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CD4 T Cell Affinities for Myelin in Clinically Relevant Models of Multiple Sclerosis

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Abstract

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By Anna Elizabeth Kersh

Multiple sclerosis is an autoimmune disease mediated primarily by myelin-specific CD4 T cells that is characterized by inflammation, demyelination and scarring in the central nervous system (CNS). As autoimmune disease has been suggested to persist and progress through the recognition of new or modified epitopes, we use the micropipette adhesion frequency assay to evaluate the frequency and two-dimensional (2D) affinity of myelin-reactive CD4 T cells in two clinically relevant mouse models of demyelinating disease: a relapsing-remitting turned secondary progressive model and a humanized mouse model.

We first explore the nonobese diabetic (NOD) mouse model in which myelin oligodendrocyte glycoprotein (MOG) immunization elicits a relapsing-remitting disease that evolves into secondary progressive phenotype, the disease course experienced by the vast majority of MS patients. Many believe that progression of relapsing-remitting disease occurs due to epitope spread, the development of reactivity to distinct CNS antigens due to the release of antigens during demyelination. In NOD mice, the vast majority of CNS-infiltrating CD4 T cells remains reactive to MOG throughout symptom onset, remission, relapse and chronic progression which limits the contribution of CD4 T cells reactive to distinct epitopes to disease progression. Cells with the highest affinity for MOG were observed during acute disease demonstrating increased TCR affinity is not required for disease progression.

As the HLA-DR4 gene is associated with an increased risk for MS, we then used a humanized mouse model of demyelinating disease in which mice express HLA DRB1*0401 (HLA-DR4) instead of murine MHC Class II genes. We explore CD4 T cell affinities for MOG and an epitope of MOG that contains citrulline in place of arginine in position 101, the result of an inflammation-induced post-translational deimination modification. We found a substantial population of cells cross-reactive to both MOG and citrullinated MOG had infiltrated the CNS before symptom onset and persisted through chronic symptoms. Further, a substantial population of CD4 T cells reactive only to citrullinated MOG was detected at symptomatic time points both in the CNS and in the periphery indicating CD4 T cell reactivity to citrulline may hold great value as a biomarker of disease in DR4+ patients.

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TABLE OF CONTENTS

INTRODUCTION

I. Overview of Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation, demyelination, and gliosis of the central nervous system (CNS). The targets of this immune response are components of the myelin sheath, which protects and insulates neurons in the CNS, as well as oligodendrocytes, the myelin-producing cells of the CNS. The pathology involves loss of oligodendrocytes as well as demyelination, which leads to axonal injury and impaired nerve conduction. Patients often present with symptoms of visual disturbances and/or sensory deficits and limb weakness. As the disease progresses, symptoms often become more severe and can include paralysis, loss of bowel/bladder control, and cognitive dysfunction. There are over 400,000 people in the United States suffering from MS and over 2.3 million affected worldwide (1)

As with most autoimmune diseases, MS is believed to be the result of a combination of predisposing genetic and environmental factors both of which contribute to disease. The incidence of MS is higher in women than in men, which has led to the investigation of the effects of sex hormones on MS (2). First-degree relatives of MS patients have been shown to have a 20- to 50-fold higher risk of developing MS than unrelated individuals (3). Many genomewide association studies have been conducted revealing the strongest predisposing genetic factor to be MHC class II genes or, in humans, histocompatibility leukocyte antigen (HLA) genes (4-6)}.

The environmental factors that contribute to MS are still the subject of investigation. The observation that MS is less prevalent in equatorial countries and more prevalent in countries that have decreased exposure to sunlight led to the hypothesized

protective effect of vitamin D in MS pathogenesis (7). This has led to the investigation into use of Vitamin D as a potential therapeutic (8). Many infectious agents have been proposed to trigger MS onset. Particularly, Epstein-Barr virus (EBV) has been associated with MS as anti-EBV antibodies have been shown to be elevated in MS patients and seropositivity among MS patients is higher than in the general population (9-11). The two major proposed mechanisms how of infectious agents are able to induce MS are by molecular mimicry, where a pathogenic antigen resembles an encephalitogenic autoantigen inducing a response against both antigens, and by bystander activation in which the general inflammatory environment resulting from infection is enough to trigger activation of autoantigen-specific cells. Contrarily, Helminth infections have been shown to negatively correlate with MS and it is believed this is due to a skewing of the CD4 T cell response into a Th2 phenotype (12).

Specific environmental triggers of MS have been investigated most recently, studies investigating the effects of salt on the Th17 CD4 T cell subset have suggested a link between high-sodium diets and MS incidence (13, 14). The implications of these papers extend to the increased prevalence of MS evolutionarily as our diets have evolved to contain more sodium and preservatives. Clearly, the search for environmental factors that trigger onset or contribute to or alleviate symptoms of MS are constantly being investigated.

In MS patients, four disease courses are observed clinically. Most commonly, patients experience relapsing remitting disease (RRMS) initially presenting with symptoms (usually mild) that resolve. These patients typically experience an asymptomatic period of disease remission followed by another symptomatic episode or

relapse. This cycle of distinct symptom-free intervals of remission followed by symptomatic relapses can continue for years. RRMS is the initial diagnosis in approximately 85% of MS patients (1). Of these patients, the vast majority eventually progress into chronic disease and no longer experience periods of remission at which point their disease is termed secondary progressive MS (SPMS). By contrast, in primary progressive MS (PPMS), patients present with symptoms that progressively worsen with no symptom-free remission periods. Roughly 10% of MS patients are diagnosed with PPMS. The fourth disease course, though rare, is termed primary relapsing disease (PRMS) in which patients present with symptoms that progressively worsen but these patients will additionally experience "relapses" of more severe symptoms. PRMS patients do not experience disease remission.

II. Genetics of MS

MHC class II genes have been shown for decades to present the greatest genetic risk of developing MS. HLA-DR15 (DRB1*1501) has shown the strongest correlation with increased risk as this gene is expressed by approximately 50-70% of MS patients compared to 20-30% in the general population (15-18). Several disease-associated single nucleotide polymorphisms (SNPs) have also been identified including a few in the IL-2 receptor locus, the IL-7 receptor locus, and many more within