well as progressive-relapsing MS (prMS), characterized by a steady decline in symptoms like ppMS, but with superimposed attacks. All forms of the disease lead to a dramatic decrease in quality of life.

Pathology

MS pathology is defined by the presence of lesions within the central nervous system correlating with disease symptoms (18). These lesions, known as plaques, are areas of demyelination that contain perivascular infiltrates composed of mononuclear cells such as T and B cells as well as macrophages releasing radical oxygen species and dendritic cells leading to the further activation

of the adaptive immune response. It is this loss of myelin that causes the symptoms of MS by impeding the conduction of electrical impulses through neurons, thereby inhibiting the transmission of signals from the CNS to the periphery and vice versa. Based on studies of animal models, it is thought that demyelination occurs by auto-reactive CNS-specific populations of T and B cells that cross over the blood-brain barrier, recognize their cognate antigens, and incite an immune response that damages the myelin sheath (19). Included in this destruction of the CNS are oligodendrocytes, the cells responsible for myelin synthesis, neurons responsible for intercellular communication, and the astrocytes maintaining the integrity of the blood-brain barrier (20, 21).

Causes

Currently, the cause of MS is unknown. However, several observations have suggested factors leading to a predisposition toward the disease. Genetics play a role, and a predilection for disease has been associated with specific HLA haplotypes as well as non-MHC immune-related genes. A correlation of disease with the HLA-DR2 locus as well as IL-7Rα has been documented (22). HLA class I genes have also been correlated with increased rates of disease, as have molecules such as CTLA-4, IL-1β, and the estrogen receptor (23-26). However, genetic factors are insufficient to completely explain the disease, as the study of identical twins has demonstrated only a 25% concordance in the disease (27).

Environmental factors also play a role. Risk for developing MS increases the further away one lives from the equator. However, individuals that move from areas of high incidence to those of lower incidence (closer to the equator) before age fifteen inherit a decreased risk for developing MS compared to those moving after that age (28). These findings have led researchers to investigate links between MS and vitamin D levels, as vitamin D in closely linked to exposure to sunlight (29, 30). In addition, pollution may also play a role in MS etiology, as areas with higher rates of MS are also notable for being industrialized nations (31).

Infectious vectors also play a role in developing MS. Pathogens such as Epstein-Barr virus may be instigating factors, as patients with MS demonstrate increased rates of infection compared to control patients (32). The wide prevalence of such viruses, however, is a confounding variable in isolating the instigating factor. The case of the Faroe Islands also suggests a transmissible vector, as MS rates increased in natives after the British occupied the islands during World War II (33). However, this could also point to an environmental factor brought in by the newly transplanted British culture. The single overarching link between each of these possible causes is that they all point out an underlying immune defect that allows inopportune inflammation to occur in the CNS, instigating demyelination and inducing disease.

TREATMENT OF AUTOIMMUNE DEMYELINATION

Immunomodulatory Therapy

While acute relapses are typically treated with a short course of corticosteroids to reduce neuroinflammation, long-term treatment of MS is centered about the disease-modifying agents. As there is no cure for the disease, therapy focuses on reducing the duration and frequency of acute exacerbations and providing reliefs of symptoms. There are currently eight FDA-approved drugs that reduce disease activity and progression in MS patients: the interferons (Avonex, Rebif, Betaseron, and Extavia), mitoxantrone (Novantrone), natalizumab (Tysabri), fingolimod (Gilenya), and glatiramer acetate (Copaxone) (34).

Interferon-β

The four β-interferon drugs are based on the naturally occurring cytokine that inhibits viral replication via a host of functions (35). In MS, these drugs are thought to have beneficial regulatory and anti-inflammatory properties, reducing relapses by 30%. These drugs are divided into two subcategories. Interferon β-1a (Avonex, Rebif) is synthesized by and purified from mammalian cells (36). It therefore possesses the glycosylation pattern as well as the exact amino acid sequence of the human protein. Interferon β-1b (Betaseron, Extavia) is produced recombinantly in *E. coli*, and are therefore non-glycosylated and contain slight variabilities in amino acid sequence (37). The two different forms of the drug have similar efficacy in MS, although Interferon β-1a may have greater biological activity due to the stabilizing effect of the carbohydrate on the protein structure (38). The predominant side effect of this class of drug is flu-like symptoms, which occur in 60% of patients that receive the drug (39). In addition, liver

function abnormalities can develop, and neutralizing antibodies raised over the course of long-term treatment can reduce the efficacy of the drug (40).

Mitoxantrone

Mitoxantrone was initially developed as a cancer treatment due to its ability to inhibit the proliferation of rapidly dividing cells by intercalating DNA and to potently inhibit topoisomerase II. This same feature led to its use in MS as the drug inhibits the activity and proliferation of T cells, B cells, and macrophages activated in the immune response to CNS antigens (41). This in turn leads to reduced levels of IFNγ, TNFα, and IL-2, Th1-associated cytokines thought to be responsible for most of the neuroinflammation in the disease. Like interferon, mitoxantrone reduces relapse rates in rrMS patients as well as improves disability scores (42). The most common adverse effects include nausea, vomiting, alopecia, and upper respiratory infections (43). Leukopenia is a potentially lethal side effect that occurs in approximately 15 percent of patients, as is cardiotoxicity (44). Therefore, mitoxantrone is usually reserved for patients with worsening disease that are not responding to other drugs.

Natalizumab

Natalizumab is a humanized monoclonal antibody (IgG_4) that binds the α_4 subunit of very-late activating antigen 4 (VLA-4) expressed on the surface of leukocytes, inhibiting adhesion and migration into the CNS (45). The drug is very effective at reducing relapses of disease, but increases the risk of progressive multifocal leukoencephalopathy (PML), an opportunistic JC

virus infection that is usually lethal (46). This risk increases with extended drug use. It therefore restricted to patients failing other forms of therapy.

Fingolimod

Fingolimod is the first oral drug approved by the FDA to reduce relapses and delay the progression of rrMS. It is a prodrug that is phosphorylated by sphingosine kinase, allowing it to reduce the emigration of lymphocytes from lymph nodes via sphingosine-1-phosphate receptor blockade (47). It is thought that this diminished egress of cells reduces the presence of pathogenic lymphocytes within the CNS. Fingolimod reduces relapses when compared to both treatment-naïve and β-interferon-treated patients (48). However, liver dysfunction is a common side effect of the drug and treatment can lead to a potentially lethal cardiotoxicity (49).

Glatiramer Acetate

Glatiramer acetate (GA) is a synthetic random polypeptide of an approximate 7.7 kDa mass (range 4.7 to 11 kDa, ~67 residues in length) composed of the amino acids alanine, lysine, glutamate, and tyrosine in a ratio reflecting that of myelin basic protein (MBP) (approximately 4.2:3.4:2.1:1) (50). When tested in MS, GA reduced the rate of attacks in rrMS patients by nearly two-thirds (51). Unlike other drugs used to treat MS, GA has very few side effects, the most common being a site reaction upon subcutaneous injection of the drug. It does not cause liver function abnormalities, immunosuppression, or a flu-like reaction, thereby making its profile of adverse effects the most acceptable of all the disease-modifying agents. The

combination of these factors has led GA to become the second most prescribed FDA-approved drug for rrMS.

THERAPEUTIC MECHANISMS OF GA

Origins and Development of Glatiramer Acetate

GA was originally synthesized to mimic MBP in the induction of EAE (52). The ratio of alanine, lysine, glutamic acid, and tyrosine residues in GA was formulated to mirror that of the hydrophobic, basic, acidic, and aromatic residues found in MBP, and its molecular mass prior to the refinement the drug has undergone over the years reflected that of the endogenous myelin-derived protein (~20kDa). However, GA reduced the severity of EAE or prevented the disease completely. Therefore, it was hypothesized that GA antagonizes MBP binding, due to two similar molecules having divergent action. This was supported by findings indicating that GA bound promiscuously to various H-2 and HLA haplotypes on a variety of antigen-presenting cells which was inhibited by anti-MHC class II antibody treatment (53). Furthermore, GA competitively antagonized MBP binding to MHC class II and could displace already bound MBP peptide epitopes (54). It was believed that this would inhibit TCR-based $CD4^+$ T cell activation, however, antagonistic activity was only demonstrated for $MBP_{82\text{-}100}$ epitope, whereas TCR activation by other epitopes of MBP and of other myelin-derived proteins such as proteolipid protein was not affected. In addition, when altered peptide ligands, molecules that antagonized myelin peptide binding to MHC class II, were tested clinically in MS, they had the unintended

effect of worsening disease symptoms (55). Therefore, while this mechanism may be important in certain circumstances, it did not appear to be a general mechanism by which GA suppressed demyelinating disease.

The focus of GA research then moved away from pure antagonism into phenotypic studies. Although GA could not inhibit the proliferation of most myelin-reactive $CD4^+$ T cells, perhaps it could convert cells with pathogenic potential into cells with non-pathogenic or anti-inflammatory characteristics. In MBP-specific T cell clones, cells shifted from putatively pathogenic Th1 phenotype to non-pathogenic Th2 phenotype (56). However, later experiments demonstrated that Th1 cells may not be responsible for the primary pathogenesis of EAE, which instead is induced by the Th17 subset of helper T cells (57), while Th2 cells were shown to induce EAE in $RAG^{-/-}$ mice (58), suggesting that the Th1-to-Th2 shift was not the inflammatory/anti-inflammatory dichotomy as originally conceived, and thus unlikely to fully encompass the anti-neuroinflammatory effects of GA.

Dispensing with myelin-reactive T cells, researchers began studying cells that were activated, rather than antagonized, by GA. Endogenous GA-specific $CD4^+$ T cells are present within the peripheral blood of untreated healthy human subjects (59). These cells were shown to secrete IL-10 and TGFβ, two canonical anti-inflammatory cytokines, and could infiltrate the CNS and inhibit inflammatory by bystander expression of these molecules. In addition, GA converted naïve $CD4^+$ T cells into $CD4^+CD25^+$ regulatory T cells by increasing Foxp3 expression (60).

This effect was mediated by an increase in IFNγ. Nonetheless, it is difficult to determine whether these effects are necessary for the beneficial effects of GA in EAE and MS, as these diseases cannot be investigated in the absence of $CD4^+$ T cells or Foxp3. Despite these difficulties, it has been demonstrated that IL-10 is unnecessary for GA action, as GA is still effective in reducing signs of disease in IL-$10^{-/-}$ mice (61). Thus, the effects of GA on $CD4^+$ T cells, while fascinating, do not appear to account for the amelioration or prevention of demyelinating disease.

$CD4^+$ T cells represent one half of the TCR/MHC class II interaction. Antigen-presenting cells (APCs), including B cells, dendritic cells, and monocytes/macrophages, are also affected by GA treatment. B cells, in addition to antigen presentation, are also responsible for the production of antibody. GA antibodies are present in low titers regardless of GA treatment status, although these antibodies increase upon treatment initiation, and are predominately of the IgG1 isotype (62). Over the course of treatment, there is often a shift from IgG1 to IgG4, which is thought to correlate with the Th1-to-Th2 shift in T helper cells (63). There is some suggestion that these antibodies, rather than reducing the clinical efficacy of the drug by neutralization, instead may be beneficial, as higher titers are found in relapse-free patients compared to patients with more active disease and lead to enhanced remyelination in murine models of MS (64). GA can also induce anti-inflammatory properties in B cells. These regulatory B cells ("Bregs") secrete IL-10, inhibiting CNS inflammation, in addition to expressing increased IL-4 and IL-13 and decreased IL-6, IL-12, TNFα, and surface levels of BAFF receptor (65). However, depletion of B cells

during the progression of EAE ameliorates disease, and treatment with rituximab, a B-cell-depleting antibody, is beneficial in rrMS patients, reducing brain lesions and clinical relapses (66, 67). Therefore, B cells can play both a therapeutic and detrimental role in regard to GA and demyelinating disease.

Dendritic cells (DCs) are the most efficient APC, and like B cells, take on an anti-inflammatory phenotype in response to GA treatment. DCs also exhibit inefficient maturation, which translates into decreased activation of pathogenic $CD4^+$ T cells and higher levels of Tregs (68). Myeloid cells like monocytes and macrophages also begin to secrete more anti-inflammatory cytokines such as IL-10 and TGFβ and less inflammatory cytokines such as IL-12 and TNFα in response to GA treatment, and also demonstrate decreased differentiation into more mature phenotypes (69-72). Moreover, these cells can induce Tregs in a non-antigen specific manner that act in the CNS to reduce neuroinflammation by the release of IL-10 and other cytokines.

$CD8^+$ T cells

More recently, $CD8^+$ T cells have been shown to play a vital role in GA treatment as regulatory cells. Although $CD8^+$ T cells are more commonly associated with anti-viral responses and tumor surveillance, their capacity in controlling aberrant immune responses has been explored since the early days of immunoregulation research. The regulatory ability of $CD8^+$ T cells was first identified in murine models of MS and myocarditis, where CD8-deficient mice were susceptible to a worsened form of the disease (73). There are currently several distinct populations of $CD8^+$

T cells, including $CD8^+CD28^-$, $CD8^+Foxp3^+$, $CD8^+CD122^+$, and Qa-1-restricted $CD8\alpha\alpha^+$, that have clearly defined roles in several autoimmune disorders, including models of diabetes, rheumatoid arthritis, and systemic lupus erythematosus (74-77).

In MS and EAE, $CD8^+$ T cells were originally believed to be a pathogenic cell population. In CNS lesions in MS patients, $CD8^+$ T cells outnumber $CD4^+$ T cells (78). In addition, increased numbers of $CD8^+$ T cells and macrophages correlate with axonal damage as well as directly kill oligodendrocytes and neurons (79). In EAE, adoptive transfer of MOG-specific $CD8^+$ T cells induces disease in both wild-type and $RAG^{-/-}$ mice in an MHCI-dependent fashion (80). However, these cells can also play a regulatory role. $CD8^{-/-}$ mice suffer more EAE relapses than littermate controls, and mice depleted of $CD8^+$ T cells by antibody injection demonstrate increased disease incidence and severity (81). Additionally, $Qa\text{-}1^{-/-}$ mice are more susceptible to PLP-induced disease, due to the inability of Qa-1-restricted $CD8^+$ T cells to inhibit pathogenic $CD4^+$ T cell responses (82).

Similarly, GA induces regulatory $CD8^+$ T cells that have a suppressive effect in demyelinating disease. GA induces $CD8^+$ T cell secretion of IFNγ in MS patients treated with the drug (83). Our group has demonstrated that both $CD4^+$ and $CD8^+$ T cells from healthy control subjects are strongly activated by GA *in vitro*, while MS patients have a deficit in $CD8^+$ T cell responses to GA (84) (Figure 1). After treatment, these GA-specific $CD8^+$ T cell responses are rescued and may correlate with disease reduction (Figure 2). These GA $CD8^+$ T cells were shown to inhibit

$CD4^+$ T cell responses to both GA and anti-CD3 stimuli via a contact-dependent interaction with HLA-E, a non-classical MHC class I molecule (85) (Figure 3).

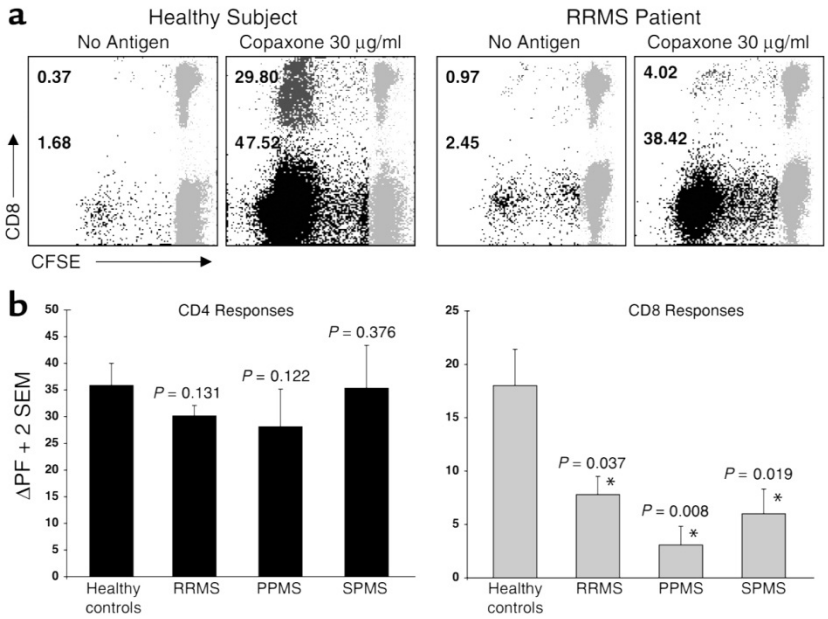

Figure 1 *Untreated MS patients have a deficient CD8$^+$ T cell response to glatiramer acetate*. CFSE-based proliferation assays were performed on PBMC specimens from 9 healthy individuals and 23 MS patients. (**a**) Representative responses from one healthy individual and one RRMS patient are shown. The data represent gated CD3$^+$ T cells, further gated for CD4$^+$/CD8$^-$ or CD8$^+$/CD4$^-$ T cells. CFSE staining is shown on the *x* axis and CD8 staining on the *y* axis (CD8$^-$ populations represent gated CD4$^+$ T cells). The gray populations represent nondividing cells. The numbers next to the darker populations represent the proliferating fraction of CD4$^+$ T cells and CD8$^+$ T cells. ΔPF is the difference between specific proliferation and the background (no antigen). Thus, the healthy control had a GA-specific ΔPF of 45.84% for the CD4$^+$ T cells and 29.43% for the CD8$^+$ T cells. The RRMS patient had a CD4$^+$ ΔPF of 35.97% and 3.05% for the CD8$^+$ response. The mean ΔPF was calculated from duplicate cultures in every experiment. (**b**) The graphs represent mean ΔPF (+ 2 SEM) of GA-specific CD4$^+$ (left panel) and CD8$^+$ (right panel) responses from 9 healthy individuals and 23 untreated MS patients (12 RRMS, 6 PPMS, and 5 SPMS). The responses from the MS patients were compared to those from the healthy individuals. The *P* values are indicated above the corresponding bars. *Significant differences ($P < 0.05$). Karandikar NJ, Crawford MP, Yan X, Ratts RB, Brenchley JM, Ambrozak DR, Lovett-Racke AE, Frohman EM, Stastny P, Douek DC, Koup RA, Racke MK. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest*. **2002** Mar; *109(5)*: 641-9.

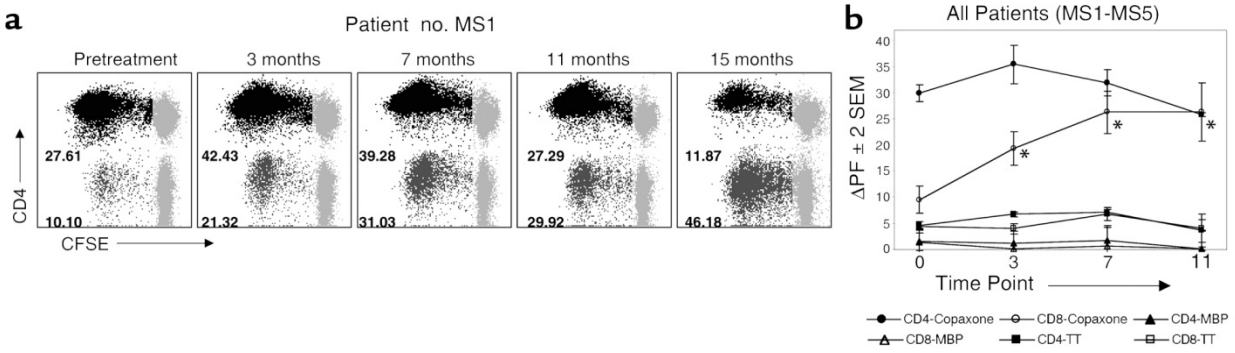

Figure 2 *GA-specific CD8$^+$ T cell responses are differentially upregulated following GA therapy*. CFSE-based proliferation assays were performed on longitudinal PBMC specimens obtained from five MS patients on daily GA therapy. (**a**) GA-specific proliferative responses from a representative patient (no. MS1) are shown at the pretreatment time point and at 3, 7, 11, and 15 months after the initiation of GA therapy. The data represent gated CD4$^+$/CD8$^-$ or CD8$^+$/CD4$^-$ T cells. CFSE staining is shown on the *x* axis and CD4 staining on the *y* axis. The gray populations represent nondividing cells. The numbers next to the darker populations represent the proliferating fraction of CD4$^+$ and CD8$^+$ T cells. The mean background proliferation ranged from 0.29 to 4.36 in various experiments. (**b**) This graph represents longitudinal mean ΔPF values (± 2 SEM) of CD4$^+$ and CD8$^+$ T cell responses to GA, MBP, and TT from all five MS patients on GA therapy (Table 1). The pretreatment time point (0) and the 3-, 7-, and 11-month time points are shown. *Statistically significant increase of GA-specific CD8$^+$ T cell responses, compared with those in the pretreatment specimens ($P < 0.05$). Karandikar NJ, Crawford MP, Yan X, Ratts RB, Brenchley JM, Ambrozak DR, Lovett-Racke AE, Frohman EM, Stastny P, Douek DC, Koup RA, Racke MK. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *J Clin Invest*. **2002** Mar; *109(5)*: 641-9.

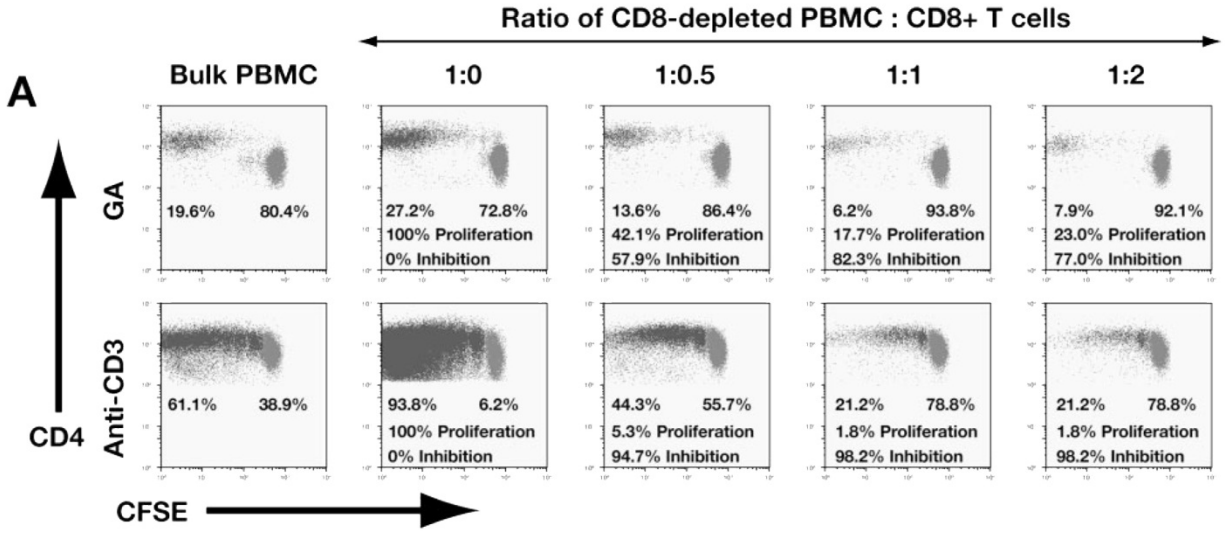

Figure 3 *Suppression of CD4$^+$ T cell proliferation by GA-reactive CD8$^+$ T cells*. A representative patient at 7 mo of daily GA therapy. An aliquot of bulk PBMC was stained with CFSE and used in a CFSE-based proliferation assay (first column). From the remaining PBMC, a purified population of CD8$^+$ T cells was obtained by positive bead selection (>95% pure; <0.1% CD4$^+$ T cell contamination). The CD8-depleted fraction was stained with CFSE and used in a proliferation assay with the addition of increasing numbers of unstained, purified CD8$^+$ T cells, with ratios ranging from 1:0 (no CD8$^+$ T cells) to 1:2. The cells were cultured with either no Ag (data not shown), GA (*top row*), anti-CD3 (*bottom row*) or CMV Ag (*B* and *C*). On day 7 of culture, cells were stained and the data gated for CD4$^+$ T cells to quantify the proliferating fraction. CD4 vs CFSE staining is shown in the dot plots. The percentages indicated are the fraction of proliferating vs nonproliferating CD4$^+$ T cells. Proliferation (and inhibition) values shown are normalized to the 1:0 ratio. Tennakoon DK, Mehta RS, Ortega SB, Bhoj V, Racke MK, Karandikar NJ. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J Immunol*. **2006** Jun 1; *176(11)*: 7119-29.

GA-Reactive CD8$^+$ T Cells in a Murine Model of MS

In response to these data, our group decided to further explore the effects of GA on CD8$^+$ T cells within the context of demyelinating disease by applying our findings to the murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). Initially identified by Thomas Rivers during investigations into the rabies vaccine, EAE has become an essential model of CNS demyelination (86). The disease is induced by immunization with myelin-derived antigens such as myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) in an emulsion with complete Freund's adjuvant (CFA) and, in some models, additional injection of pertussis toxin (87). Several species of animals have been shown to be susceptible to EAE, including mice, rats, guinea pigs, rabbits, dogs, and non-human primates. As in MS, several different forms of the disease exist, including relapsing-remitting and chronic progressive. Like MS, EAE demonstrates characteristic CNS pathology, including demyelinating plaques and perivascular mononuclear inflammation, although the course of the disease differs, in that EAE is characterized by an ascending paralysis. The disease is scored on a 0 to 5 scale, with each point representing an increase in paralysis, beginning from tail weakness, moving through the lower limbs, and ending with the forelimbs (88). Disease can also be associated with cerebellar signs including ataxia and tremor (89).

Our initial experiments were to determine whether GA could induce CD8$^+$ T cell responses in mice as it could in humans. This would validate use of the murine model, as any further findings in mice would accurately represent that found in humans. Mice were immunized with GA/IFA.

After 10 days, bulk splenocytes and lymph node cells were isolated, stained with CFSE, and incubated with GA for 5 days. $CD8^+$ and $CD4^+$ T cells responded to GA in a dose-wise manner, reflecting our human findings and confirming our decision to use the murine model (Figure 4).

Our next step was to determine whether $CD8^+$ T cells played a role in the disease-modifying abilities of GA. By utilizing mice deficient in CD8α, we could determine whether $CD8^+$ T cells were necessary for GA-mediated inhibition of EAE. Disease was induced in wild-type C57BL/6 and $CD8^{-/-}$ mice by subcutaneous injection of MOG_{35-55}/CFA with intraperitoneal injection of *Bordetella pertussis* toxin immediately following immunization and two days later. These mice were subjected to three different treatment regimens: a subcutaneous injection of GA in IFA before disease induction (day -10), daily subcutaneous injection of GA after disease induction but prior to clinical signs of disease (day 2 to 15), and a therapeutic protocol during clinical disease (day 11 to 25). While each protocol was effective in wild-type mice, none of the protocols limited disease in $CD8^{-/-}$ mice. In some cases, treatment actually worsened disease symptoms (Figure 5). Examination of the CNS of these animals revealed lower levels of demyelination in GA-treated wild-type mice compared to controls, whereas no such decrease was noted in $CD8^{-/-}$ mice. These findings promote a vital and necessary role for $CD8^+$ T cells in the mechanism of GA in diminishing the signs and symptoms of demyelinating disease.

$CD8^+$ T cells play a crucial part in mediating GA action *in vivo*. However, it remains unclear whether disease amelioration is due to direct effects of GA-reactive $CD8^+$ T cells. While

regulatory populations of $CD8^+$ T cells have been described in the literature, GA-reactive $CD8^+$ T cells are notable in that they are activated by a synthetic molecule with no known natural analog. To test the regulatory capabilities of GA-reactive $CD8^+$ T cells, donor C57BL/6 mice were immunized with GA or control antigen (OVAp) in IFA. After twenty days, lymph node cells and splenocytes were isolated and restimulated *in vitro*, followed by $CD8^+$ T cells isolation. Cells were then injected intravenously into wild-type and $CD8^{-/-}$ recipient mice followed by disease induction one day later. In both wild-type and $CD8^{-/-}$ mice, GA-reactive $CD8^+$ T cells inhibited signs of clinical disease, demonstrating that GA-reactive $CD8^+$ T cells are a viable therapy for autoimmune demyelinating disease (Figure 10).

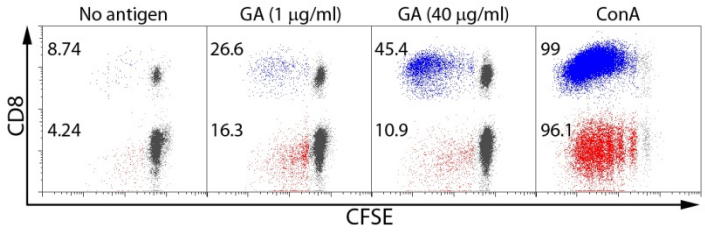

Figure 4 *$CD8^+$ T cells respond to GA treatment in vivo*. Wild-type C57BL/6 mice were immunized with 2 mg GA/IFA. At day 20 post-immunization, bulk splenocytes and draining lymph node cells were isolated, stained with CFSE, and cultured *in vitro* for 5 days with vehicle, GA (20 µg/ml), or concanavalin A (1 µg/ml). Data are gated for $CD4^+$ and $CD8^+$ T cells, with proportion of proliferating cells indicated.

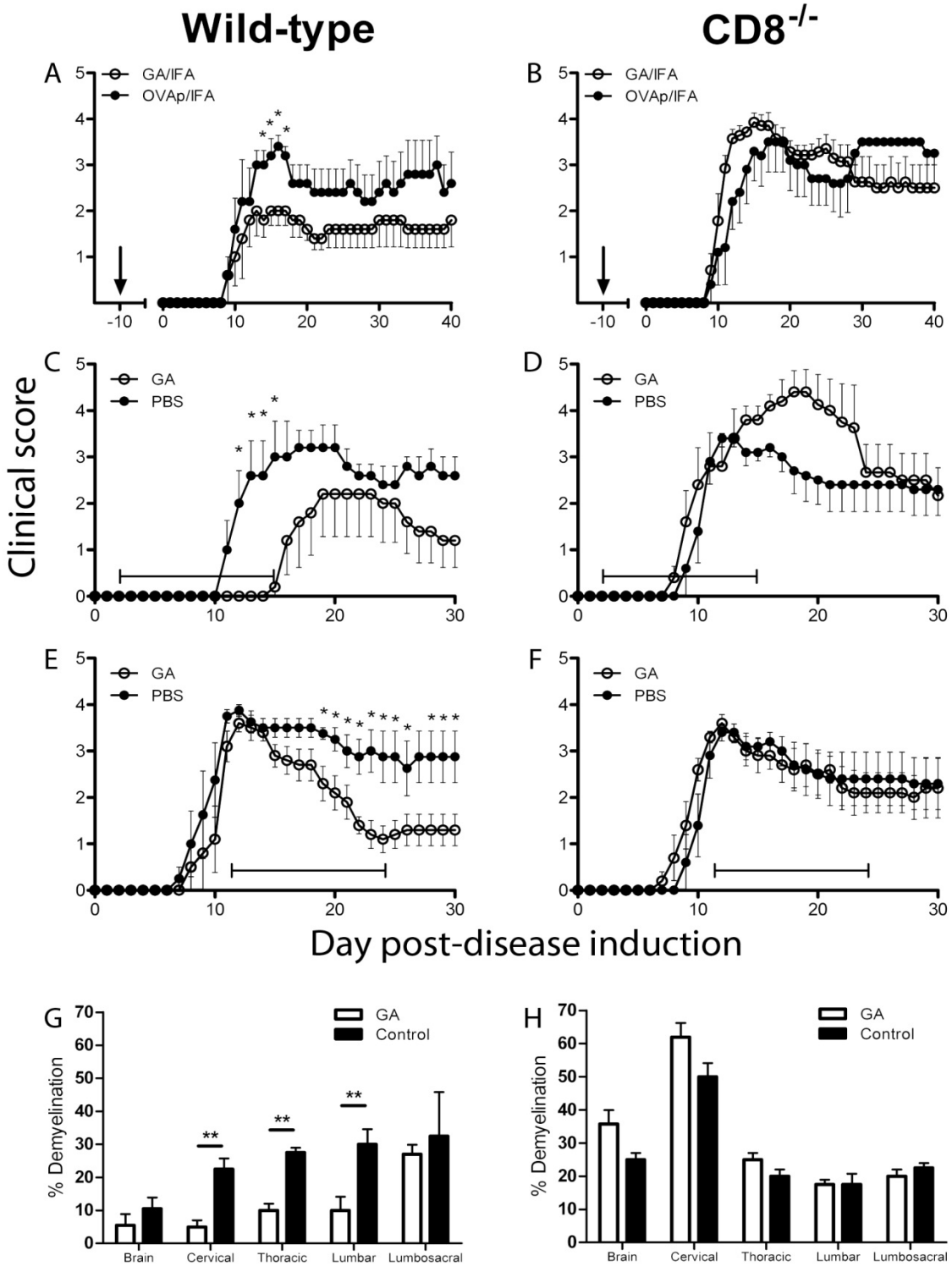
Wild-type
CD8-/-
A
B
C
D
E
F
G
H
GA/IFA
OVAp/IFA
GA
PBS
Clinical score
Day post-disease induction
% Demyelination
Control
Brain
Cervical
Thoracic
Lumbar
Lumbosacral

Figure 5 *$CD8^+$ T cells are required for GA action in ameliorating demyelinating disease in mice*. GA treatment was administered to wild-type and $CD8^{-/-}$ mice by three treatment regimens: GA/IFA emulsion (2 mg GA) on day -10 (A, B), daily subcutaneous GA treatment (20 μg/mouse/day) from day 2 to 15 (C,D), or daily subcutaneous GA treatment from day 11 to 25 (E, F). Disease was induced on day 0 by subcutaneous immunization of MOG_{35-55} (200 μg) in CFA followed by intraperitoneal injection of pertussis toxin (200 ng) on day 0 and 2. (G, H) Mice from (A) and (B) were euthanized 28 days after disease induction. Brains and spinal cords were processed, stained, and analyzed for percent demyelination. * represents $p < 0.05$, ** represents $p < 0.01$.

SPECIFIC AIMS

These findings form the basis of my dissertation work surrounding the hypothesis that GA therapy induces a population of MHC class I-restricted regulatory cytotoxic $CD8^+$ T cells that are essential mediators of drug action. To explore this idea, I have decided to focus my work into three specific aims, representing the three subsections of the Results chapter:

1) Explore the functional requirements of suppressive GA-induced $CD8^+$ T cells

2) Investigate the cellular populations necessary for the $CD8^+$ T cell-mediated suppression of demyelinating disease by GA

3) Isolate the MHC class I-binding moiety of GA that induces suppressive $CD8^+$ T cells

The results garnered through examination of these aims will lead to improved treatment modalities for MS as well as a better understanding of the role of $CD8^+$ T cells in the mechanism of GA in ameliorating autoimmune demyelinating disease.

CHAPTER TWO
Methodology

Mice

Female C57BL/6 mice aged 6-8 weeks were purchased from Taconic Farms and the UTSW Mouse Breeding Core Facility. SJL, CD8$\alpha^{-/-}$, perforin$^{-/-}$, IFN$\gamma^{-/-}$, $\beta_2$microglobulin$^{-/-}$, OT-II, and OT-I mice were purchased from Jackson Laboratories. 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).

EAE Induction

Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice by subcutaneous immunization with 200 μg of MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK, Protein Chemistry Technology Center, UT Southwestern) in emulsified CFA (Difco Laboratories) supplemented with 4 mg/ml M. tuberculosis strain H37Ra (Difco) followed by intraperitoneal injection of 250 ng of Bordetella pertussis toxin (Difco) in phosphate-buffered saline (PBS) at the time of and 2 days after immunization. In the SJL model, EAE was induced by subcutaneous injection of 100 μg of $PLP_{139-151}$ (HSLGKWLGHPDKF, Protein Chemistry Technology Center, UT Southwestern) in emulsified CFA supplemented with 4 mg/ml M. tuberculosis H37Ra. Clinical disease severity was monitored daily and scored according to the following scale: 0 – no clinical disease, 1 – limp tail, 2 – hind limb weakness, 3 – severe hind

limb weakness and/or partial hind limb paralysis, 4 – complete hind limb paralysis, 5 – moribund/death (88).

CFSE-Based Proliferation Assay

Carboxyfluorescein succinimidyl ester (CFSE) dilution assays were performed as previously described (90). Briefly, cells were suspended at 1 x 10^6 cells/ml in PBS and incubated at 37°C for 7 minutes with 0.25 μM carboxyfluorescein diacetate N-succinimidyl ester (CFDA-SE). Cells were then washed 3X with 10% fetal bovine serum (FBS, Gemini Bio-Products) in PBS. Cells were then resuspended in complete media (RPMI 1640 supplemented with 10% FBS, L-glutamine (2 mM), penicillin/streptomycin (100 IU/ml / 100 μg/ml), HEPES (10 μM), sodium pyruvate (1 mM), non-essential amino acids (all from Mediatech), and β-mercaptoethanol (50 μM) (Sigma)) and incubated for 120 hours at 37°C and 5% CO_2. In some experiments, anti-Qa-1 (5 μg/ml) (clone 6A8.6F10.1A6) or control IgG_2 were added to cultures. Cells were washed with FACS buffer (PBS with 1% bovine serum albumin (BSA) and 0.1% sodium azide) and stained with anti-TCRβ, anti-CD8α, anti-CD4, and anti-CD25 fluorescent antibodies (BD Biosciences). Flow cytometric data were acquired on a BD LSR II running FACSDiva software. Data were analyzed by FlowJo (TreeStar). Proliferation was considered significant if the Δ Proliferation (percent proliferation of sample – percent background proliferation) was >1% and the stimulation index (percent proliferation of sample / percent background proliferation) was >2.

Glatiramer Acetate

Glatiramer acetate (Copaxone®, GA) (Teva Neuroscience) was graciously donated by the Multiple Sclerosis Center of St. Paul University Hospital, UT Southwestern. Mice were immunized by subcutaneous injection of either GA (2 mg) or hen ovalbumin residues 323-339 (OVAp, ISQAVHAAHAEINEAGR, Protein Chemistry Technology Center, UT Southwestern) emulsified in 200 μl incomplete Freund's adjuvant (IFA, Difco). For daily regimens, GA was subcutaneously administered at 20 μg per mouse per day in 100 μl PBS. For experiments involving IDO inhibition, 1-methyltryptophan-D (MTD, Sigma) (5 mg/ml) was included in the emulsion.

Media

Unless otherwise noted, all *in vitro* cell culture used RPMI 1640 (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products), HEPES (10 mM) (Cellgro), MEM non-essential amino acids (Cellgro), GlutaMAX-I (Gibco), sodium pyruvate (1mM) (Cellgro), penicillin/streptomycin solution (Gibco), and 2-mercaptoethanol (50 μM) (Sigma).

Adoptive Transfer of $CD8^+$ T Cells

Donor mice were subcutaneously immunized with GA or OVAp emulsified in IFA, as above. Twenty days post immunization, splenocytes were harvested and stimulated *in vitro* with GA or OVAp (20 μg/ml) and hIL-2 (10 pg/ml) in complete media at 7.5 x 10^6 cells/ml for 72 hours at 37°C in 5% CO_2. Live cells were separated by density gradient (Lympholyte-M, Cedarlane

Labs). $CD8^+$ T cells were purified with CD8α (Ly-2) microbeads (Miltenyi Biotec). Purity was >90% by flow cytometry. Cells (5×10^6) were transferred intravenously.

In Vivo Induction of Type 2 Monocytes

Mice were treated with daily subcutaneous injections of GA (150 μg) for six days. Splenocytes were isolated and $CD11c^-CD11b^+$ cells were purified with CD11c and CD11b microbeads (Miltenyi Biotec). Cells were resuspended at 1×10^6 cells/ml in complete media and treated with 0, 10, or 100 U IFNγ (Sigma Aldrich) for 48 (TNFα), 72 (IL-12p40), and 120 (IL-10) hours. Cytokines were analyzed by ELISA.

Preparation of Clodronate- and PBS-loaded Liposomes

Liposomes were prepared as previously described (91). Briefly, cholesterol (8 mg) (Sigma) was dissolved in chloroform (10 ml), followed by addition of 0.86 ml of a solution of phosphatidylcholine (Sigma) in chloroform (100 mg/ml). Low vacuum rotovap was applied to remove chloroform. The remaining phospholipid film was dispersed in 10 ml of either PBS (for control liposomes) or a 0.6 M solution of dichloromethylene-bisphosphonate (clodronate, Sigma) in water, and kept under nitrogen for 2 hours at room temperature. Solutions were sonicated in a waterbath for 3 minutes and swelled under nitrogen overnight at 4^oC. Prior to use, liposomes were centrifuged at 10000 x g, collected, then further washed and resuspended in PBS.

Depletion of Phagocytic Macrophages

Mice were intraperitoneally treated with PBS- or clodronate-loaded liposomes (200 μl) beginning on day -3 and continuing every four days. Disease was induced by subcutaneous injection of 100 μg MOG_{35-55} with or without 2 mg GA emulsified in CFA and followed by intraperitoneal injection of B pertussis toxin following emulsion injection and on day 2. On day 20, splenocytes were isolated and stained with anti-MHCII and anti-F4/80 antibodies (BD Biosciences). Cells were analyzed as above.

In Vitro Induction of Suppressive Macrophages

Suppressive macrophages were induced as previously described. Briefly, bone marrow cells were isolated from wild-type C57BL/6 mice and incubated at 1 x 10^6 cells/ml in six-well plates (5 ml per well) in complete media supplemented as above plus 10 ng/ml M-CSF (Sigma) and 100 U/ml IFNγ with or without 50 μg/ml GA. Half of culture supernatants were replaced with media, M-CSF, and IFNγ on day 3. On day 6 cells were washed twice with ice cold PBS and scraped off of plates using a rubber policeman. Cells were transferred to recipient mice (1 x 10^6 cells per mouse) or used for *in vitro* studies.

In Vitro Proliferation of $CD8^+$ T Cells by Macrophages

$CD4^+$ T cells from naïve OTII mice and $CD8^+$ T cells from naïve OTI and GA-immunized mice were isolated by magnetic bead separation. OVA macrophages were derived as above, replacing GA with hen egg ovalbumin (50 μg/ml, Sigma). Cells were stained with CFSE and incubated in

a 1:4 ratio ($CD8^+$ T cells to monocytes) for five days. Exogenous antigen was added at 20 μg/ml. Cells were stained and analyzed as above.

Quantitative Real-Time PCR Assay

Total RNA was extracted from total splenocytes using RNeasy Mini Kit (Qiagen) followed by reverse transcription using Superscript II reverse transcription kit (Qiagen). Quantitative real-time PCR assays were performed using Brilliant SYBR Green QPCR Master Mix on an MX3000p thermocycler. The following primer pairs were used: β-actin: (F) GTGGGCCGCTCTAGGCACCAA, (R) CTCTTTGATGTCACGCACGATTTC; IDO: (F) CACTGATACGCCTGAGTG, (R) GTGAGCGCTGAATCGAAA.

Epitope Immunoprecipitation

Immunoprecipitation and LC/MS-MS were performed as previously described (92). Briefly, eighteen wild-type C57Bl/6 mice were immunized with 2 x 10^6 B16-Flt3L cells (a kind gift of Todd Eagar, Ph.D.). Sixteen days later, mice were intraperitoneally injected with 50 μg poly I:C (high molecular weight, In Vivo Gen). Five hours later spleens were dissected, treated with 400 U/ml collagenase D (Roche) in HBSS (with Ca^{2+} and Mg^{2+}) (Cellgro) for 25 minutes at 37°C. Red blood cells were lysed with ACK buffer and the remaining cells were resuspended at 10 x 10^6 per ml and placed in complete media in 125 cm^2 flasks and treated with PBS or GA (20 μg/ml). After 5 hours, cells were pelleted and lysed with 1% CHAPS, 0.1 mM iodoacetamide, 5 mM EDTA, 1:100 Protease Inhibitors Cocktail, 1 mM PMSF in 50 mM Tris-HCl (pH 8) at 4°C

for 45 min on a rotator at 400 x 10^6/ml. Lysate was cleared by 30 minutes of centrifugation at 15000 rpm. MHC class I molecules from cleared lysate were immunoaffinity purified with 15 mg of purified antibody M1/42 bound to CNBr-activated sepharose (GE Healthcare) at a ratio of 40 mg sepharose per mg of antibody following manufacturer's protocol. The affinity column was washed first with 3 column volumes of lysis buffer, followed by 6 column volumes of 250 mM NaCl, 50 mM Tris-HCl (pH 8) then 6 column volumes of 50 mM Tris-HCl (pH 8). The MHC I molecules were eluted at room temperature for 4 min on rotator by adding 1 mL of 10% acetic acid. MHC I peptide complexes were boiled at 70 °C for 10 min. MHC I peptides were separated from the denatured protein subunits of the HLA molecules and the contaminating antibody by ultrafiltration through a 10 kDa cutoff membrane filter (Sartorius Stedim, Aubagne, France) and centrifugation at 3000× *g*. The filters were washed three times with 2 mL water to remove contaminants interfering with the mass spectrometry. Recovered peptide mixtures (5–6 mL) were concentrated and desalted with C-18 cartridge (Waters, Medford, MA). The C-18 cartridge was first washed three times with 50% acetonitrile (1.5 mL), equilibrated with 0.1% trifluoroacetic acid (TFA) in water, and then loaded with the peptide mixture. The cartridge was then washed by an additional 3 mL 0.1 TFA%, and the peptides were eluted with 0.1% TFA in 50% acetonitrile in (1.5 mL). The eluted MHC peptides were reduced to near dryness and then reconstituted at 20 µl 0.1% TFA/water. Half of the peptide mixture, corresponding to approximately 3.5–4 × 10^8 cell equivalents, was injected for LC–MS/MS analysis.

LC/MS-MS

The MHC peptide mixture was separated on the Dionex U3000 capillary/nano-HPLC system (Dionex, Sunnyvale, California) that is directly interfaced with the Thermo-Fisher LTQ-Orbitrap mass spectrometer (Thermo Fisher, San Jose, CA). Prior to the analysis by tandem LC–MS/MS, the complex mixture was passed through a 10 kDa filter to separate the peptides bound to the MHC I complex from other higher molecular weight peptides and proteins that might be present in the mixture eluted from the antibody column. The analytical column was a homemade fused silica capillary column (75 μm ID, 100 mm length; Upchurch, Oak Harbor, WA) packed with C-18 resin (300 A, 5 mm, Varian, Palo Alto, CA). To optimize the separation of peptides bound to the MHC I complex, the mixture was run on capillary/nano HPLC system with a shallow gradient of an aqueous mobile phase A (0.1% formic acid in water) and organic mobile phase B (0.1% formic acid in 100% acetonitrile) formed in 180 min with a flow rate of 250 nl/min under the following conditions: 0–55% B formed in 120 min, followed by 25 min gradient from 55–80% solvent B. Solvent B was maintained at 80% for another 10 min and then decreased to 0% in 10 min. Another 15 min interval was used for equilibration, loading and washing. The HPLC system was interfaced with the Thermo-Fisher LTQ Orbitrap XL mass spectrometer. The LTQ-Orbitrap mass spectrometer was operated in the data-dependent acquisition mode using the Xcalibur 2.0.7 software. The experiment consisted of a single MS full-scan in the Orbitrap (620–1200 *m*/*z*, 30 000 resolution) followed by 6 data-dependent MS/MS scans in the ion trap at 35% normalized collision energy. The most intense 6 masses from each full mass spectrum with doubly and triply charge states were selected for fragmentation by collision-induced dissociation

in the linear ion-trap. The dynamic exclusion parameters were as follows: Repeat count = 1; Repeat Duration = 30 s; Exclusion list = 100; and Exclusion time = 90 s.

Database Search

The MS/MS spectra from each LC–MS/MS run were converted from the .RAW file format to .DTA files using the Bioworks 3.3.1 software. DTA files were analyzed using the MASCOT software search algorithm against IPI mouse database. The following search parameters were used in all MASCOT searches: the digestion enzyme was set as none and methionine oxidation as the variable modification. The maximum error tolerance for MS scans was 10 ppm for MS and 1.0 Da for MS/MS. Peptide were identified by comparing the found sequences with the sequences deposited in the International Protein Index mouse (IPI) database. Only peptides with Mascot Score more than 20 and mass deviation less than 5 ppm were considered.

CHAPTER THREE
Results

MOLECULAR REQUIREMENTS OF GA CD8$^+$ T CELLS

Mechanisms of Regulatory CD8$^+$ T Cells

To explore the mechanism by which CD8$^+$ T cells exercise the suppressive effects of GA, I first aimed to dissect the necessary molecules responsible for the activation of the suppressive subset of CD8$^+$ T cells as well as the effector molecules necessary for inducing immune regulation. While CD8$^+$ T cells are generally considered to be responsible for the clearance of intracellular pathogens such as virus as well as tumor, regulatory behavior by CD8$^+$ T cells has been observed in a number of autoimmune disorder models, including diabetes, arthritis, and systemic lupus erythematosus, and often depends on the same molecules associated with cytotoxic lymphocyte action (93-95). One of the most pertinent of these molecules is the major histocompatibility complex class I (MHC I). These molecules are present on almost every nucleated cell in both humans and mice, and are the end product of a complex network of proteins responsible for sampling intracellular proteins and displaying them on the cell surface, although some cell types, including subsets of dendritic cells, can cross-present exogenous antigen on MHC I (96). CD8$^+$ T cells recognize MHC I/peptide complexes via interaction with the CD8 molecule and the T cell receptor (TCR), a polymorphic molecule capable of recognizing a host of peptide sequences due

to the mutability of its variable (V) region, and are activated by a set of downstream effector molecules, including CD3, NF-κB, and NFAT (97).

Once activated, $CD8^+$ T cells begin to secrete cytokines such as interferon-γ and cytotoxic effector molecules like perforin. IFNγ is a type II interferon first discovered in PHA-activated lymphocyte supernatants (98). Produced predominantly by NK cells, Th1 type $CD4^+$ T helper cells, and activated $CD8^+$ T cells, IFNγ exerts its effects on cells by binding cell surface receptors and activating JAK-STAT-type kinases and interferon regulatory factors (IRFs). This results in endogenous protein synthesis and increased cell surface expression of MHC class I and II molecules. The cytokine also induces innate immune responses, such as macrophage activation and the respiratory burst (99).

Perforin is produced predominantly by NK cells and $CD8^+$ cytotoxic T lymphocytes (100). Stored within cytoplasmic granules of these cells, the molecule is released in response to a calcium-dependent mechanism linked to TCR activation. Perforin then "perforates" target cell membranes by forming transmembrane pores upon polymerization, inducing osmotic lysis similar to the membrane attack complex (MAC) of complement components C5b-C9.

Both IFNγ and perforin have been associated with the control of autoimmune disorders. Anti-IFNγ treatment *in vivo* aggravates several disease models, including diabetes, autoimmune uveitis, collagen-induced arthritis, and experimental autoimmune encephalomyelitis (101). In

addition, IFNγ treatment has proven to be effective in reducing signs of demyelination in EAE. Perforin regulates the elimination of $CD8^+$ T cells after an acute exposure to foreign antigen, while perforin-deficient mice maintain an increased clonal persistence of superantigen- and virus-specific T cells that may induce autoimmunity through molecular mimicry (102). Additionally, perforin-deficient mice also had higher rates of disease in certain lupus models (103). Finally, both IFNγ and perforin, but not Fas/FasL, are vital for the control of demyelinating disease by Qa-1 restricted, $CD8\alpha\alpha^+$ Treg to induce depletion of encephalitogenic Vβ8.2 $CD4^+$ T cells (104). In addition, $CD8^+$ T cell secretion of IFNγ has been associated with GA therapeutic action (83). Treatment with GA increased levels of IFNγ and IL-4 in the blood of MS patients. The increase in IFNγ was linked to expression by $CD8^+$ T cells.

IFNγ, Perforin, and MHC Class I Are Required for GA Action *In Vivo*

In order to test whether these molecules were necessary for the suppression of demyelinating disease by GA-reactive $CD8^+$ T cells, I first tested the effects of GA directly in mice deficient in each of these molecules. MOG_{35-55} disease was induced in wild-type, $IFN\gamma^{-/-}$, $perforin^{-/-}$, and $MHCI^{-/-}$ ($\beta_2 microglobulin^{-/-}$) mice, which were then treated with daily subcutaneous injections of GA (20 μg/ml) from day 2 to 15 (Figure 6) or immunized with GA in IFA ten days prior to disease induction (Figure 7). While wild-type mice treated with GA showed decreased levels of disease, GA had no significant effect on the knockouts, and in one case ($perforin^{-/-}$) increased disease. Therefore, these molecules are essential for the suppression of disease by GA, and in conjunction with earlier data demonstrating the requirement of $CD8^+$ T cells in GA treatment,

are likely involved in either activating GA-reactive suppressive $CD8^+$ T cells or in the effector mechanisms used by such cells.

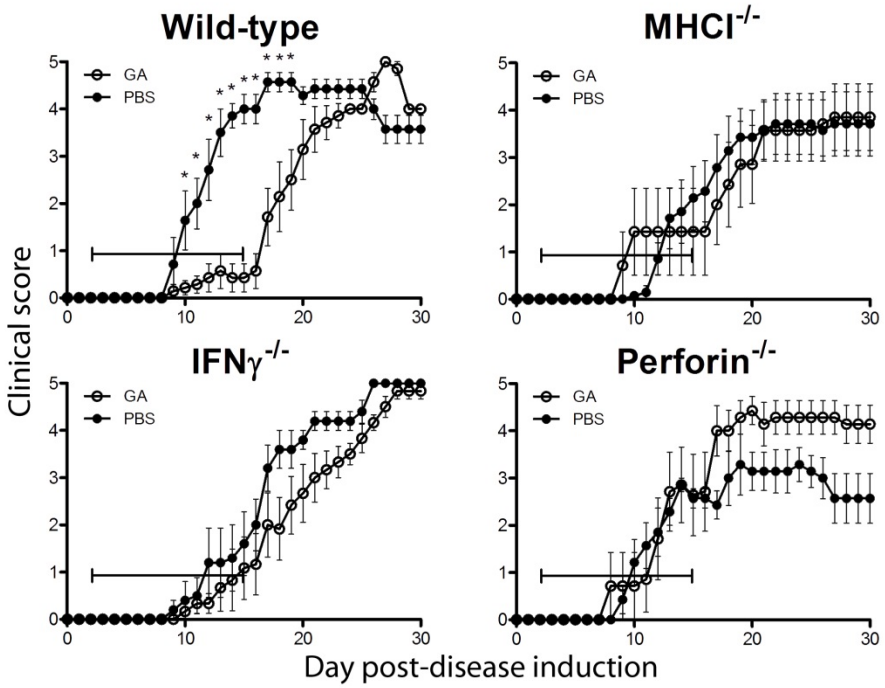

Figure 6 *Daily GA treatment does not reduce demyelinating disease in MHCI$^{-/-}$, IFNγ$^{-/-}$, or perforin$^{-/-}$ mice*. Wild-type, MHCI$^{-/-}$, IFNγ$^{-/-}$, and perforin$^{-/-}$ mice on the C57BL/6 background strain were subcutaneously immunized with MOG_{35-55} (200 μg) in CFA (100 μl/mouse) followed by intraperitoneal injection of pertussis toxin (200 ng/mouse/day) on day 0 and 2 of disease. Mice were treated by subcutaneous injection of GA (20 μg/mouse/day) in the rear flanks. * denotes $p < 0.05$.

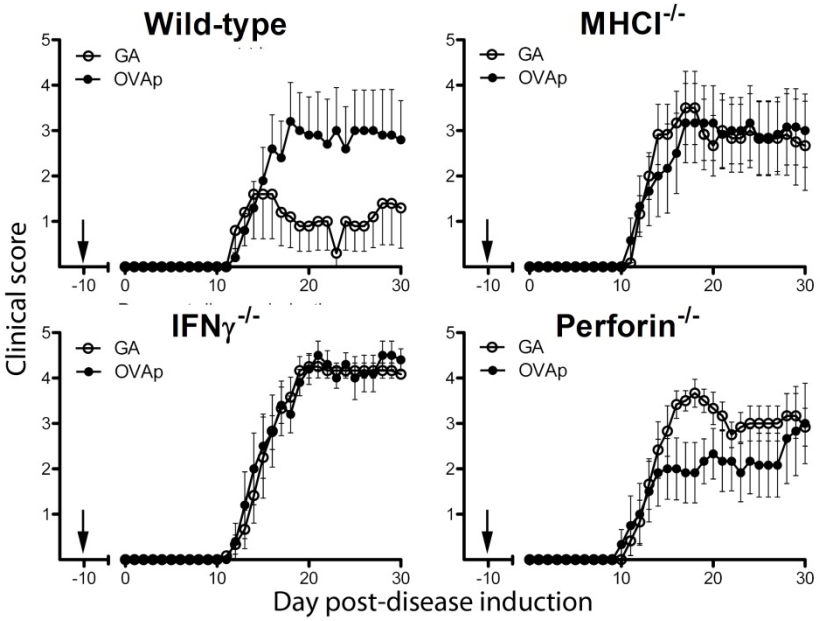

Figure 7 *Pre-immunization with GA does not reduce demyelinating disease in MHCI$^{-/-}$, IFNγ$^{-/-}$, or perforin$^{-/-}$ mice*. Wild-type, MHCI$^{-/-}$, IFNγ$^{-/-}$, and perforin$^{-/-}$ mice on the C57BL/6 background strain were subcutaneously immunized with GA (1 mg) in IFA (100 µl/mouse) unilaterally in the rear flank and shoulder. Ten days later mice were immunized in the contralateral flank and shoulder with MOG_{35-55} (200 µg) in CFA (100 µl/mouse) followed by intraperitoneal injection of pertussis toxin (200 ng/mouse/day) on day 0 and 2 of disease.

GA-Reactive $CD8^+$ T Cells Use IFNγ and Perforin as Effector Molecules in Reducing Signs of Demyelinating Disease

Based on earlier reports describing regulatory $CD8^+$ T cells utilizing IFNγ and perforin to suppress autoimmune reactions, I hypothesized that these molecules were likely effectors used directly by $CD8^+$ T cells to induce regulation of disease. Therefore, the adoptive transfer of GA $CD8^+$ T cells deficient in these molecules should abrogate the suppressive effects of these cells. To test this, I immunized wild-type, $IFN\gamma^{-/-}$, and $perforin^{-/-}$ mice with GA as GA $CD8^+$ T cell donors. Cells were injected one day prior to disease induction. While wild-type GA $CD8^+$ T cells suppressed signs of disease, cells lacking IFNγ did not ameliorate disease, while perforin-deficient cells showed reduced capacity to abrogate disease (Figure 8). As a further control, I also immunized $IL\text{-}10^{-/-}$ mice with GA for donor $CD8^+$ T cells. IL-10 had previously been shown to be unnecessary for GA action (61); therefore, IL-10-deficient $CD8^+$ T cells should be able to suppress disease as well as wild-type cells. When transferred into wild-type recipient mice one day prior to disease induction, these $IL\text{-}10^{-/-}$ $CD8^+$ T cells suppressed disease equally to wild-type cells, demonstrating that IL-10 is unnecessary for GA $CD8^+$ T cell-mediated control of demyelinating disease, as well as arguing against a generalized immunosuppressive mechanism for GA.

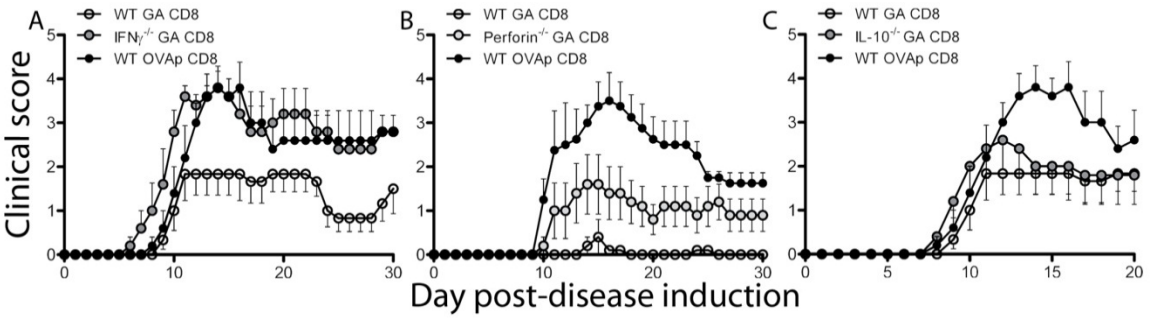

Figure 8 *IFNγ and perforin, but not IL-10, are required for suppression of EAE by GA-induced CD8$^+$ T cells*. Donor wild-type, IFNγ$^{-/-}$, perforin$^{-/-}$, and IL-10$^{-/-}$ mice on the C57BL/6 background were immunized with GA (1 mg) or OVAp (200 μg) in IFA (100 μl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension then incubated with cognate antigen (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 5 x 10^6 cells were injected intravenously into wild-type recipient mice 24 hours before disease induction with MOG_{35-55}, as above.

Non-Classical MHC Class I Is Responsible for GA CD8$^+$ T Cell Activation *In Vitro* and *In Vivo*

Unlike IFNγ and perforin, MHC class I would not act as an effector molecule for suppressive CD8$^+$ T cells but would instead activate these cells in response to their cognate antigen. MHC I can be divided into two categories: classical and non-classical (105). Classical MHC I (K, D, and L in mice; HLA-A, -B, and –C in humans) is composed of a polymorphic α-chain that contains the peptide binding region and $\beta_2$microglobulin (106). These molecules are responsible for activating anti-viral and anti-tumor responses, and are the more widely expressed forms of MHC I. Non-classical MHC I (including Qa-1, Qa-2, and CD1d in mice; HLA-E, -F, and -G in humans) are similar to classical molecules in terms of their molecular formation (composed of an α-chain paired with $\beta_2$microglobulin, although the α-chain demonstrates less polymorphism than the classical variant) but differ in terms of their cellular expression and function (107). Unlike classical MHC I, non-classical MHC I is expressed on a more restricted subset of cells, including activated CD4$^+$ T cells and APCs. Non-classical MHC I is associated with activation of NK cells, NK T cells, and can also bind peptide to activate CD8$^+$ T cells. Several ligands have been described which induce regulatory behavior in CD8$^+$ T cells, including the Qdm peptide derived from MHC I leader peptide sequences, as well as heat shock protein sequences (108). CD8$^+$ T cells restricted for Qa-1 have been shown to have regulatory properties in several autoimmune disorders. Additionally, our group has demonstrated that GA-induced CD8$^+$ T cells in MS patients inhibit CD4$^+$ T cell proliferation via HLA-E interactions. Therefore, it is likely that GA-reactive CD8$^+$ T cells in mice are also dependent on non-classical MHC I for activation and

disease suppression. To test activation, GA $CD8^+$ T cells were stained with CFSE and incubated *in vitro* with T cell-depleted wild-type, MHC $I^{-/-}$, and $K^{b-/-}D^{b-/-}$ splenocytes. After 5 days, proliferation was analyzed by flow cytometry. Incubation with MHC $I^{-/-}$ APCs completely abrogated proliferation when compared to wild-type APCs, as expected (Figure 9). However, when GA $CD8^+$ T cells were incubated with APCs deficient of classical MHC class I, proliferation was equivalent to wild-type. This demonstrates that GA $CD8^+$ T cells require non-classical MHC I for activation.

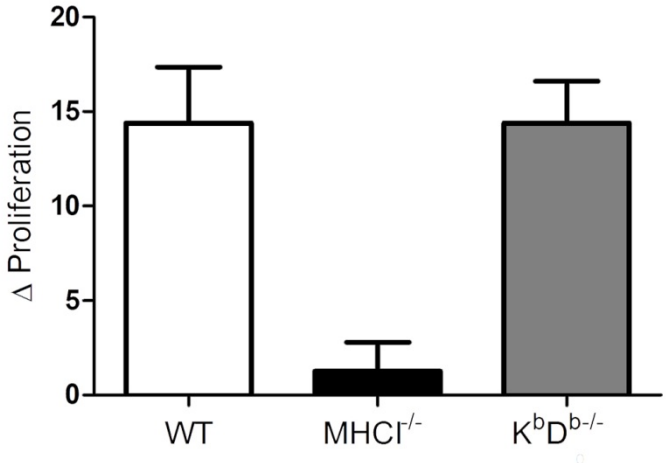

Figure 9 *GA CD8$^+$ T cells require non-classical MHC class I for activation in vitro*. Wild-type C57BL/6 mice were subcutaneously immunized with GA (1 mg) in IFA (100 µl/mouse) in the rear flanks. After 20 days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension, then stained with CFSE. APCs were derived from the spleens of naïve wild-type, $\beta_2$microglobulin$^{-/-}$ (MHC I$^{-/-}$), and K$^{b-/-}$ D$^{b-/-}$ mice after depletion of T cells with magnetic bead-labeled anti-CD3 antibody. CFSE-stained GA CD8$^+$ T cells were incubated with APCs in a 1:4 ratio (1 x 10^6 cells/tube) with vehicle or GA (20 µl/ml). Δ proliferation represents the difference in percentage of CFSE-low CD8$^+$ T cells between GA-treatment and vehicle control.

The next step was to determine whether the difference in activation was also reflected in GA $CD8^+$ T cell's ability to inhibit demyelination. GA $CD8^+$ T cells were adoptively transferred to wild-type, MHC $I^{-/-}$, and $K^{b-/-}D^{b-/-}$ recipient mice one day prior to disease induction. In MHC $I^{-/-}$ hosts, GA $CD8^+$ T cells were completely ineffective in reducing signs of disease. However, GA $CD8^+$ T cells were still able to suppress disease in $K^{b-/-}D^{b-/-}$ mice in a manner similar to wild-type hosts (Figure 10). Therefore, the activation and suppressive ability of GA $CD8^+$ T cells are dependent on a non-classical MHC I molecule. Furthermore, it appears that $CD4^+$ T cells are unnecessary for the activation of these cells. This may suggests that GA $CD8^+$T cells are initially activated by non-classical MHC I-expressing APC upon adoptive transfer, and are then able to recognize other cells expressing non-classical MHC I.

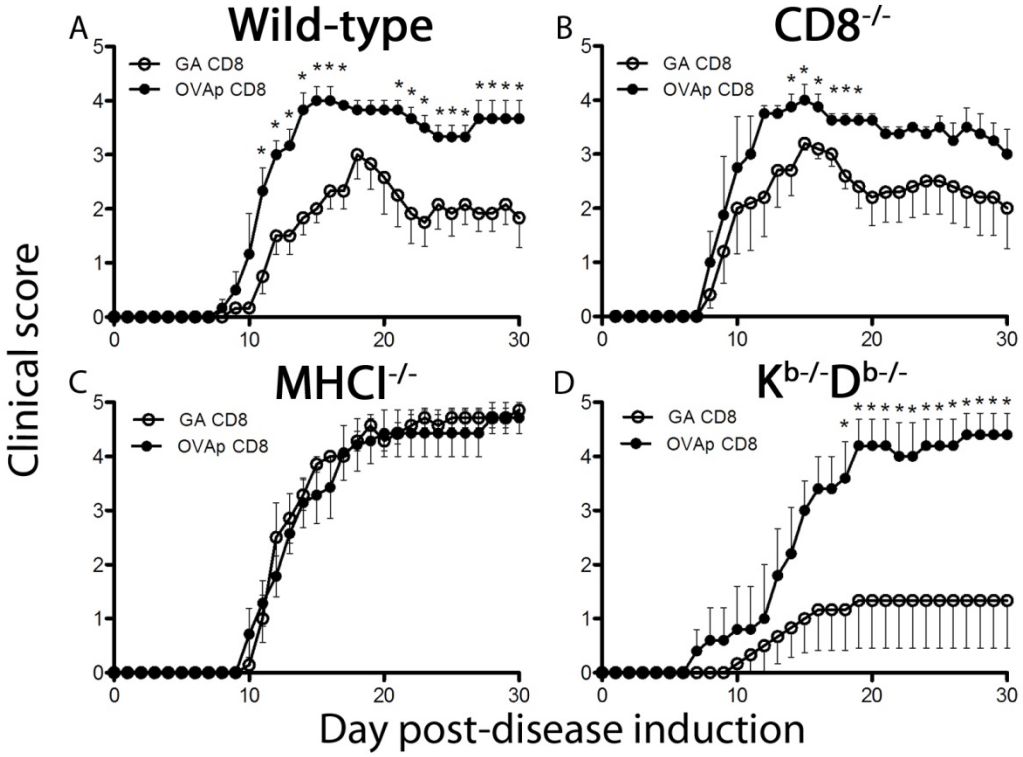

Figure 10 *GA-induced CD8$^+$ T cells suppress disease upon adoptive transfer by acting on non-classical MHC class I.* Donor C57BL/6 wild-type mice were immunized with GA (1 mg) or OVAp (200 μg) in IFA (100 μl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension then incubated with cognate antigen (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 5 x 10^6 cells were injected intravenously into wild-type, CD8$^{-/-}$, $\beta_2$microglobulin$^{-/-}$ (MHC I$^{-/-}$), and K$^{b-/-}$D$^{b-/-}$ recipient mice 24 hours before disease induction with MOG_{35-55}, as above. * represents $p < 0.05$.

GA CD8$^+$ T Cells Do Not Require Qa-1 for Activation

GA-mediated amelioration of a model of colitis has been shown to require Qa-1 expression (109). To test whether Qa-1 expression was required for activation of GA CD8$^+$ T cells, cells from draining lymph nodes and splenocytes were stained with CFSE and incubated with GA with or without anti-Qa-1 blocking antibody. Cells incubated with GA proliferated strongly both in the presence of control and anti-Qa-1 antibody (Figure 11). This demonstrates that Qa-1-mediated peptide presentation is not required for the activation of GA CD8$^+$ T cells, contrasting with human data in which HLA-E, the human homolog of Qa-1, is necessary for proliferative responses to GA (85). Therefore, a different non-classical MHC class I molecule, such as Qa-2, is needed for GA CD8$^+$ T cell activation.

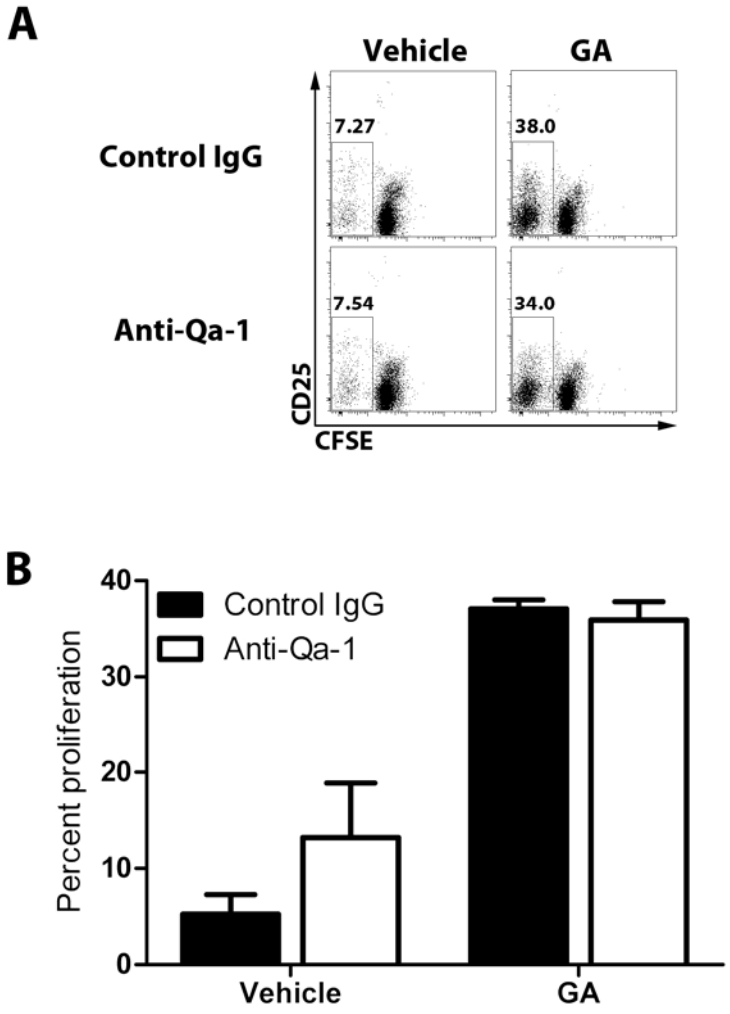

Figure 11 *Qa-1-mediated peptide presentation is not required for GA CD8$^+$ T cell activation*. Wild-type mice were immunized with GA (1 mg) in IFA. Fifteen days post-immunization, draining lymph nodes and spleens were processed to single-cell suspension, stained with CFSE, and cultured with vehicle or GA (1 μg/ml) in the presence of control IgG or anti-Qa-1 antibody (5 μg/ml, clone 6A8.6F10.1A6) for 5 days, then analyzed by flow cytometry. (A) Flow diagrams. (B) Graphical representation of combined data.

GA CD8$^+$ T Cells Do Not Respond to HSP60-Derived Peptides Presented by Qa-1

Qa-1-restricted regulatory CD8$^+$ T cells respond to a peptide sequence derived from heat shock protein 60 (HSP60) (108). To determine whether GA CD8$^+$ T cells are the same population of regulatory T cells as Qa-1-restricted CD8αα$^+$ T cells, responses to regulatory HSP60 epitopes were assayed and compared. Cells from draining lymph nodes and splenocytes were stained with CFSE and incubated with GA or the regulatory HSP60 epitope (GMKFDRGYI). While CD8$^+$ T cells responded strongly to GA, HSP60 did not induce proliferation (Figure 12). This indicates that Qa-1-restricted regulatory CD8$^+$ T cells are not the same population as GA CD8$^+$ T cells, and are thus responding to a distinct peptide.

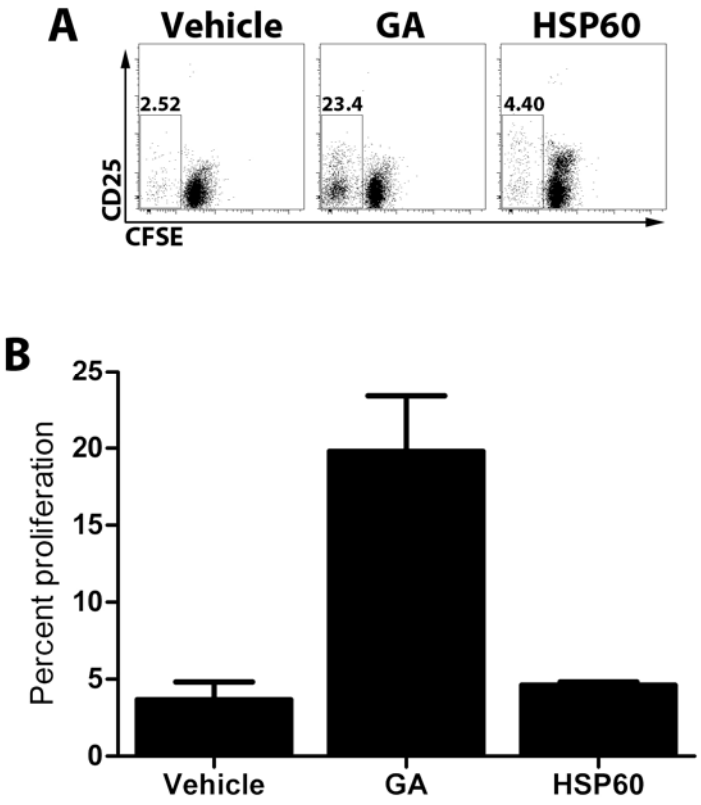

Figure 12 *HSP60 regulatory epitopes do not induce proliferation in GA CD8$^+$ T cells*. Wild-type mice were immunized with GA (1 mg) in IFA. Fifteen days post-immunization, draining lymph nodes and spleens were processed to single-cell suspension, stained with CFSE, and cultured with vehicle, GA (20 μg/ml), or HSP60 peptide GMKFDRGYI (20 μg/ml) for 5 days, then analyzed by flow cytometry. (A) Flow diagrams. (B) Graphical representation of combined data.

GA CD8$^+$ T Cell-Mediated Suppression of Disease Is Not Limited to a Single Model

Different strains of mice respond different to myelin derived proteins and peptides. Whereas MOG_{35-55} induces a chronic form of demyelinating disease in C57BL/6 mice, it does not in the SJL strain, which respond to $PLP_{139-151}$ immunization with a relapsing/remitting form of EAE. However, both strains are susceptible to chronic disease upon $PLP_{178-191}$ injection (110). Therefore, it is possible that the findings concerning GA's ability to induce regulatory CD8$^+$ T cells are restricted to the MOG_{35-55}/C57BL/6 form of EAE, and are not generalizable to other models. To test this hypothesis, GA CD8$^+$ T cells were derived from SJL mice by GA immunization and adoptively transferred into SJL recipient mice. One day later, disease was induced with $PLP_{139-151}$ immunization. As in the MOG_{35-55}/C57BL/6, GA CD8$^+$ T cells were able to suppress signs of disease, reducing symptoms of demyelination in both the initial acute stage as well as in relapse (Figure 13). This demonstrates that GA CD8$^+$ T cells are effective in both other strains of mice as well as other peptide models, suggesting that the adoptive transfer of these cells as a therapy for demyelination takes advantage of a general immune mechanism.

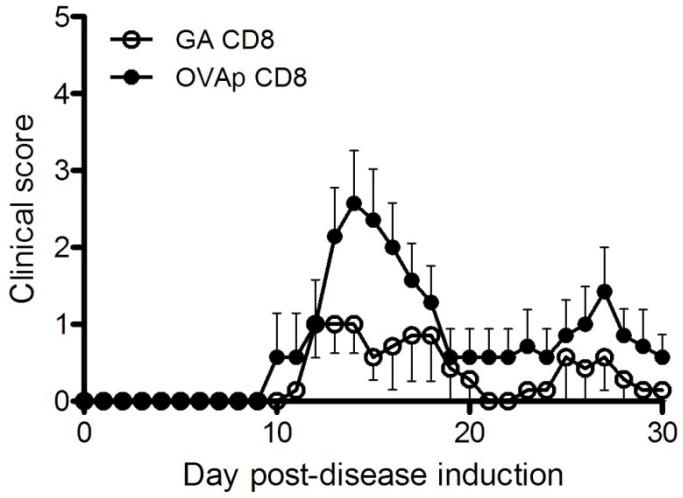

Figure 13 *GA CD8$^+$ T cells suppress demyelinating disease in the PLP$_{139-151}$/SJL model of EAE*. Donor SJL wild-type mice were immunized with GA (1 mg) or OVAp (200 μg) in IFA (100 μl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension then incubated with cognate antigen (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 5 x 10^6 cells were injected intravenously into wild-type SJL mice 24 hours prior to disease induction with PLP$_{139-151}$ (100 μg) in CFA.

Passive Disease Is Reduced by GA $CD8^+$ T Cells

In addition, it is possible that GA $CD8^+$ T cells are only effective in treating active disease. If this were the case, then it would suggest that these cells are acting on the initial stages of the immune reaction, including antigen uptake and presentation to $CD4^+$ T cells in the periphery, as well as differentiation toward different T helper subsets. While the ability of GA $CD8^+$ T cells to suppress disease in a therapeutic manner after the onset of disease argues against this idea, it is still occurring in an artificial inflammatory environment containing adjuvant and exogenous antigen. A better model would be the passive model of disease, in which MOG_{35-55}-specific $CD4^+$ T cells are adoptively transferred into non-immunized recipient mice. These mice succumb to demyelinating disease without the need for strong adjuvant, although pertussis toxin must still be used. Therefore, GA $CD8^+$ T cells were intravenously transferred into wild-type mice. After one day, these mice received $CD4^+$ T cells isolated from the draining lymph nodes of donor mice immunized with MOG_{35-55}. GA $CD8^+$ T cells were still able to suppress disease as compared to control $CD8^+$ T cells (Figure 14). This suggests that these cells do not require the artificial inflammatory environment present in active disease. Additionally, it suggests that the mechanism of action is not dependent on abrogating the initial priming event of the pathogenic $CD4^+$ T cells, as these cells have already been primed and skewed toward an inflammatory phenotype prior to adoptive transfer. This may mean that GA $CD8^+$ T cells may act directly on activated $CD4^+$ T cells expressing a non-classical MHC I molecule such as Qa-1. Otherwise, these cells could be acting on the antigen-presentation occurring within the periphery or the CNS

that reactivates the pathogenic $CD4^+$ T cells, and may thus reduce the levels of free neuroantigen induced by destruction of the CNS.

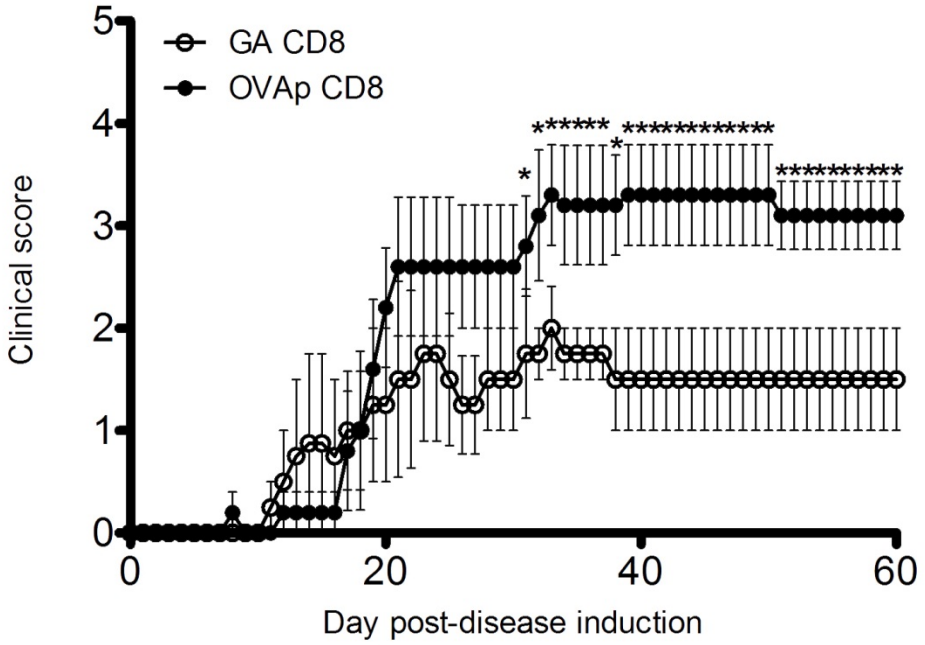

Figure 14 *GA CD8$^+$ T cells can suppress passive EAE*. GA CD8$^+$ T cells (5 x 10^6/mouse) were derived and injected intravenously as outlined in previous figures. Pathogenic CD4$^+$ T cells were derived by immunizing wild-type C57BL/6 mice with MOG_{35-55} (200 μg) in CFA. After ten days draining lymph nodes were isolated and processed to single cell suspension, then cultured *in vitro* with MOG_{35-55} (30 μg/ml) and IL-12 (20 ng/ml) for 72 hours. CD4$^+$ T cells were isolated by magnetic bead cell sorting and 5 x 10^6 cells were injected intraperitoneally one day after CD8$^+$ T cell injection followed by intraperitoneal pertussis toxin injection (200 ng) on day 0 and 2.

GA CD8$^+$ T Cells Traffic to the Central Nervous System

There are several sites at which GA CD8$^+$ T cells could interact with the other cell types involved in the suppression of demyelinating disease. While secondary lymphoid organs would be the most obvious place where GA-reactive CD8$^+$ T cells would encounter an activated neuroantigen-specific CD4$^+$ T cell as well as APCs capable of presenting autoantigen, these actions could also occur within the CNS itself. To test whether GA CD8$^+$ T cells could access the CNS, GA CD8$^+$ T cells isolated from congenic CD45.1$^+$ C57BL/6 mice were injected into naïve wild-type hosts. After twelve days, spleens, inguinal lymph nodes, and spinal cords were isolated, processed, and stained for CD45.1$^+$CD8$^+$ T cells. These cells appeared in all three tissues tested, demonstrating that GA CD8$^+$ T cells could access the CNS to induce suppression of disease (Figure 15).

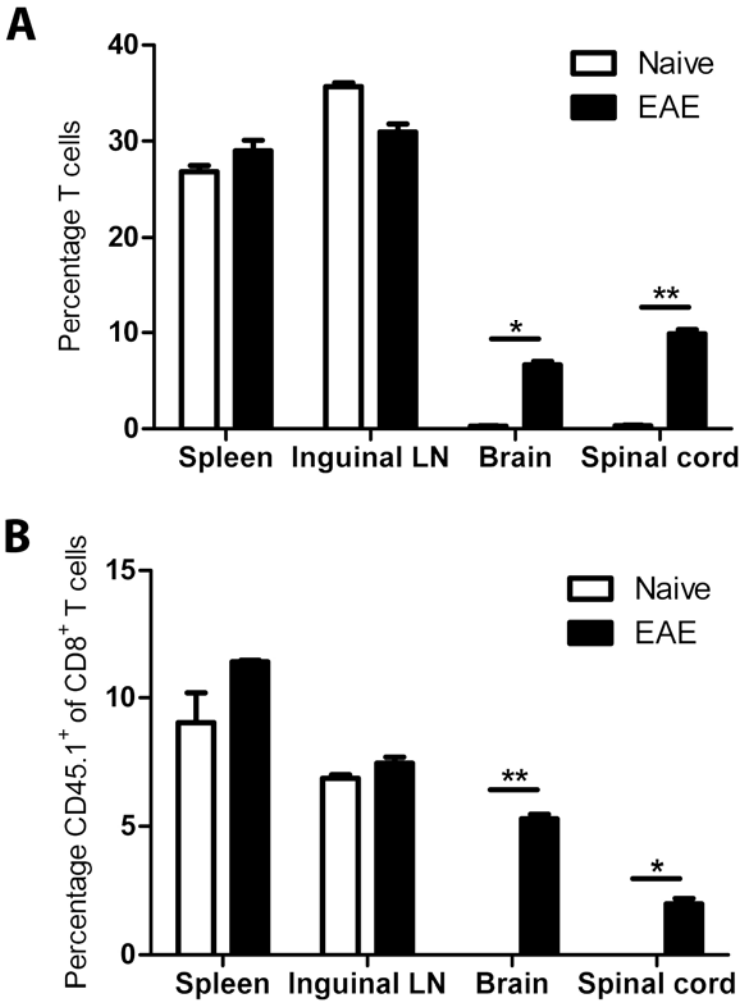

Figure 15 *GA CD8$^+$ T cells traffic to the CNS during demyelinating disease*. Donor CD45.1$^+$ C57BL/6 mice were immunized with GA (1 mg) in IFA (100 µl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single cell suspension then incubated with GA (20 µg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 10 x 10^6 cells were injected intravenously for 24 hours into either naïve or MOG_{35-55}-EAE-induced wild-type C57BL/6 mice at the peak of disease (day 12 post-immunization). Spleens, inguinal lymph nodes, brains, and spinal cords were isolated from recipients, processed, and stained for CD45.1$^+$CD8$^+$ T cells. (A) Percentage T cells of total cells processed. (B) Percent CD45.1$^+$ of CD8$^+$ T cells. ** represents $p < 0.01$, * represents $p < 0.05$.

IN VIVO REQUIREMENTS OF GA CD8$^+$ T CELLS

Effects on Proliferation of CD4$^+$ T Cells

One possible mechanism by which GA-reactive CD8$^+$ T cells reduce neuroinflammation is by reducing numbers of myelin-reactive CD4$^+$ T cells responsible for the pathology of the disease. Early data by our group showed that GA CD8$^+$ T cells could directly kill GA-loaded splenocytes *in vitro*. My own work demonstrates that IFNγ and perforin can both induce apoptosis and cell death, and are both necessary for GA CD8$^+$ T cell-mediated suppression. Therefore, I wanted to determine how myelin-reactive CD4$^+$ T cell populations were affected by GA-reactive CD8$^+$ T cells *in vivo*. Bulk splenocytes from mice treated with GA- or OVAp-CD8$^+$ T cells were isolated 25 days after induction of disease and stained with CFSE. Proliferation of myelin-reactive CD4$^+$ T cells was assayed via CFSE dilution in response to the disease-inducing immunizing antigen (MOG_{35-55}) to determine if there was any change in reactivity of these putatively pathogenic cells. While cells from mice treated with OVAp CD8$^+$ T cells demonstrated high levels of specific proliferation to myelin-derived antigens, splenocytes from mice treated with GA CD8$^+$ T cells demonstrated greatly reduced antigen-specific CD4$^+$ T cell proliferation to neuroantigen (Figure 16). The reduced responsiveness of the pathogenic CD4$^+$ T cell populations suggests that there are either fewer pathogenic cells present in mice treated with GA CD8$^+$ T cells, or that these cells are less able to be activated by myelin antigens, and are therefore less pathogenic. In addition, there is less proliferation to mitogenic stimulus, by approximately the same degree (Figure 17). However, it is unclear by these data alone whether GA CD8$^+$ T cells are acting

directly on myelin-specific $CD4^+$ T cells, or mediate their effects via an intermediate cell type such as an APC. In addition, the earlier cell cytoxicity assay does not translate exactly to the *in vivo* situation, as host mice receiving GA $CD8^+$ T cells do not express GA. This means that if cell-mediated cytotoxicity is indeed a viable mechanism of GA $CD8^+$ T cells, some form of molecular mimicry may be present, in that the target myelin-reactive $CD4^+$ T cells are expressing an antigen closely resembling the GA sequence expressed on MHC class I molecules.

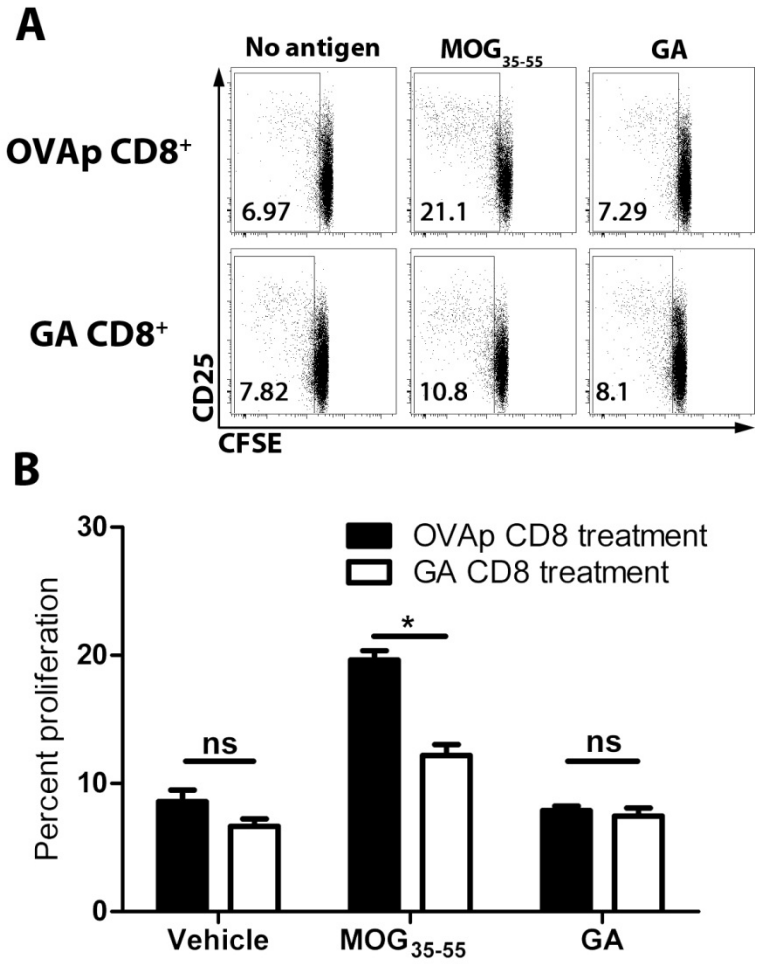

Figure 16 *Splenic CD4$^+$ T cells from GA CD8$^+$ T cell-treated mice are less reactive to neuroantigen*. Donor wild-type, IFNγ$^{-/-}$, perforin$^{-/-}$, and IL-10$^{-/-}$ mice on the C57BL/6 background were immunized with GA (1 mg) or OVAp (200 μg) in IFA (100 μl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension then incubated with cognate antigen (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 5 x 10^6 cells were injected intravenously into wild-type recipient mice 24 hours before disease induction with MOG_{35-55}. Twenty-five days after disease induction, spleens were isolated, processed to single cell suspension, stained with CFSE, and incubated at 1 x 10^6 cells/ml for five days with vehicle, MOG_{35-55} (20 μg/ml), or GA(20 μg/ml). Cell proliferation was analyzed by flow cytometry. (A) Flow plots. (B) Combined data. Percent proliferation represents percentage of $CFSE_{low}$ CD4$^+$ T cells. * denotes $p < 0.05$.

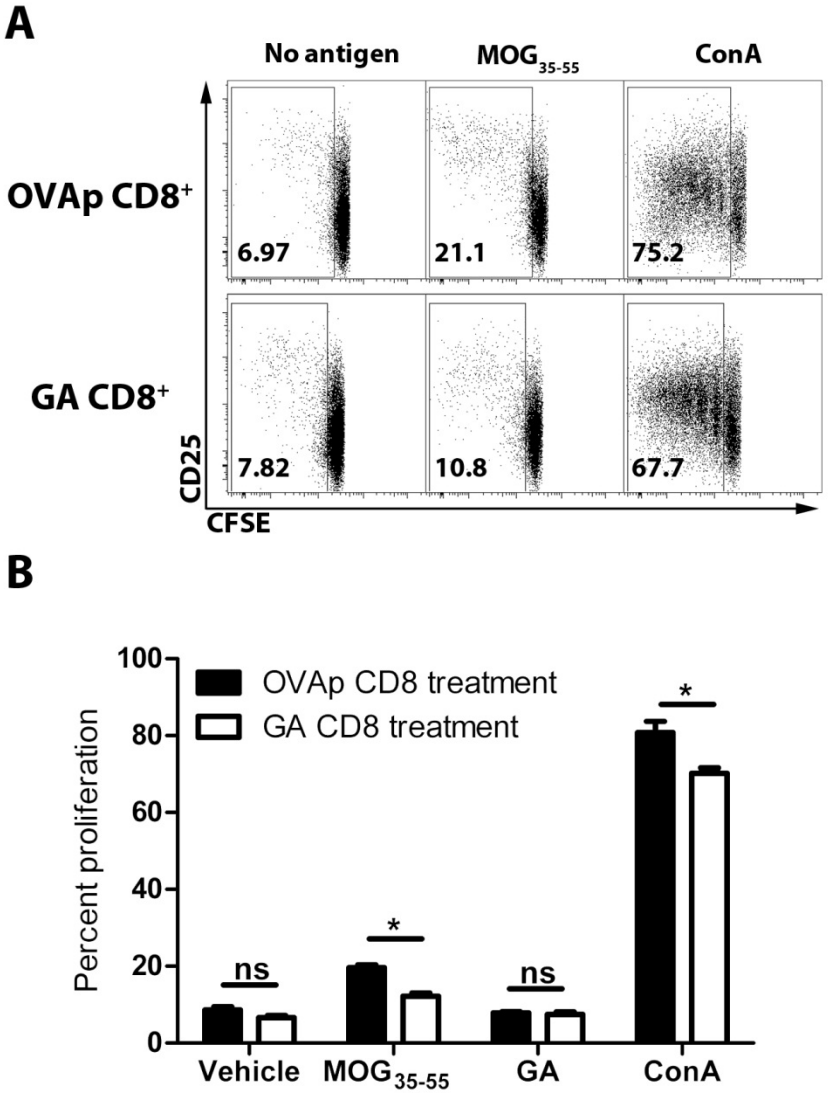

Figure 17 *Splenic CD4$^+$ T cells from GA CD8$^+$ T cell-treated mice are less reactive to neuroantigen and mitogen stimuli*. Donor wild-type C57BL/6 mice were immunized with GA (1 mg) or OVAp (200 μg) in IFA (100 μl/mouse) in the rear flanks. After twenty days, draining lymph nodes (inguinal, deep axillary, superficial axillary) and spleens were isolated and processed to single-cell suspension then incubated with cognate antigen (20 μg/ml) and IL-2 (10 pg/ml) for 72 hours. CD8$^+$ T cells were purified by magnetic bead-activated cell sorting and 5 x 10^6 cells were injected intravenously into wild-type recipient mice 24 hours before disease induction with MOG_{35-55}. Twenty-five days after disease induction, spleens were isolated, processed to single cell suspension, stained with CFSE, and incubated at 1 x 10^6 cells/ml for five days with vehicle, MOG_{35-55} (20 μg/ml), GA(20 μg/ml), or ConA (1 μg/ml). Cell proliferation

was analyzed by flow cytometry. (A) Flow plots. (B) Combined data. Percent proliferation represents percentage of $CFSE_{low}$ $CD4^+$ T cells. * denotes $p < 0.05$.

Effects on Antigen-Presenting Cells

A second mechanism by which GA may decrease symptoms of MS is through the induction of tolerogenic and anti-inflammatory antigen-presenting cells (APCs), including dendritic cells (DCs), B cells, microglia, and monocytes/macrophages. In DCs, GA diminishes expression of CD40, a vital costimulatory molecule involved in T cells activation, and can modulate cytokine secretion (111). GA can induce regulatory B cells to secrete more IL-4, IL-10, and IL-13 and less IL-6, IL-12, and TNFα while down-regulating expression of B cell-activating factor (BAFF) (112). Patients treated with GA have shown decreased microglial activation accompanying attenuated neuroinflammation (69). GA was shown to induce a "type 2", anti-inflammatory phenotype in monocytes both *in vivo* and *in vitro* (72). Furthermore, these monocytes induced Tregs that suppressed disease in a non-antigen-specific manner.

Clodronate Depletion Does Not Affect GA-Mediated Disease Suppression

While these data demonstrate that anti-inflammatory APCs are sufficient to reduce signs of neuroinflammation, they do not clarify whether these cell populations are necessary for therapeutic action. Although several APC populations are clearly affected during the course of GA therapy, I chose to focus on myeloid lineage cells due to the strong data concerning their role in GA therapy. Therefore, my initial experiment was to determine if macrophages were necessary for GA-mediated suppression of disease. While many cell types can be depleted by administration of cell type-specific antibodies, there is currently no commercially available antibody that specifically and selectively depletes macrophages. Currently, the best way to

deplete macrophages *in vivo* is by administration of liposomes loaded with clodronate. Clodronate is ineffective as a drug in the free aqueous form; however, when loaded in liposomes it is readily phagocytosed by macrophages *in vivo*. Upon phagocytosis, the liposomes are degraded and free clodronate is released into the cytoplasm, where it induces apoptosis and quickly depletes macrophages, though cells are quickly regenerated and return to baseline levels after approximately one week. Therefore, C57BL/6 mice were treated with clodronate- or PBS-loaded (control) liposomes beginning four days prior to disease induction and repeated every three days in order to maintain depletion of phagocytic cell populations. Disease was induced with a mixed emulsion of MOG_{35-55} peptide with or without GA (1 mg/ml) and CFA in order to minimize the number of injections. Although myeloid cell populations were clearly diminished, as demonstrated by flow cytometry of splenocytes from treated mice, clodronate-liposome treatment did not affect GA's ability to reduce signs of demyelinating disease (Figure 18). Therefore, myeloid cells such as macrophages, while sufficient to induce suppression of disease when treated with GA, are unnecessary for the mechanisms of GA and likely serve a redundant function with other antigen presenting cells.

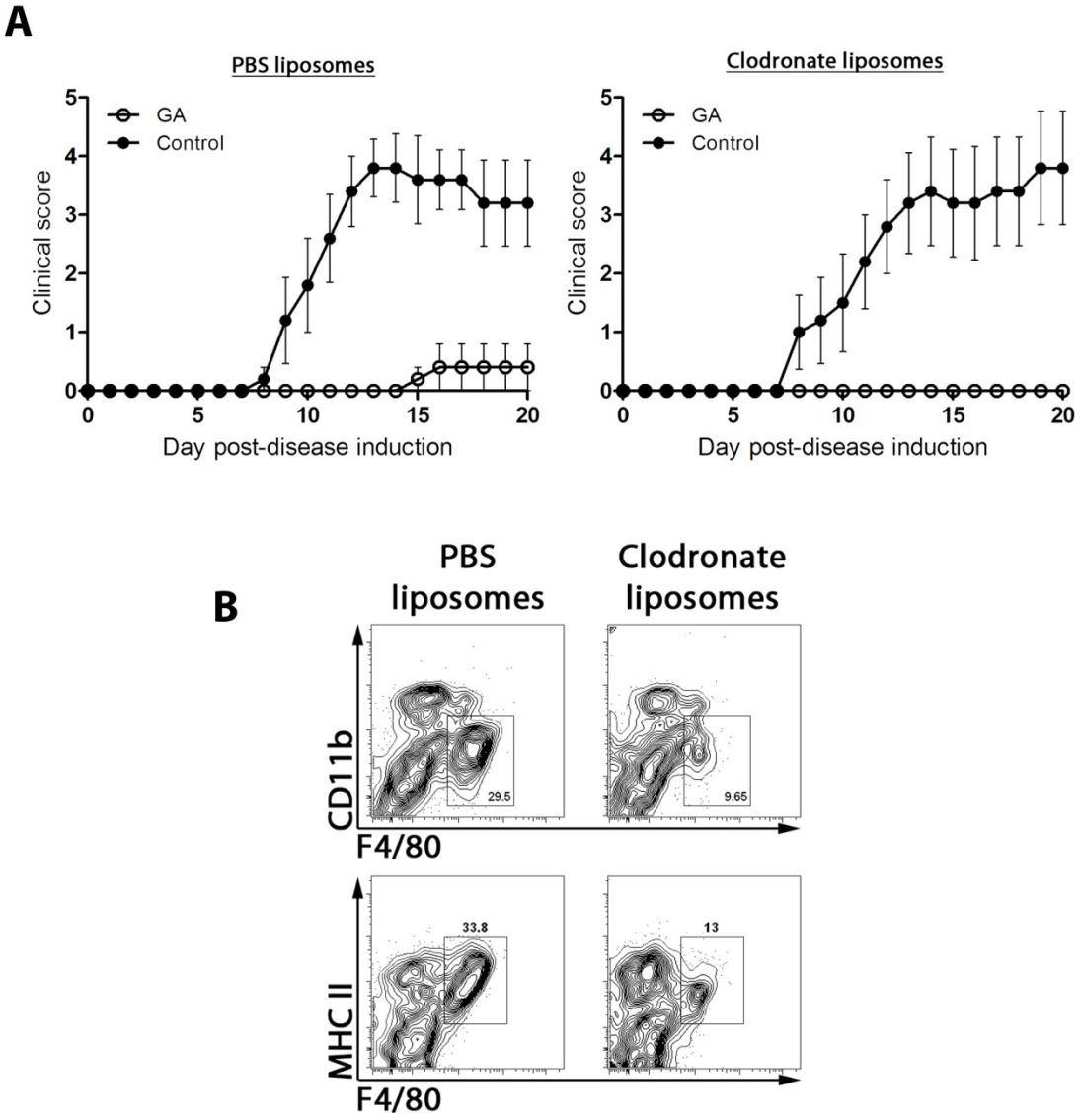

Figure 18 *Macrophage depletion does not affect GA-mediated amelioration of EAE*. (A) C57BL/6 mice were intraperitoneally injected with PBS- or clodronate-loaded liposomes, beginning on day -4 and repeated every 3 days. Disease was induced by subcutaneous injection of emulsion containing MOG_{35-55} (200 μg) and GA (1 mg) or PBS in CFA, followed by intraperitoneal injection of pertussis toxin on day 0 and 2. (B) Splenocytes were isolated from mice in (A) on day 15 and stained with MHCII ($I\text{-}A^b$) -Alexa Fluor 700, F4/80-Alexa Fluor 647, and CD11b-PE then analyzed by flow cytometry.

GA Induces Type 2 Monocytes in Wild-Type and CD8-Deficient Mice

While macrophages do not appear to be necessary for the mechanism of GA, it remains possible that the pertinent myeloid cell type is non-phagocytic, but secretes anti-inflammatory cytokines that induce suppression of disease. While this seems unlikely to be the true mechanism of GA given data demonstrating that IL-10 is unnecessary for disease suppression (61), I ventured to determine whether this "type II" phenotype was pertinent in a mouse model in which GA was ineffective; namely, one deficient in $CD8^+$ T cells. To dissect the respective roles of monocytes and $CD8^+$ T cells and investigate the interactions between these cell types, wild-type and $CD8^{-/-}$ mice were treated daily with subcutaneous injections of GA (150 μg/mouse/day). After 6 days, splenocytes were isolated and immature myeloid populations ($CD11c^-CD11b^+$) were isolated by magnetic bead cell-sorting. These cells were activated with IFN-γ to increase cytokine expression then assayed by ELISA for production of cytokines demonstrating a shift to the type II phenotype (IL-12, IL-10, and TNFα). In parallel with previous findings from other groups, myeloid cells from wild-type C57BL/6 mice treated with GA secrete less IL-12 and TNFα and more IL-10 than cells from control-treated mice, demonstrating that GA treatment can induce the anti-inflammatory, type II phenotype. Cells from $CD8^{-/-}$ mice treated with GA demonstrated an identical cytokine profile (Figure 19). However, our data have repeatedly demonstrated that GA does not reduce neuroinflammation in mice deficient of $CD8^+$ T cells. Therefore, the shift toward the anti-inflammatory phenotype in splenic myeloid cells is unnecessary for disease modulation, and therefore insufficient to explain the mechanism of the drug. Instead, it is likely an epiphenomenon that comes about in response to the central action of the drug that induces

disease suppression, such as cell-mediated cytoxicity via perforin expression or induction of differential cytokine profile in response to IFNγ secretion. Regardless, the idea that anti-inflammatory cytokines mediates the disease suppression seen in GA treatment is too simplistic of an explanation.

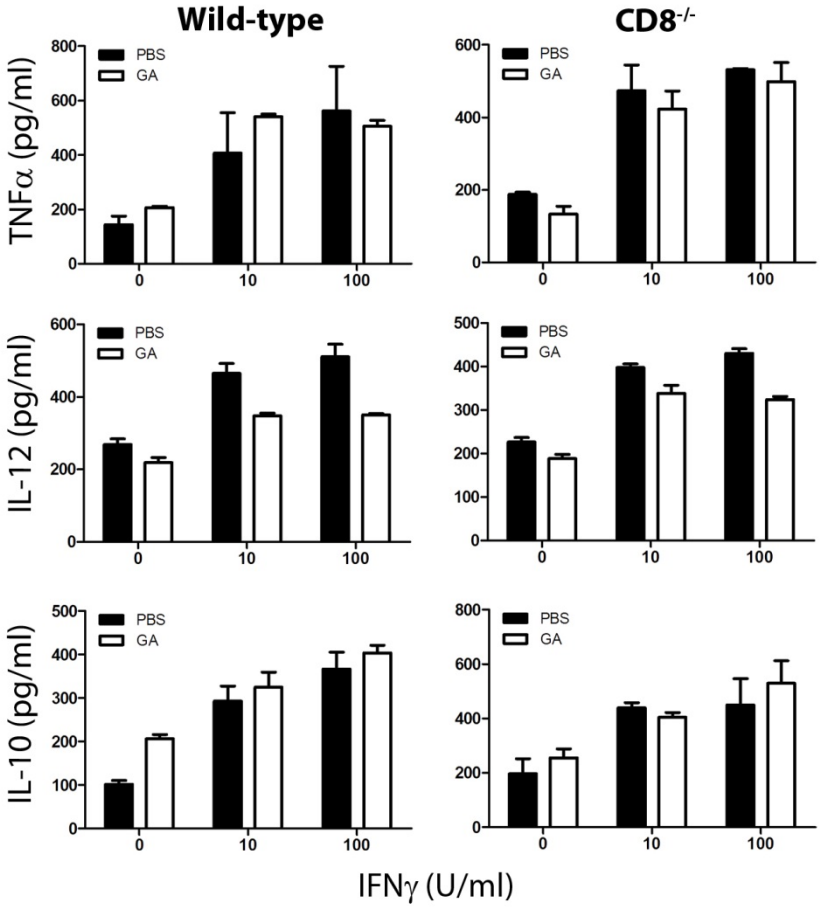

Figure 19 *Splenic monocytes from wild-type and CD8$^{-/-}$ mice acquire an anti-inflammatory phenotype after GA treatment in vivo*. Wild-type and CD8$^{-/-}$ mice were subcutaneously injected with GA (150 μg) or PBS daily for 6 days. Splenocytes were isolated and cultured *in vitro* with IFNγ (0, 10, or 100 U/ml). Supernatant cytokines were analyzed by ELISA after 48 (TNFα), 72 (IL-12), and 120 (IL-10) hrs.

Splenic Myeloid Cells from GA-Treated Mice Do Not Mature Efficiently In Vitro

However, the effects of GA on myeloid cell populations were not limited to cytokine secretion. GA treatment also has effects on the ability of myeloid cells to mature into more differentiated cells types such as TipDCs, regulatory myeloid cells, and inflammatory macrophages (71). As before, mice were treated for 6 days with GA (150 μg/mouse/day), splenocytes were isolated, and immature myeloid populations ($CD11b^{+}CD11c^{-}$) were purified via magnetic bead cell-sorting. When these cells were stained *ex vivo* with a panel of myeloid differentiation markers including CD11b, CD11c, MHC class II, and Ly-6C, there was no noticeable difference in surface marker expression (Figure 20). This suggests that, at least on the surface, GA does not appear to activate myeloid populations or skew them toward a certain phenotype. These cells were then subjected to a 48 hour *in vitro* culture in the presence of increasing levels of IFNγ to activate the cells. After culture, cells were again assayed by flow cytometry for myeloid markers. Cells from control- (PBS-) treated mice demonstrated a strong increase in CD11c, MHC class II, and Ly-6C expression, reflecting maturation to an inflammatory macrophage, as would be expected after IFNγ treatment. However, cells from mice treated with GA *in vivo* did not express CD11c, and showed a diminished ability to upregulate surface expression of MHC class II and Ly-6C when compared to cells from control mice, although increased IFNγ concentrations reversed the retarded expression of MHCII and Ly-6C (Figure 21). In addition, these immature myeloid cells were also assayed for intracellular cytokine expression to determine the extent to which these cells could induce inflammation. $CD11b^{+}CD11c^{-}$ cells were incubated *in vitro* for 4 hours with LPS (10 ng/ml) and monensin. After culture, cells were stained for IL-12, IL-10, and

TNFα. More cells from control-treated mice expressed IL-12, IL-10, and TNFα than did cells from GA-treated mice. These data mean that GA treatment *in vivo* has affects which decrease the ability of myeloid cells to convert into an inflammatory cell type. This would reduce the localization of inflammatory myeloid cells to the CNS and inhibit the pathogenicity of these same cells, which play an essential role in the pathogenesis of experimental autoimmune demyelination.

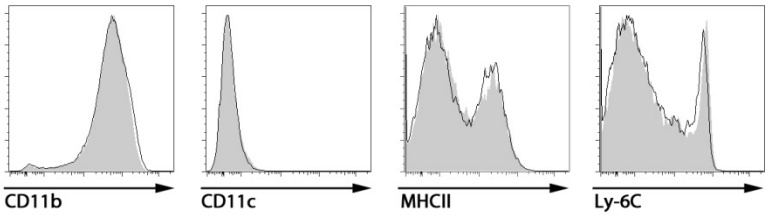

Figure 20 *Splenic myeloid cells do not show differential expression of cell surface markers*. Wild-type mice were subcutaneously injected with GA (150 μg) or PBS daily for 6 days. Splenocytes were isolated and $CD11b^{+}CD11c^{-}$ cells were purified by magnetic bead cell sorting. Cells were stained with CD11b-PE, CD11c-Pacific Blue, I-A-Alexa Fluor 700 (MHCII), and Ly-6C-APC-Cy7 then analyzed by flow cytometry. GA-treated cells are shown as grey, control cells are shown as a solid black line.

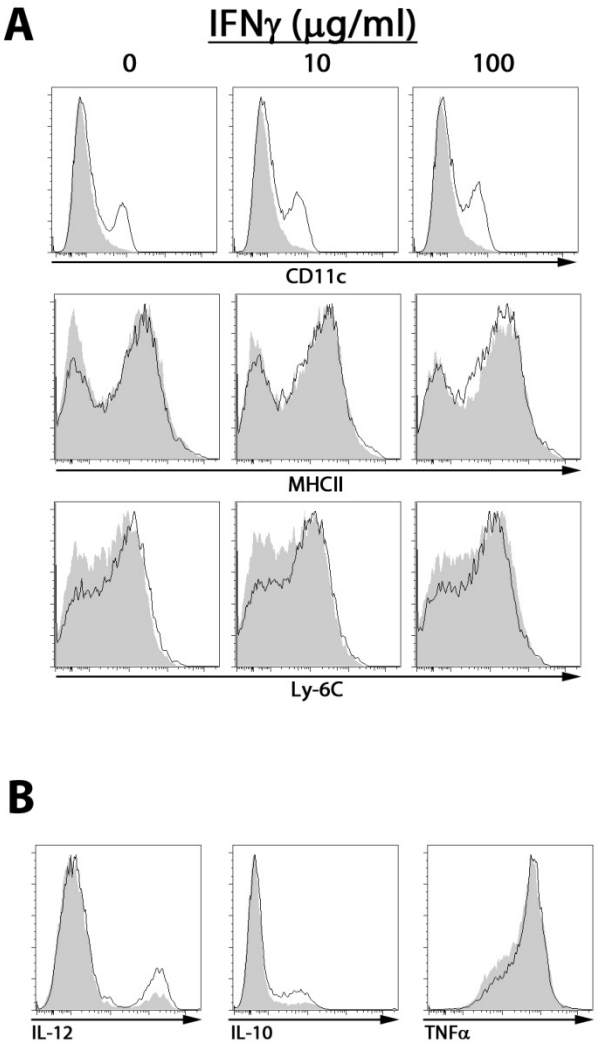

Figure 21 *Splenic myeloid cells do not show differential expression of cell surface markers*. Wild-type mice were subcutaneously injected with GA (150 μg) or PBS daily for 6 days. Splenocytes were isolated and $CD11b^{+}CD11c^{-}$ cells were purified by magnetic bead cell sorting then incubated *in vitro* for 48 hours with IFNγ (0, 10, or 100 U/ml). Cells were stained with CD11b-PE, CD11c-Pacific Blue, I-A-Alexa Fluor 700 (MHCII), and Ly-6C-APC-Cy7, then analyzed by flow cytometry (A), or stimulated with LPS (10 ng/ml) and monensin (2 μM) for 4 hours, then stained with IL-12-PE, IL-10-APC, and TNFα-PE-Cy7 and analyzed (B). GA-treated cells are shown as grey, control cells are shown as a solid black line.

However, while this phenotype is readily appreciated in cells from mice treated with GA *in vivo*, it was uncertain whether GA exerts its effects directly on myeloid cells, activated cell-intrinsic factors that reduce the ability of cells to mature, or if the drug requires the presence of other cell types to induce its effects. To test this, I used myeloid cells derived from murine bone marrow. Bone marrow was isolated from C57BL/6 mice and purified by density gradient centrifugation to remove red blood cells, dead cells, and polluting cells types such as fibroblasts. The remaining cells were treated *in vitro* for 6 days in culture media containing M-CSF (10 ng/ml) and IFNγ (100 U/ml) with or without GA (50 μg/ml). After culture, cells were stained with the same panel of myeloid markers used for the splenic myeloid cells. GA-treated cells showed no difference in cell surface marker expression from control-treated cells. In addition, cells were activated with LPS (10 ng/ml) to determine if stimulation would increase differences in cell surface marker expression between the two populations. However, even with exogenous stimulation, GA- and control-treated cells demonstrated identical expression of myeloid markers (Figure 22). Therefore, the inhibition of maturation of myeloid cells types is specific to *in vivo* treatment. This may suggest that other cells types, such as $CD8^+$ T cells, are required for the induction of the cellular programs responsible for the decrease in cell maturation. This could be due to changes in the cellular microenvironment through the release of various cytokines, or by the presence of apoptotic bodies created by a GA-specific cell-mediated cytotoxicity. However, there is also the possibility that GA is only effective on a specific cell type that is present *in vivo* but not in bone-marrow derived macrophage cultures. Either way, the fact remains that the

change in maturation ability is likely an epiphenomenon to the pertinent mechanism that reduces signs of demyelinating disease.

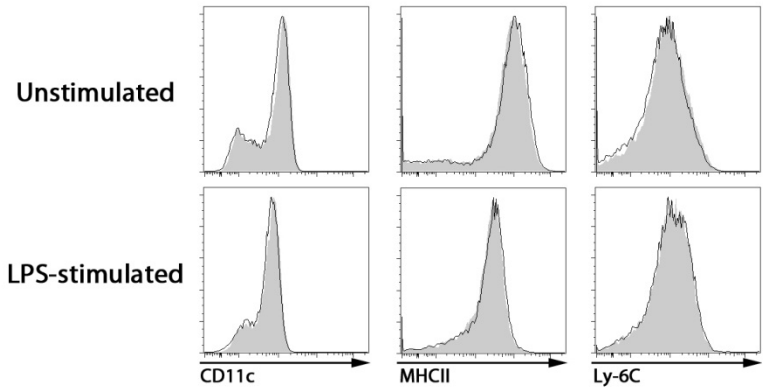

Figure 22 *Bone marrow macrophages' cell surface marker expression is not affected by GA*. Wild-type bone marrow cells were cultured with M-CSF (10 ng/ml), IFNγ (100 U/ml). After 6 days macrophages were isolated and incubated for 24 hours with GA (50 μg/ml) or vehicle (PBS) with or without LPS (100 ng/ml). Cells were stained with CD11b-PE, CD11c-Pacific Blue, I-A-Alexa Fluor 700 (MHCII), and Ly-6C-APC-Cy7 and analyzed by flow cytometry. GA-treated cells are shown as grey, control cells are shown as a solid black line.

GA-Treated Macrophages Require CD8$^+$ T Cells and MHC Class I to Induce Disease Suppression

One of the landmark discoveries in GA biology was the finding that bone-marrow derived macrophages treated with GA *in vitro* could adoptively transfer disease suppression (72). This phenotype was attributed to the fact that these cells could induce CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells (Tregs) that suppressed disease in an antigen-nonspecific manner (i.e. naïve CD4$^+$ cells cultured with GA-treated macrophages and any antigen including ovalbumin, GA, and MOG, converted to Tregs and suppressed MOG_{35-55}-induced demyelinating disease). The authors attributed disease suppression to the idea that GA-treated, "type II" macrophages were scavenging endogenous neuroantigen and inducing myelin-specific Tregs. They also demonstrated that the effect was not due to the transfer of GA into recipient mice via antigen phagocytosed by macrophages, as GA-treated cells were unable to induce GA-specific CD4$^+$ T cell proliferation outside of the presence of exogenous GA.

However, the data completely neglect the role of CD8$^+$ T cells in inducing disease suppression. Therefore, to test whether the adoptive transfer of suppressive macrophages was dependent on their ability to interact with CD8$^+$ T cells, GA-treated and control macrophages were transferred intravenously into wild-type and CD8$^{-/-}$ mice at the peak of acute disease (approximately 12 days after disease induction with MOG_{35-55}). While GA-treated macrophages suppressed disease in wild-type mice as previously demonstrated, these cells were completely ineffective in reducing disease in mice deficient of CD8$^+$ T cells (Figure 23). Additionally, when GA-treated and

control macrophages deficient in MHC class I were transferred into wild-type recipient mice, they also did not have any therapeutic effect. This means that GA-treated macrophages require MHC class I expression and the presence of $CD8^+$ T cells *in vivo* in order to have any beneficial effect. Therefore, GA macrophages likely take up GA and express epitopes via surface expression of MHC class I. These molecules in turn activate suppression GA-reactive $CD8^+$ T cells that are then responsible for the observed downstream effects such as reduced neuroantigen-specific $CD4^+$ T cell proliferation, the shift toward an anti-inflammatory phenotype in myeloid cells, and disease suppression.

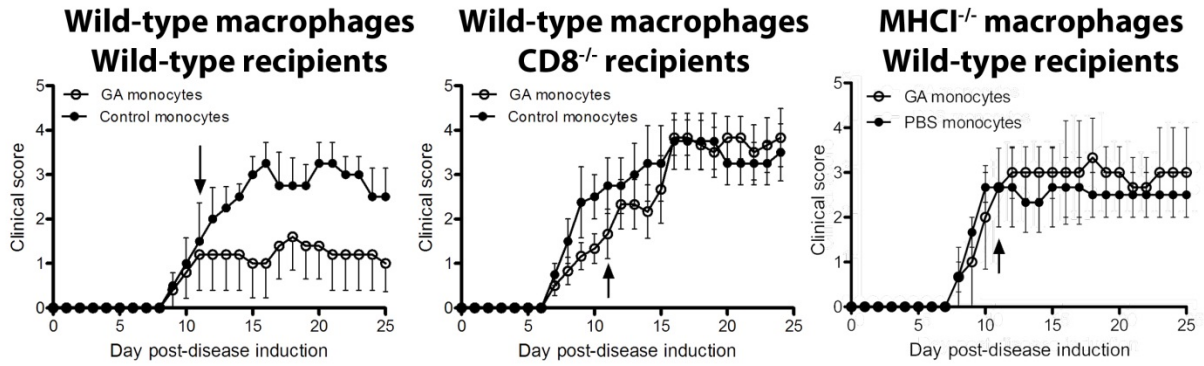

Figure 23 *$CD8^+$ T cells are required for the action of GA-induced suppressive macrophages*. Wild-type and $MHCI^{-/-}$ bone-marrow cells were cultured with M-CSF (10 ng/ml), IFNγ (100 U/ml), and GA (50 μg/ml) or vehicle (PBS). After 6 days macrophages (1 x 10^6) were injected into wild-type and $CD8^{-/-}$ mice at peak disease. Wild-type recipient mice receiving $MHC^{-/-}$ cells were depleted of NK cells by administration of anti-NK1.1 antibody (250 μg).

Bone Marrow Macrophages Induce Antigen-Specific $CD8^+$ But Not $CD4^+$ T Cell Responses After Antigen Pretreatment

In order to be able to induce a GA-specific $CD8^+$ T cell response, it is necessary that macrophages are able to transfer antigen to recipient mice via phagocytosis and intracellular processing. While the authors of the original report demonstrated that this did not occur with respect to GA-specific $CD4^+$ T cells, they did not test whether these cells could activate GA-specific $CD8^+$ T cells. In order to test whether antigen-treated macrophages could induce the proliferation of antigen-specific $CD8^+$ T cells, bone marrow-derived macrophages grown in M-CSF and IFNγ were exposed to hen egg ovalbumin (50 μg/ml) or vehicle (PBS) for six days. After culture, cells were thoroughly washed with PBS to remove any antigen still remaining within the culture medium. OVA-treated and control macrophages were then incubated *in vitro* with CFSE-stained OTII and OTI cells specific for epitopes within ovalbumin. After five days, cells were assayed for CFSE dilution by flow cytometry. While $CD4^+$ OTII cells did not proliferate to either OVA-treated or control macrophages, OTI cells proliferated strongly to OVA-treated macrophages (Figure 24). This suggests that while bone marrow-derived macrophages cannot induce activation of antigen-specific $CD4^+$ T cells without the addition of exogenous antigen, they can activate $CD8^+$ T cells.

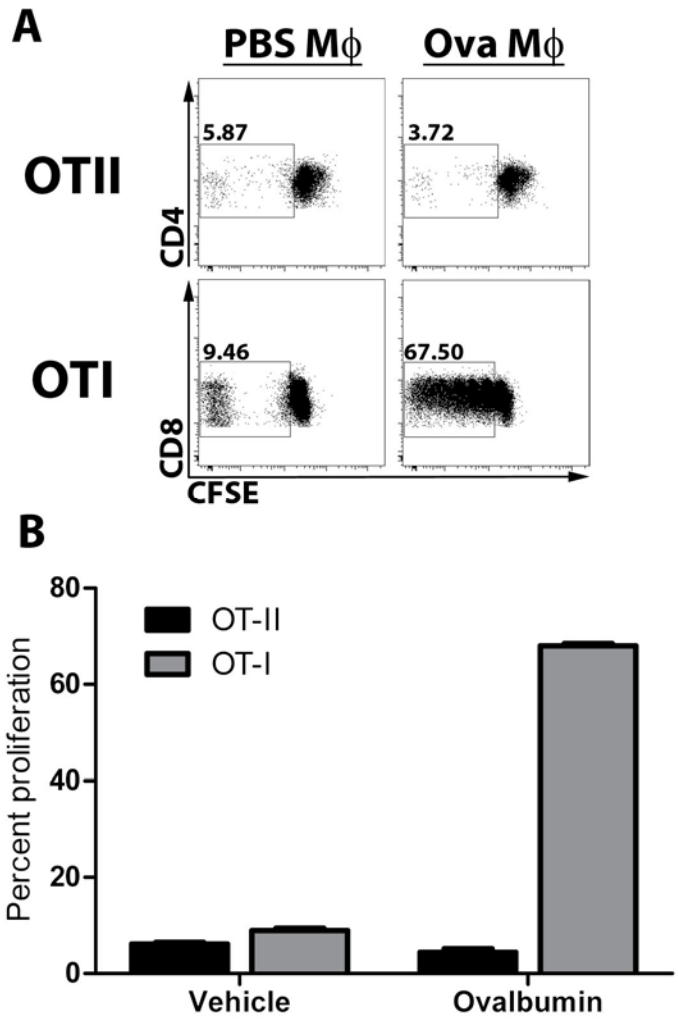

Figure 24 *Bone marrow macrophages induce proliferation in OVA-specific CD8$^+$ T cells*. (A) Bone marrow cells were cultured *in vitro* for 6 days with M-CSF, IFNγ, and hen egg ovalbumin (50 μg/ml) or vehicle (PBS). Cells were then combined *in vitro* with CFSE-stained OTI or OTII cells in a 1:4 ratio (T cells : APCs) for 5 days. (A) Flow plot. (B) Combined data.

While the fact that antigen-treated macrophages can activate ovalbumin-specific $CD8^+$ T cells without additional antigen is interesting, it is not relevant to demyelinating disease. Therefore, it was necessary to test whether these cells could also induce GA-specific $CD8^+$ T cells, which could in turn induce disease suppression. Macrophages were treated *in vitro* for six days with GA (50 μg/ml), OVA (50 μg/ml), or vehicle in the presence of M-CSF and IFNγ. After culture, these cells were incubated with either OTI cells specific for ovalbumin or GA-specific $CD8^+$ T cells derived from mice immunized with GA/IFA and stained with CFSE. While both cell types proliferated strongly to vehicle-treated mice in the presence of exogenous antigen, as expected, they proliferated even more strongly to cells they had been pre-treated *in vitro* with their respective antigen (OVA or GA) (Figure 25). This demonstrates that unlike $CD4^+$ T cells, $CD8^+$ T cells can be activated by macrophages treated *in vitro* with GA, and suggests that GA-treated macrophages can induce GA-specific $CD8^+$ T cell responses *in vivo* that are then responsible for the effects of GA treatment.

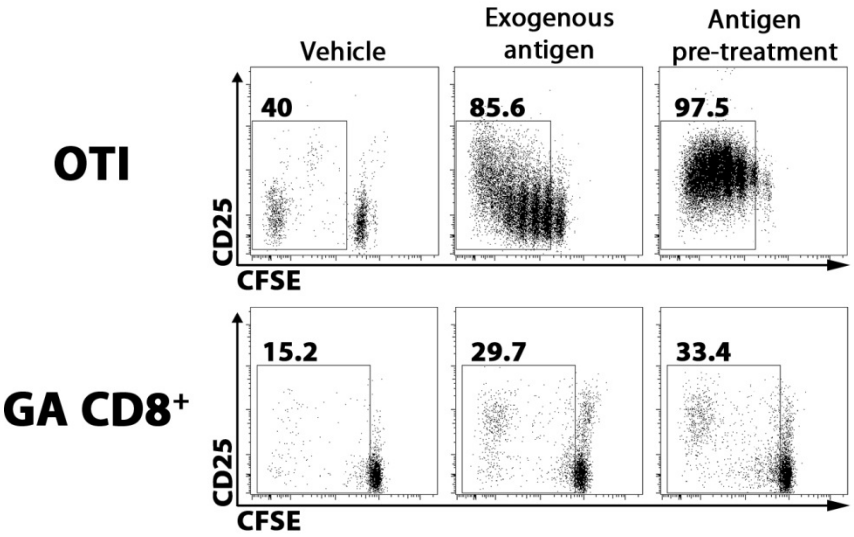

Figure 25 *Suppressive bone-marrow macrophages induce proliferation in GA-specific CD8$^+$ T cells*. Bone marrow cells were cultured *in vitro* for 6 days with M-CSF, IFNγ, and GA (50 μg/ml), hen egg ovalbumin (50 μg/ml), or vehicle (PBS). Cells were then combined *in vitro* with CFSE-stained CD8$^+$ OTI cells or CD8$^+$ T cells derived from GA/IFA immunized mice 10 days post-immunization in a 1:4 ratio (T cells : APCs) for 5 days. (B) Wild-type mice were immunized with GA/IFA (2 mg) for 20 days. Splenocytes were isolated and CD8$^+$ T cell were purified by magnetic bead sorting and stained with CFSE. OTI and GA CD8$^+$ T cells were cultured *in vitro* with monocytes at a 1:4 ratio (1 x 10^6 total cells/ml) for 5 days with vehicle (PBS) or additional cognate antigen (ovalbumin or GA, 20 μg/ml).

GA Does Not Affect IDO Expression in Bone Marrow Macrophages

It is still possible that GA may have some direct effects on macrophages that cause differential responses to antigen, i.e. anti-inflammatory instead of pro-inflammatory. One factor that causes just such a switch is indoleamine-2,3-dioxygenase, or IDO. IDO is an enzyme responsible for the rate-limiting step in tryptophan catabolism (113). By reducing the levels of tryptophan within the microenviroment, antigen-presenting cells can reduce the activation and proliferation of pathogenic $CD4^+$ T cells responding to autoantigens. In addition, several of the metabolites produced downstream of IDO in tryptophan catabolism have immunomodulatory effects of their own, such as inducing anti-inflammatory cytokines and regulatory T cells. IDO is strongly upregulated by IFNγ, and is therefore expressed as a direct result of Th1-type inflammatory reactions. Our group has demonstrated that GA-treatment induces IDO expression in the draining lymph nodes of treated mice. We have also shown that IDO expression is necessary for GA-mediated suppression of demyelinating disease. Finally, while GA-specific $CD4^+$ T cells from $IDO^{-/-}$ mice show no difference in proliferation when compared to wild-type cells, proliferation of GA-specific $CD8^+$ T cells is greatly reduced. Therefore, I wanted to test whether GA induced IDO expression in bone marrow-derived macrophages, which in turn led to the difference in suppressive ability. As before, bone marrow-derived cells were treated *in vitro* with GA or vehicle for six days in the presence of M-CSF and IFNγ. After six days, cells were lysed and mRNA was isolated followed by rtPCR. Levels of IDO transcript in vehicle and GA-treated cells were identical, suggesting that GA does not induce a phenotype in bone marrow-derived macrophages that would induce regulatory behavior in T cells (Figure 26). Therefore,

while IDO is required for the induction of regulatory $CD8^+$ T cells, GA does not increase IDO expression *in vitro*, supporting the earlier findings concerning the effects of *in vitro* and *in vivo* GA treatment on the cell surface marker expression of myeloid cells and suggests that many of the effects of GA require several cell types to be present.

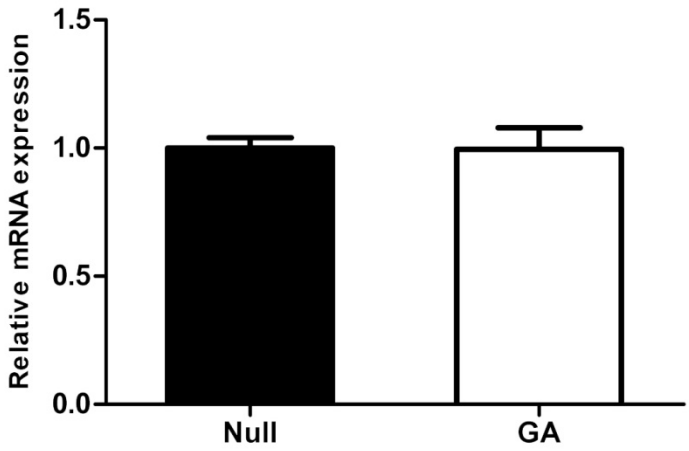

Figure 26 *GA does not affect IDO expression in bone marrow macrophages*. Wild-type bone marrow cells were cultured with M-CSF (10 ng/ml), IFNγ (100 U/ml), and GA (50 μg/ml) or vehicle (PBS). After 6 days, mRNA was extracted from cells followed by reverse transcription using Superscript II reverse transcription kit (Qiagen). Quantitative real-time PCR assays were performed using Brilliant SYBR Green QPCR Master Mix on a MX3000p thermocycler.

Induction of $CD4^+$ Regulatory T Cells

Another of the main mechanisms by which GA exerts its anti-inflammatory effects is through the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs). Tregs were initially described by Sakaguchi and colleagues in the mid-1990s; they showed that while $CD25^-CD4^+$ T cells induce autoimmunity in athymic nude mice, replenishing the $CD25^+$ fraction prevents autoimmunity (114). It was later found that the transcription factor Forkhead box 3 (Foxp3) was necessary and sufficient for the induction of murine $CD4^+$ regulatory T cells (115). GA itself induces the expression of Foxp3 in patients with initially low levels, while the adoptive transfer of GA-specific Tregs suppresses demyelinating disease in antigen-specific and non-specific manners (60).

GA-Treated Macrophages Do Not Induce $CD4^+$ Tregs Without $CD8^+$ T Cells

GA-treated macrophages are thought to induce disease suppression through the induction of Tregs, which in turn release anti-inflammatory cytokines such as IL-10 and TGFβ that reduce CNS inflammation. However, while GA macrophages require $CD8^+$ T cells to suppress demyelinating disease, it is unknown whether these cells are also required for the induction of Tregs. To test this, murine bone marrow was treated with GA (50 μg/ml) or vehicle in the presence of M-CSF and IFNγ. After six days, cells were transferred into wild-type and $CD8^{-/-}$ recipient mice at the peak of disease induced with MOG_{35-55}. Twenty-five days after disease induction (13 days after adoptive transfer), spleens were isolated from mice and stained for Tregs. While wild-type mice treated with GA macrophages demonstrate an increase in the

number of splenic Tregs compared with those treated with control macrophages, CD8-deficient recipients showed no such increase (Figure 27A). In addition, baseline levels of Tregs are lower in CD8-deficient mice after the induction of disease, although there is no difference in Treg levels in naïve wild-type and $CD8^{-/-}$ mice. These data suggest that $CD8^+$ T cells are required for the induction of peripheral Tregs in response to GA treatment as well as neuroinflammation.

GA $CD8^+$ T Cells Induce $CD4^+$ Tregs In Vivo

While $CD8^+$ T cells are necessary for the induction of Tregs in response to GA macrophage treatment, it remained unknown whether they were sufficient to induce Tregs. Therefore, wild-type mice were treated with GA-specific $CD8^+$ T cells one day prior to disease induction. Twenty five days after disease induction, spleens were isolated and stained for Tregs. Mice that received GA $CD8^+$ T cells demonstrated an increase in splenic Tregs compared to control $CD8^+$ T cells specific for OVAp (Figure 27B). Combined with the macrophage data, this shows that GA $CD8^+$ T cells are both necessary and sufficient to induce Tregs *in vivo*. However, the mechanism by which this occurs remains uncertain. It is possible that the IFNγ expression by GA $CD8^+$ T cells induces factors such as IDO that are able to induce Tregs *in vivo*. GA $CD8^+$ T cells could also kill pathogenic $CD4^+$ T cells, not only indirectly increasing the percentages of Tregs but also creating apoptotic bodies that are phagocytosed by APCs and induce Tregs.

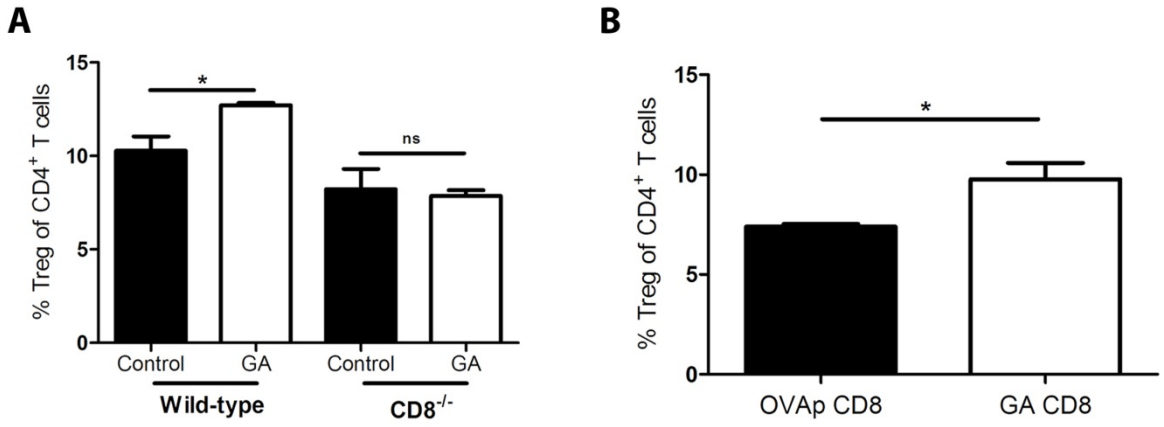

Figure 27 *GA CD8$^+$ T cells are necessary and sufficient to induce CD4$^+$ Tregs after GA macrophage treatment*. Splenocytes from mice in Figure 7 (A) or Figure 4A (B) were isolated 25 days after disease induction and stained for CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells using Foxp3 staining kit (eBioscience). * signifies $p < 0.05$. "ns" signifies not statistically significant.

DETERMINING THE ACTIVE MOIETY OF GA

Isolating MHC Class I Epitopes Induced by GA

Based on our earlier work, it was evident that GA-induced antigen-specific $CD8^+$ T cells proliferate via MHC class I (specifically non-classical MHC I) engagement. This suggested one of three possibilities: (a) GA is degraded in serum, and activated epitopes are then bound with cell-surface MHC I, (b) GA is taken up by APCs, processed in the cytoplasm, then loaded onto MHC I and presented on the cell surface, or (c) GA binds to a heretofore unknown factor that activates a cellular response which in turn loads a specific register of MHC I-binding peptides onto non-classical MHC class I. The first two possibilities suggest a peptide based on the GA backbone, and would therefore contain only alanine, lysine, glutamic acid, and tyrosine residues. The third possibilities would suggest an endogenous peptide, likely one already associated with suppressive behavior. However, even a peptide derived from GA prohibits a recombinant library approach – the number of possible 10-mer peptides derived from four amino acids is 4^{10}, or 1,048,576 possible sequences. Therefore, the proper approach is to capture MHCI molecules expressing the desired antigen via solid-state antibody chemistry, isolate peptide fragments, then characterize and sequence these peptides with LC/MS-MS (Figure 28). This approach has been used successfully in determining protein-derived epitopes from both MHC I and MHC II molecules. LC/MS-MS works by first separating peptides by liquid chromatography, or LC. LC operates by running the dissolved peptides over a column that contains either a hydrophilic (normal phase) or a hydrophobic (reverse phase) residue. As different peptides will contain

different proportions of aliphatic side chains, charges, and number of amino acid residues, they will migrate through the column at different rates. After moving through the column, the peptides are exposed to the first mass spectrometer, which analyzes the whole mass of the peptide and allows for secondary discrimination of the peptides after LC. These peptides are then "broken" by electronically shattering the peptide bonds between amino acids, producing smaller fragments. By analyzing the fragmentation data derived from the whole peptide mass, one can determine the sequence of a particular peptide and correlate it with a particular peak on the chromatograph. By comparing these results with a database, this allows for the protein sources of the peptides to be determined.

However, there are a number of difficulties in using this approach that apply specific to GA. The primary difficulty with immunoprecipitation is finding the requisite number of cells. Most protocols involving immunoprecipitation require at least 10^9 cells. As such, the most common approach is to express the antigen of choice in a common cell line intracellularly by encoding the protein of choice in a gene construct. This allows the endogenous protein processing machinery to operate efficiently and allows for the use of a non-APC cell type. As GA is a random polypeptide, this approach is impossible, as the antigen cannot be encoded in a construct. A second approach is to "shock" the cells with a hyperosmotic solution containing the antigen of choice followed by a hypotonic treatment, in effect perforating the cellular membrane and allowing the entrance of exogenous protein. However, this approach may unintentionally bypass specific cellular mechanisms that are required for GA processing. Regardless, I first attempted

GA epitope sequencing by osmotically shocking EL4 cells, a thymoma cell line, with GA (116). Due to difficulties with maintaining viability in these cells, I abandoned this approach.

Instead, I chose to expose APCs to GA *in vitro*, allowing them to uptake and process the antigen, followed by lysis and immunoprecipitation. Initially, I considered bone-marrow derived macrophages, as I had earlier shown these to be able to support GA-specific proliferation of $CD8^+$ T cells. However, a high enough yield of these cells would require a highly labor-intensive procedure to derive bone marrow in addition to the expense incurred by growing up these cells *in vitro*. However, as T cell-depleted splenocytes had served as a viable APCs in proliferation assays, I chose to expand the APC population in the spleens of donor mice. This type of epitope determination experiment had been undertaken previously using large numbers (~100) mouse spleens to derive the requisite number of cells. To again avoid this type of labor, I chose to inject mice subcutaneously with the B16-Flt3L cell line, a melanoma cell line the constitutively expresses Flt3 ligand, a dendritic cell growth factor (117). This led to the explosive growth of splenic DCs expressing MHC class II, CD11c, DEC205, and CD86, which were isolated and culture *in vitro* with GA or vehicle. Cells were lysed and MHCI/epitope complexes were purified from the lysate by M1/42 antibody (specific for $\beta_2$microglobulin) covalently attached to sepharose beads. After washing the column, MHCI complexes (classical and non-classical) were eluted from the column. Peptides were separated from the larger protein complexes by boiling the eluent then running the mixture over a size-barrier cellulose column. The peptide fraction was concentrated by lyophilization then resuspended in water and run on an

LC/MS-MS. The run produced unique LC spectrographs for both control and GA-treated cells (Figure 29). In addition, the GA-treated lysate contained several peptide epitopes that were not present in the control lysate (Figures 30 and 31). These included several ribosomal proteins as well as epitopes of heat shock protein of 60 kDa (HSP60) (Figure 32). HSP60 has been associated with Qa-1 dependent $CD8^+$ T cell-mediated immune regulation, and was therefore of great interest (108). However, the epitope most commonly associated with immunoregulation (GMKFDRGYI) was not found in the mass spectrograph. Instead, several epitopes from the C-terminal aspect of the protein, which possesses unknown functionality, were found. Based on the association of HSP60 with Qa-1 activated $CD8^+$ T cells, I decided to test one of the HSP60 epitopes for disease suppression and activation of GA-specific $CD8^+$ T cells.

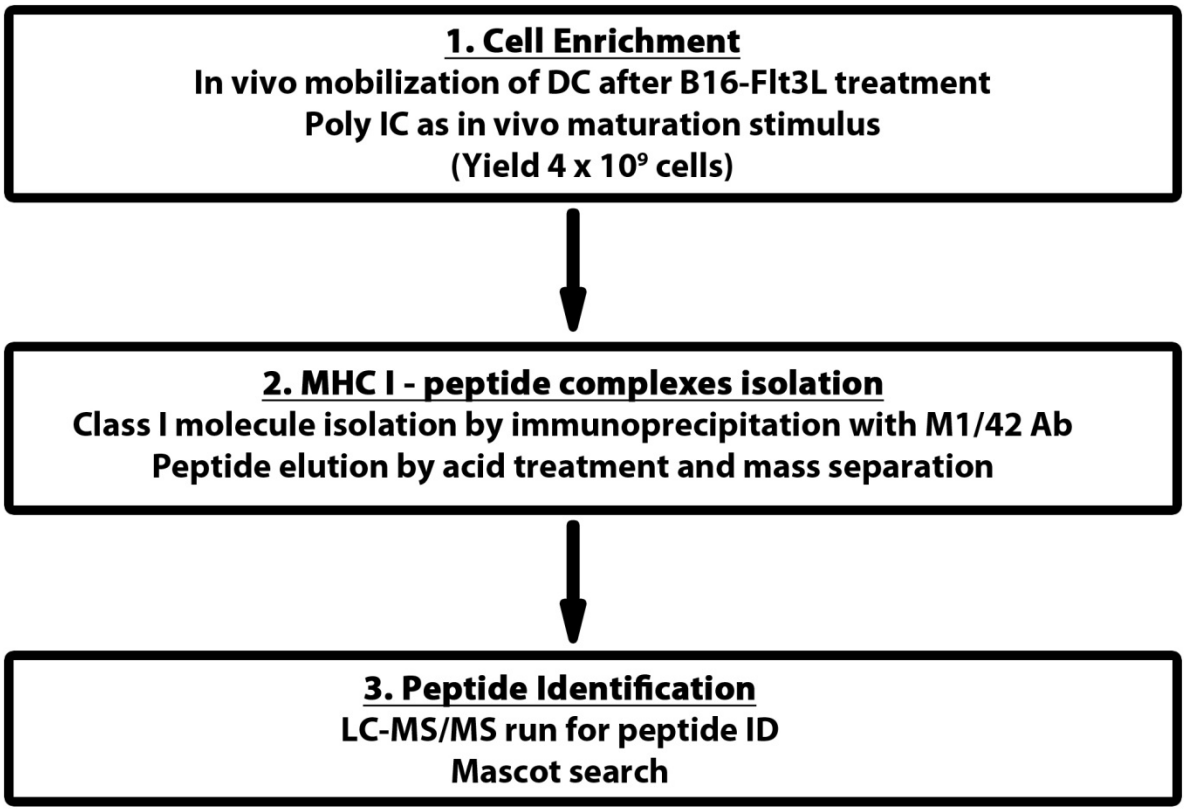

Figure 28 *GA epitope search protocol.*

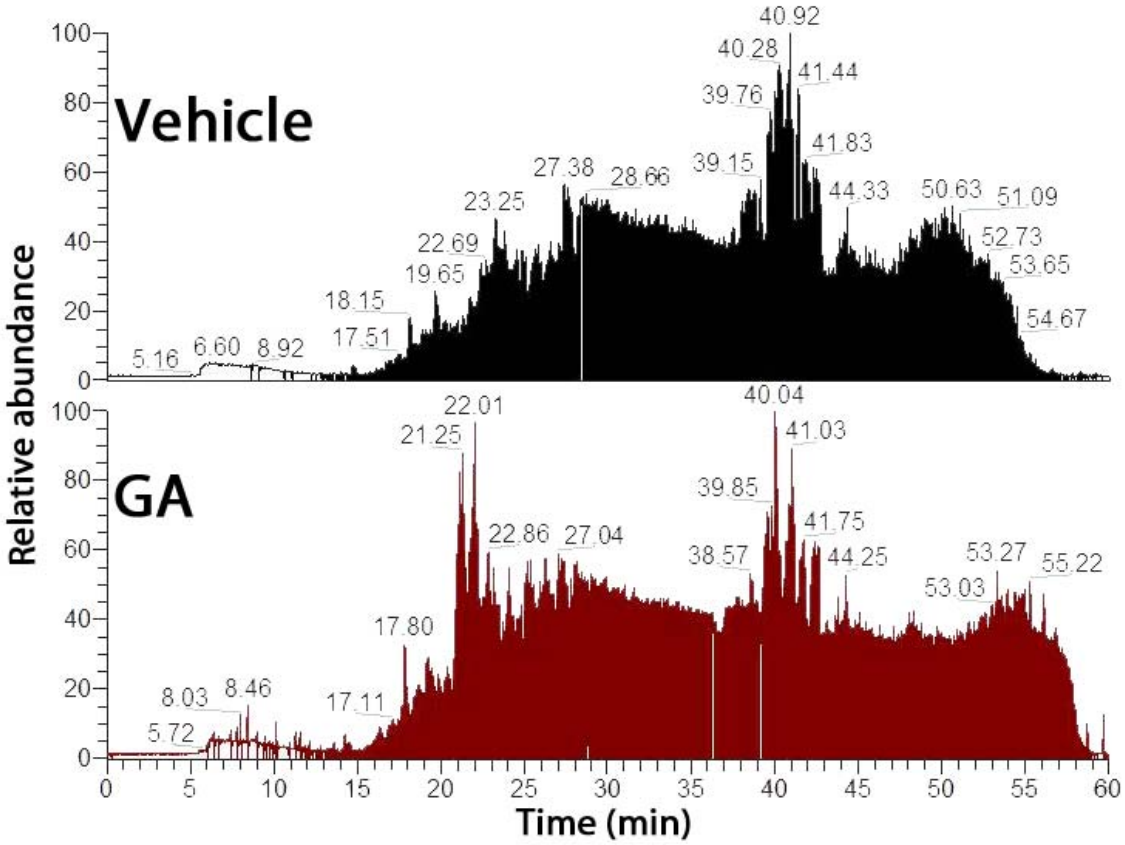

Figure 29 *Comparison of LC/MS-MS spectra of GA- and vehicle-treated Flt3L-induced DCs*. Wild-type C57BL/6 mice were subcutaneously injected with 2 x 10^6 B16-Flt3L cells. Sixteen days post-injection, mice were intraperitoneally injected with poly I:C (50 μg). Five hours later, spleens were treated with collagenase D to isolate DCs. Cells (10 x 10^6 per ml) were treated with PBS or GA (20 μg/ml) for 5 hours, then pelleted and lysed. Lysate was cleared, MHC class I molecules were immunoaffinity purified with MHC I-specific antibody (clone M1/42) bound to CNBr-activated sepharose. The affinity column was washed and MHC I molecules were eluted at by addition of 10% acetic acid. MHC I peptides were separated from the denatured protein subunits of the MHC molecules and the contaminating antibody by ultrafiltration through a 10 kDa cutoff membrane filter. The filters were washed three times with 2 mL water to remove contaminants interfering with the mass spectrometry. Recovered peptide mixtures (5–6 mL) were concentrated and desalted. The eluted MHC peptides were reduced to near dryness and then reconstituted at 20 μl 0.1% TFA/water. Half of the peptide mixture, corresponding to approximately 3.5–4 × 10^8 cell equivalents, was injected for LC–MS/MS analysis.

gi|74207178	unnamed protein product [Mus musculus]
gi|6680836	**calreticulin precursor [Mus musculus]**
gi|122441	RecName: Full=Hemoglobin subunit alpha; AltName: Full=Hemoglobin alpha chain; AltName: Full=Alpha-globin
gi|12845853	unnamed protein product [Mus musculus]
gi|12846963	**unnamed protein product [Mus musculus]**
gi|156257677	**beta-globin [Mus musculus]**
gi|136580	**RecName: Full=Thymosin beta-4; Short=T beta 4; Contains: RecName: Full=Hematopoietic system regulatory peptide; AltName: Full=Seraspenide**
gi|1200246	**MRP8 protein [Mus musculus]**
gi|4506631	**ribosomal protein L30 [Homo sapiens]**
gi|74220435	unnamed protein product [Mus musculus]
gi|94377377	**PREDICTED: similar to ribosomal protein L38 [Mus musculus]**
gi|1399919	TAP2
gi|1401252	**mlrq-like protein [Mus musculus]**
gi|55451	**Y box-binbing protein [Mus musculus]**
gi|124378005	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 4 [Mus musculus]
gi|82801284	PREDICTED: similar to heterogeneous nuclear ribonucleoprotein A3, isoform 12 [Mus musculus]
gi|149259652	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [Mus musculus]
gi|56541102	**Ribosomal protein L9 [Mus musculus]**

Figure 30 *MASCOT search of vehicle-treated sample*. Proteins in bold were found in both vehicle- and GA-treated samples.

gi|12846963	**unnamed protein product [Mus musculus]**
gi|49868	put. beta-actin (aa 27-375) [Mus musculus]
gi|156257677	**beta-globin [Mus musculus]**
gi|136580	**RecName: Full=Thymosin beta-4; Short=T beta 4; Contains: RecName: Full=Hematopoietic system regulatory peptide; AltName: Full=Seraspenide**
gi|1200246	**MRP8 protein [Mus musculus]**
gi|6680836	**calreticulin precursor [Mus musculus]**
gi|71664	calmodulin - salmon
gi|12860603	unnamed protein product [Mus musculus]
gi|94377377	**PREDICTED: similar to ribosomal protein L38 [Mus musculus]**
gi|51829956	**PREDICTED: similar to ribosomal protein L30 [Mus musculus]**
gi|30519911	transgelin 2 [Mus musculus]
gi|55451	**Y box-binbing protein [Mus musculus]**
gi|4506643	ribosomal protein L37a [Homo sapiens]
gi|1167510	TI-225 [Mus musculus]
gi|38074163	PREDICTED: similar to ribosomal protein L10a [Mus musculus]
gi|51452	unnamed protein product [Mus musculus]
gi|71059757	Sgpl1 [Mus musculus]
gi|255940140	Pc16g04890 [Penicillium chrysogenum Wisconsin 54-1255]
gi|55491	unnamed protein product [Mus sp.]
gi|1401252	**mlrq-like protein [Mus musculus]**
gi|56541102	**Ribosomal protein L9 [Mus musculus]**
gi|53103	unnamed protein product [Mus musculus]
gi|4506685	ribosomal protein S13 [Homo sapiens]
gi|4506621	ribosomal protein L26 [Homo sapiens]
gi|13385044	ribosomal protein L35 [Mus musculus]
gi|82885362	PREDICTED: hypothetical protein [Mus musculus]
gi|82801284	PREDICTED: similar to heterogeneous nuclear ribonucleoprotein A3, isoform 12 [Mus musculus]

Figure 31 *MASCOT search of GA-treated sample*. Proteins in bold were found in both vehicle- and GA-treated samples. HSP60 is listed in red as "unnamed protein product".

APHLTRAYAKDVKFGADARALMLQGVDLLADAVAVTMGPKGRTVIIE
QSWGSPKVTKDGVTVAKSIDLKDKYKNIGAKLVQDVANNTNEEAGD
GTTTATVLARSIAKEGFEKISKGANPVEIRRGVMLAVDAVIAELKKQS
KPVTTPEEIAQVATISANGDKDIGNIISDAMKKVGRKGVITVKDGKTLN
DELEIIEGMKFDRGYISPYFINTSKGQKCEFQDAYVLLSEKKFSSVQSI
VPALEIANAHRKPLVIIAEDVDGEALSTLVLNRLKVGLQVVAVKAPGF
GDNRKNQLKDMAIATGGAVFGEEGLNLNLEDVQAHDLGKVGEVIVT
KDDAMLLKGKGDKAHIEKRIQEITEQLDITTSEYEKEKLNERLAKLSD
GVAVLKVGGTSDVEVNEKKDRVTDALNATRAAVEEGIVLGGGCALL
RCIPALDSLKPANEDQKIGIEIIKRALKIPAMTIAKNAGVEGSLIVEKILQ
SSSEVGYDAMLGDFVNMVEKGIIDPTKVVRTALLDAAGVASLLTTAE
AVVTEIPKEEKDPGMGAMGGMGGGMGGGMF

Figure 32 *HSP60 protein sequence*. Peptide associated with Qa-1-restricted $CD8^+$ regulatory T cells in listed in green. Peptides derived from immunoprecipitation are listed in red and yellow; red is epitope used in experiments.

HSP60

HSP60 Epitopes Reduce Signs of EAE

First, I tested whether the HSP60-derived peptide could suppress demyelinating disease. Emulsions of GA, OVAp, or HSP60 in IFA were injected subcutaneously into wild-type mice ten days prior to disease induction. The HSP60 epitope suppressed disease as well as GA compared to controls (Figure 33). This reflects reports of similar sequences derived from mycobacterial HSP60 that can also suppress inflammation. However, the mechanism of action appears to vary; the mycobacterial antigen was given intranasally, thus inducing tolerance to the antigen, while the murine HSP60 was given in a manner to induce an active immune response, possibly to induce a suppressive $CD8^+$ T cell response.

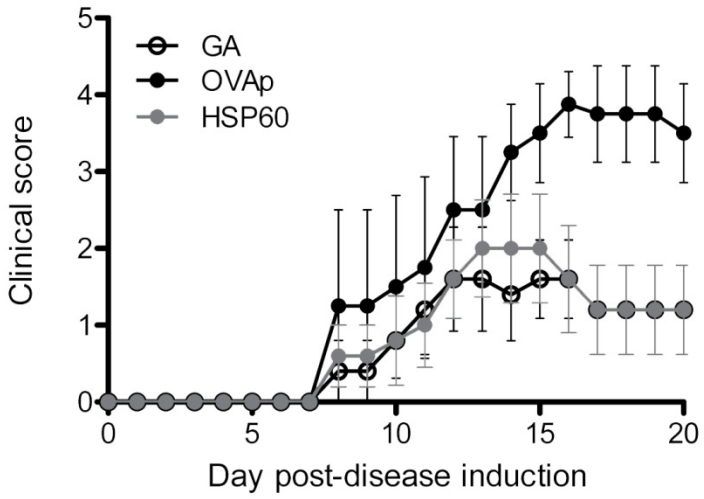

Figure 33 *HSP60 epitopes derived from MHC class I immunoprecipitation of GA-treated DCs can suppress EAE*. GA (1 mg), HSP60 (200 μg), or OVAp (200 μg) were emulsified in IFA and injected into wild-type C57BL/6 mice. Ten days post-immunization disease was induced with MOG_{35-55}.

HSP60 Does Not Induce GA-Specific Proliferation

As the HSP60 epitope reflected GA in its ability to suppress EAE, I next wanted to determine whether GA-specific $CD8^+$ T cells could be activated by the peptide. If this were the case, it could suggest that C-terminal aspects of HSP60 act as molecular mimics of GA in activating $CD8^+$ T cell responses. Therefore, GA-specific $CD8^+$ T cells were isolated from GA/IFA-immunized mice, stained with CFSE, and cultured with T cell-depleted splenocytes as APCs to determine responses toward vehicle, GA, OVAp, HSP60, and ConA. After five days, antigen-specific proliferation was analyzed by flow cytometry. While GA was able to induce proliferative responses, HSP60 responses were comparable to controls (Figure 34). Thus, while an endogenous protein that appears to be completely unrelated to GA is able to suppress disease, it does not appear to mimic GA in terms of antigen-specific responses, though it may be utilizing a similar mechanism. In addition, it is unclear why HSP60 appeared in the LC/MS-MS spectra of GA-treated DCs. HSP60 is released from the mitochondria and presented in MHC class I in response to cellular stress. Therefore, it is possible that GA acts as a stressor within these cells, and may reflect an epiphenomenon of GA treatment unassociated with its effects on GA.

While the approach appears to work, it clearly requires further fine-tuning, particularly during culture, to maximize yields. In addition, it would be prudent to immunoprecipitate only Qa-1, as opposed to all MHCI, as ensuing experiments have clearly demonstrated that classical MHCI molecules are unnecessary for the activation of GA $CD8^+$ T cells.

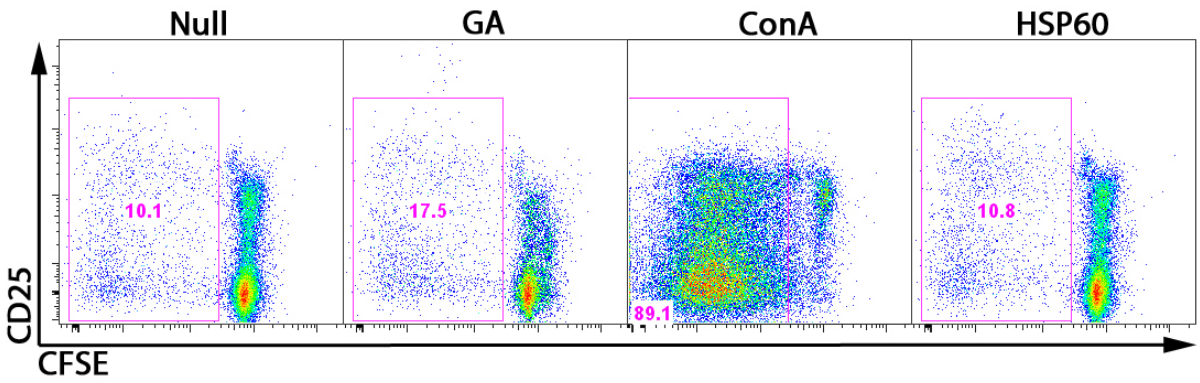

Figure 34 *HSP60 peptide epitopes do not induce proliferation of GA CD8$^+$ T cells*. Wild-type C57BL/6 mice were immunized with GA (1 mg) emulsified in IFA. Ten days post-immunization, CD8$^+$ T cells were isolated from draining lymph node and spleens by magnetic bead cell sorting and stained with CFSE. APCs were derived from T cell-depleted spleens of naïve wild-type mice. Cells were coincubated at a 1:4 ratio of CD8$^+$ T cells : APCs with vehicle (null, PBS), GA (20 μg/ml), ConA (1 μg/ml), or HSP60 peptide (20 μg/ml) for five days. Cells were analyzed by flow cytometry and gated on CD8$^+$ T cells.

TAP-Binding Sequences

Additionally, several reports have explored polyalanine sequences for MHC class I binding, and have found that several of these sequence are able to bind the transporter associated with protein binding (TAP), one of the steps in MHC class I surface expression of antigenic peptides (118). Therefore, in addition to exploring HSP60-derived peptides, two peptides were also chosen from these synthetic sequences. The first (AYAKAAAAY, "official") possesses a binding capacity for TAP similar to influenza derived peptides, thus suggesting that it can also be processed by a similar mechanism. The second (AKYKAEAAY, "theoretical") is based upon site-specific mutagenesis of backbone peptide. The residues in this peptide represent the strongest binding interactions found. In addition, both peptides possess only the amino acids alanine, lysine, glutamic acid, and tyrosine, as well as slightly positive charges, reflecting the molecular characteristics of GA.

Some Substituted Polyalanine Sequences Suppress EAE

As with the HSP60 peptide, the first experiment was to determine if these TAP-binding peptides could suppress EAE. Equimolar amounts of the two peptides as well as GA and OVAp were injected subcutaneously in a mixed emulsion with MOG_{35-55} followed by intraperitoneal pertussis toxin injections on day 0 and 2. While AYAKAAAAY ("official") suppressed disease, AKYKAEAAY ("theoretical") did not (Figure 35). This suggests that MHC class I-binding sequences resembling GA can suppress disease. In addition, while AYAKAAAAY has been empirically demonstrated to bind TAP, AKYKAEAAY is a purely theoretical peptide that has

not been tested for TAP binding. Therefore, it is possible that it is not processed in the same manner as the "official" peptide, and cannot act to induce suppressive behavior via $CD8^+$ T cells.

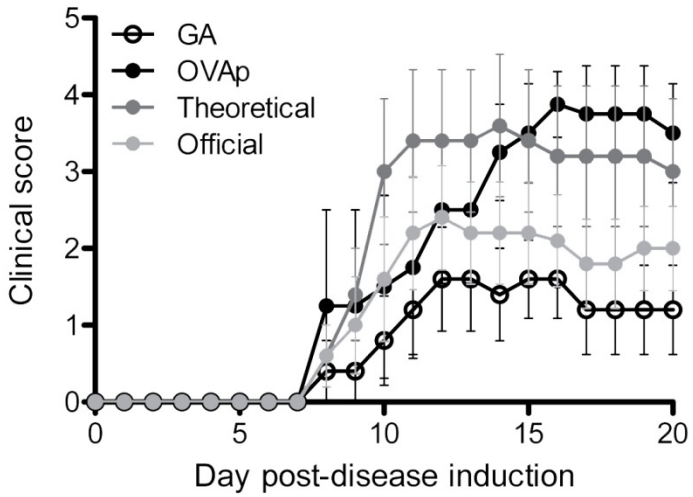

Figure 35 *HSP60 epitopes derived from MHC class I immunoprecipitation of GA-treated DCs can suppress EAE*. GA (1 mg, 650 nmol), AYAKAAAAY ("Official", 650 nmol), AKYKAEAAY ("Theoretical", 650 nmol), and OVAp (100 μg) were emulsified in CFA with MOG_{35-55} (100 μg) and injected into wild-type C57BL/6 mice followed by pertussis toxin injection on day 0 and 2.

Substituted Polyalanine Sequences Do Not Induce GA-Specific CD8$^+$ T Cell Responses

I next wanted to determine whether these epitopes could induce activation of GA-specific CD8$^+$ T cells, thereby representing the active moiety of the drug. To test the polyalanine sequences, bulk splenocytes were isolated from GA-immunized mice, stained with CFSE, and incubated *in vitro* with vehicle, GA, OVAp, and the two peptides. After 5 days, cells were analyzed by flow cytometry. While GA was able to induce a proliferative response in CD8$^+$ T cells, OVAp, AYAKAAAAY, and AKYKAEAAY did not (Figure 36). While this was not unexpected for OVAp and AKYKAEAAY, in the case of AYAKAAAAY, it suggests that epitopes exist which can suppress demyelinating disease but are unrelated to GA, and like the HSP60 peptides, may use a similar mechanism of action.

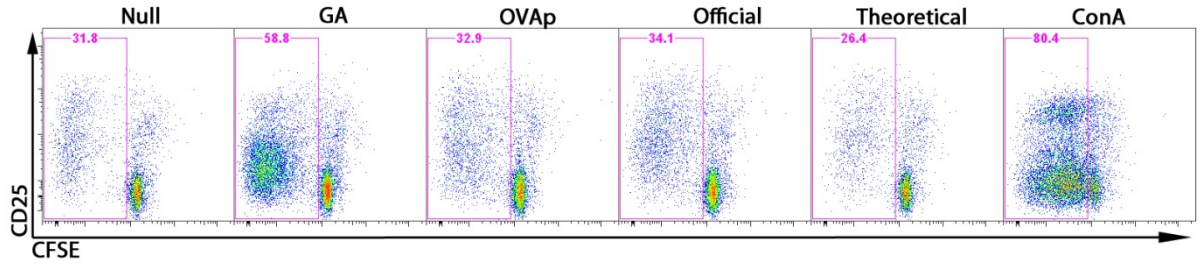

Figure 36 *Substituted polyalanine peptide epitopes do not induce proliferation of GA CD8$^+$ T cells*. Wild-type C57BL/6 mice were immunized with GA (1 mg) emulsified in IFA. Ten days post-immunization, CD8$^+$ T cells were isolated from draining lymph node and spleens by magnetic bead cell sorting and stained with CFSE. APCs were derived from T cell-depleted spleens of naïve wild-type mice. Cells were coincubated at a 1:4 ratio of CD8$^+$ T cells : APCs with vehicle (null, PBS), GA (20 μg/ml), "Official" peptide (20 μg/ml), "Theoretical" peptide (20 μg/ml), or ConA (1 μg/ml) for five days. Cells were analyzed by flow cytometry and gated on CD8$^+$ T cells.

CHAPTER FOUR

Discussion

MECHANISMS OF GLATIRAMER ACETATE

Effects of GA on $CD4^+$ T Cells and Antigen-Presenting Cells

Like other MS therapies, the precise mechanism of action of GA is unknown (119). GA can bind to various MHC II alleles and displace bound MBP epitopes from the MHC II complex as well as antagonize $MBP_{82\text{-}100}$ TCR activation (54). Drug treatment can reduce proliferation of myelin-specific T cell lines, shift T helper cells phenotypes from pathogenic Th1 to anti-inflammatory Th2, and induce regulatory $CD4^+CD25^+$ T cells by activation of the factor Foxp3 (60, 120, 121). GA induces regulatory B cells that secrete IL-10 and reduce neuroinflammation via bystander suppression (65). Myeloid lineages are also affected. Monocytes/macrophages and dendritic cells increase IL-10 secretion and decrease IL-12 and TNFα secretion in response to drug treatment (122). Differentiation to a more mature inflammatory state is also decreased.

Effects of GA on $CD8^+$ T Cells

However, the effects of GA on $CD8^+$ T cells have only recently come to light. $CD8^+$ T cells were generally regarded as pathogenic in MS and EAE due to their high numbers within the CNS plaques, outnumbering $CD4^+$ T cells 2:1, and the association of certain MHC I haplotypes with increased risk of disease. Additionally, some models of MS have demonstrated that $CD8^+$ T

cells can induce demyelination; however, these models included a viral encephalitis as well as several on genetic backgrounds not representative of human biology (transgenic expression of hen egg ovalbumin by oligodendrocytes, $RAG^{-/-}$) (79, 123, 124). Therefore, there are few studies demonstrating a truly pathogenic role for $CD8^+$ T cells in MS.

On the contrary, there is much stronger data suggesting that $CD8^+$ T cells are regulatory in nature in MS and EAE. $CD8^+Foxp3^+$ regulatory T cells have been found to reduce $CD4^+$ T cell activation in MS (125). Additionally, $CD8^+$ T cells restricted for the non-classical MHC class I molecule Qa-1 can inhibit disease through the suppression of $CD4^+$ T cells with affinity toward autoantigens such as PLP. They do so by sensing the signal strength of the TCR/MHC class II interaction of the responding $CD4^+$ T cell. Cells with high or low avidity express the Qdm peptide within Qa-1, to which the $CD8^+$ T cells do not respond. However, cells that are activated by intermediate avidity antigens, such as autoantigens, express HSP60 epitopes. The $CD8^+$ T cells recognize these cells and eliminate them by a variety of mechanisms.

GA has also been associated with increasing $CD8^+$ T cell regulatory capacity. Hohlfeld and colleagues compared immunological responses to GA in the PBMCs of healthy donors, untreated MS patients, and GA-treated MS patients (83). GA-treated patients showed a significant reduction in GA-induced proliferation, increased IL-4 secretion mediated by $CD4^+$ T cells, and elevated IFNγ responses mediated by $CD8^+$ T cells, each of which was specific to GA, as these effects were not observed with tuberculin-purified protein or tetanus toxoid. Our own group

expanded on these findings by utilizing a novel flow cytometric approach examining the GA-specific responses in the PBMCs of healthy control and MS patients as they initiate GA treatment (84). Healthy controls demonstrate robust $CD4^+$ and $CD8^+$ T cell responses to GA. While untreated MS patients mount equivalently strong $CD4^+$ T cell responses to GA, $CD8^+$ T cell responses are significantly lower. However, over the course of GA treatment, these $CD8^+$ T cell responses increased to healthy control levels, while $CD4^+$ T cell responses began to decline.

Mechanistically, this results in improved immune regulatory function. When increasing numbers of $CD8^+$ T cells were placed in culture with anti-CD3-stimulated CD8-depleted PBMCs from healthy controls, a dose-dependent increase in suppression of proliferation was observed (85). This regulatory function of $CD8^+$ T cells was impaired in untreated MS patients, however, after several months of therapy, suppressive ability significantly improved. This finding also appears to apply to GA-stimulated cells as well, suggesting that GA is able to correct a deficiency in MS patients to levels seen in healthy controls.

Interactions Between GA and $CD8^+$ T Cells in a Murine Model of MS

It was from this starting point that studies into the regulatory role of GA-induced $CD8^+$ T cells commenced using the murine model of EAE. Our initial data demonstrating that GA immunization induced both $CD4^+$ and $CD8^+$ T cell responses indicated that murine biology reflected our human observations in terms of GA reactivity. Therefore, it was likely that GA, discovered to ameliorate demyelinating disease first in animals, then in humans, has similar

effects and a similar mechanism of action. This was an important question to answer if we wanted to utilize the murine model to answer questions about human disease. Indeed, several drugs useful in murine EAE that were translated to MS (such as altered peptide ligands or anti-TNFα therapy) actually exacerbated disease and provoked stronger CNS inflammation. Thus, the analogous proliferation data signaled a commonality in GA treatment between mouse and human.

Based on our earlier data, which demonstrating a correlation between GA treatment and $CD8^+$ T cell reactivity in MS patients, we wanted to determine whether $CD8^+$ T cells were a necessary subtype of leukocyte for amelioration of disease. By comparing several treatment protocols in wild-type and $CD8^{-/-}$ mice, we observed that GA was ineffective in reducing signs of disease in the absence of $CD8^+$ T cells. This unequivocally demonstrates that these cells are necessary for GA's primary action in EAE. Additionally, our group also demonstrated that transfer of $CD8^+$ T cells from mice treated with GA into diseased mice also reduces symptoms of EAE, and is thus sufficient for inducing the regulatory aspects of GA therapy.

Present Studies

These findings serve as the foundation for this thesis. Within this work, I have aimed to explain the mechanism by which $CD8^+$ T cells are involved in the suppression of demyelinating disease in response to GA treatment. By analyzing the molecular mediators used by these cells, characterizing their interactions with other cell types affected by GA therapy, and finally, by

searching for the active moiety responsible for the induction of these effects, I have begun to explain GA's relationship to and reliance on $CD8^+$ T cells.

Molecular Requirements of GA $CD8^+$ T Cells

I first examined the role of molecules associated with $CD8^+$ T cells action, particularly those linked with the regulatory capacities of these cells. GA treatment of mice deficient in MHC class I, IFNγ, and perforin did not decrease signs of EAE. Therefore, these molecules are vital for the suppressive action of GA. I furthered tested each of these molecules within the context of GA $CD8^+$ T cell-mediated suppression, and each proved to be necessary for amelioration of disease. Moreover, the MHC class I-dependence of these cells was localized to non-classical variants of the molecule such as Qa-1, Qa-2, and CD1d for both activation and disease suppression. Disease suppression is effective in other models of EAE, such as the SJL/PLP model as well as passive disease. Thus, upon adoptive transfer into diseased hosts, GA $CD8^+$ T cells become activated by antigen-presenting cells (possibly including activated $CD4^+$ T cells) expressing non-classical MHC class I. Upon activation, these $CD8^+$ T cells express IFNγ and perforin, thereby inducing IDO, Foxp3, and other regulatory proteins, as well as lysing pathogenic target cells. As IL-10 is not required for GA or GA $CD8^+$ T cell action, bystander suppression in the form of activated regulatory cells types including, but not limited to, $CD8^+$ T cells is likely not a pathway by which disease is ameliorated. This mechanism is not restricted to the C57BL/6/MOG model of disease, as disease in SJL mice immunized with PLP peptides can be suppressed by GA $CD8^+$ T cells. In addition, the suppression of passive disease indicates that

GA $CD8^+$ T cells do not require the artificial inflammatory environment present in the immunization models, and can also act to prevent severe disease in the presence of autoimmune $CD4^+$ T cells alone, representing a therapeutic approach likely to be effective in MS.

Cellular Interactions of GA $CD8^+$ T Cells

I next investigated the cellular interactions between $CD8^+$ T cells and other cell types to determine how suppressive GA $CD8^+$ T cells are induced and on which cell types they act. $CD4^+$ T cells from diseased mice treated with GA $CD8^+$ T cells showed decreased proliferative ability to MOG_{35-55}, the immunizing antigen, as well as concanavalin A, a mitogenic stimulus, while no differences were observed in response to vehicle or GA stimulation. MOG-reactive $CD4^+$ T cells are pathogenic and cause disease upon adoptive transfer. By limiting the amount of proliferation, GA $CD8^+$ T cells reduce CNS inflammation. The decrease in mitogen-induced proliferation can also be associated with the removal of highly active cells from the $CD4^+$ T cell pool. In this situation, less easily activated cells would remain, which would be less likely to proliferate. The reduction in proliferation may be due to cell-mediated cytolysis, or it may also be due to the conversion of MOG-reactive cells to a regulatory phenotype, which can be anergic and unresponsive to both antigen-specific and mitogenic stimuli. $CD4^+CD25^+Foxp3^+$ T cells are induced by GA $CD8^+$ T cells, reflecting the loss of pathogenic cells prone to proliferation, as well as a gain in cells that secrete regulatory factors such as IL-10. It remains unclear whether regulatory T cells are necessary for disease suppression by GA; unfortunately, current techniques do not easily allow for this study. For example, while inducible Foxp3 knockout mice are

available, they quickly succumb to lethal lymphoproliferation shortly after the Foxp3 gene is removed. This greatly reduces the amount of time available to observe any effects on disease course such that any significant conclusions cannot be draw.

GA induces a type 2, anti-inflammatory phenotype in myeloid cell types such as monocytes and macrophages, leading to reduced EAE levels upon adoptive transfer. However, mice treated with clodronate-loaded liposomes still respond to GA, even though macrophage levels are clearly decreased. In addition, splenic myeloid cells express the type 2 phenotype after GA treatment in both wild-type and CD8-deficient mice, although GA is ineffective in the latter. Therefore, phagocytic myeloid cells are unnecessary for disease suppression, and while GA may induce regulatory phenotypes in these cells, including the inhibition of maturation, the secretion of anti-inflammatory cytokines is insufficient for inducing disease suppression. GA-treated macrophages are likely acting on a downstream effector cell type, such as $CD8^+$ T cells, responsible for the inhibition of demyelinating disease. GA-treated macrophages do not suppress disease if transferred to CD8-deficient hosts, while macrophages lacking MHC class I cannot induce disease inhibition after GA treatment. This suggests that GA-treated macrophages phagocytose, process, and express GA peptide on non-classical MHC class I, thereby activating GA-specific $CD8^+$ T cells that then institute further suppressive action. While these cells cannot activate $CD4^+$ T cells in this manner, $CD8^+$ T cells are strongly activated by macrophages preincubated with antigen. However, these macrophages can induce $CD4^+$ regulatory T cells in a non-antigen specific manner, which then suppress disease through bystander mechanisms.

However, GA-treated macrophages do not induce $CD4^+$ regulatory T cells *in vivo* without $CD8^+$ T cells, and GA $CD8^+$ T cells can induce $CD4^+$ T cells.

Mechanisms of *In Vivo* GA Treatment

Our current model of GA therapy begins with subcutaneous drug administration, where it is rapidly delivered to APCs in the draining lymph nodes (Figure 37). Here, GA is presented via non-classical MHC class I molecules to GA-specific $CD8^+$ T cells. Upon activation, these cells begin to secrete IFNγ, increasing IDO expression in APCs. IDO signaling, including the decrease in tryptophan and increase in kynurenines in the local microenvironment as well as intracellular signaling cascades, begin to modulate the behavior of GA $CD8^+$ T cells, and endow regulatory properties upon these cells. While the exact properties acquired by these cells are currently unknown, they likely include the upregulation of costimulatory molecules such as PD-1 associated with $CD8^+$ T cell-mediated immune regulation and cellular trafficking markers. The GA $CD8^+$ T cells then leave the lymph node and migrate to sites of immune activation, including the CNS and secondary lymph organs, where they come into contact with autoantigen specific $CD4^+$ T cells and inflammatory APCs. Here, GA $CD8^+$ T cells suppress $CD4^+$ proliferation by APC modulation as well as direct cytotoxicity. Th1-type helper cells are likely the main focus of this suppression, leading to a decreased ratio of Th1:Th2 helper cells. In addition, the modulation of APCs by GA $CD8^+$ T cells causes a decrease in cellular maturation, leading to lower levels of IL-12 and TNFα and increased secretion of IL-10 and TGFβ. The combination of these effects decreases CNS demyelination, leading to improvement in disease.

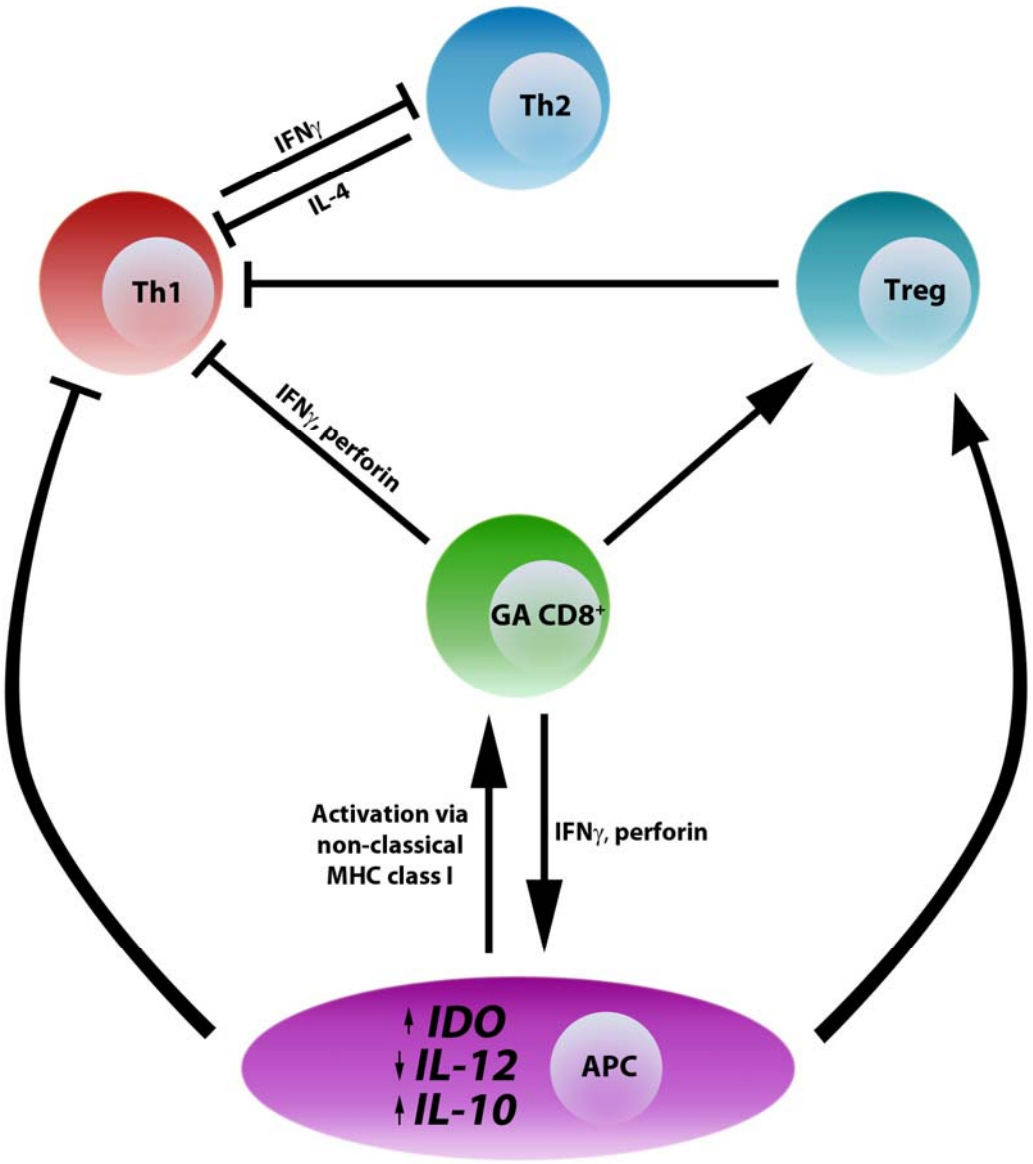

Figure 37 *Model of CD8$^+$ T Cell-Mediated Action of GA Therapy*

Similarities to ACAID

Anterior chamber-associated immune deviation (ACAID), first described by Kaplan and Streilein, is a unique form of immune regulation characterized by antigen-specific suppression of systemic Th1 immune responses (126). In this mechanism, antigen placed in the anterior chamber of the eye is taken up by $F4/80^+$ APCs, which then migrate via aqueous outflow tracts into the blood, eventually reaching the spleen (127, 128). Here, in conjunction with other cell types such as marginal zone B cells, γδ T cells, and NK T cells, these APCs induce the differentiation of $CD4^+$ afferent and $CD8^+$ efferent regulatory T cells (129) through the immunomodulatory effects of the anti-inflammatory cytokines (130). Like ACAID, GA treatment induces both $CD4^+$ and $CD8^+$ regulatory T cells, activates $CD8^+$ T cells via non-classical MHC class I molecules, and can rely on $F4/80^+$ APCs such as macrophages to induce disease suppression by promoting the development of anti-inflammatory phenotypes. However, while ACAID depends on IL-10 secretion by invariant NK T (iNKT) cells for immunoregulation, GA does not. Therefore, while GA and ACAID may share effector mechanisms, there are differences in the differentiation of the necessary cell types.

4-1BBL Treatment

Treatment with 4-1BBL or other 4-1BB-stimulating molecules has also been shown to induce regulatory $CD8^+$ T cell in autoimmune disease. Upon 4-1BB activation, $CD8^+$ T cells demonstrate increased proliferation, survival, cytokine production, and cytotoxic killing activity (131-133). This leads to eradication of tumors cells as well as autoimmune $CD4^+$ T cells in such

diseases as autoimmune demyelination and lupus through to production of IFNγ (134-136). Other molecules important for GA $CD8^+$ T cell-mediated suppression of disease, such as IDO, also have documented roles in 4-1BBL treatment (94). Further research is needed to determine if GA acts by this pathway.

Clinical Translation

Unlike many experimental therapies, GA $CD8^+$ T cell adoptive immunotherapy can easily and rapidly be adapted to human use. GA is already approved for human use, and our current findings closely reflect our own human studies in GA-treated MS patients. Autologous cell transfer of regulatory T cells has already been tested in several autoimmune disorders, including type 1 diabetes, as well as graft-versus-host disease (137). However, cellular manipulation and purification of the palliative subset remain obstacles to the widespread use of such therapies. Once these obstacles are overcome, GA $CD8^+$ T cell transfer should prove a safe and effective therapy.

Limitations of the Current Study

This thesis attempts to analyze the role of $CD8^+$ T cells in the mechanism of GA in the treatment of demyelinating disease. However, several aspects remain unclear. Although it is clear that $CD8^+$ T cells are activated in a non-classical MHC class I-dependent manner, the peptide epitope of either GA or the endogenous protein mimic responsible for stimulation of these cells remains unknown. The precise cellular target of suppression is also undetermined, though modulation

and inhibition of several cell types may be responsible for the therapeutic effects of GA $CD8^+$ T cells. Furthermore, the role of $CD4^+$ T regulatory cells in GA action as well as their interactions with regulatory $CD8^+$ T cells remain difficult to isolate, as mice deficient in these cells are prone to lethal lymphoproliferative disorders. The basis of the anti-inflammatory phenotype observed in several populations of APCs is also unclear. However, it remains possible that GA or GA $CD8^+$ T cells may have effects not directly associated with disease amelioration though immune-related. Finally, the exact subset of $CD8^+$ T cells responsible for disease suppression is undefined, but examination of proliferating cells should unveil associated cell surface markers and further phenotypic characterization.

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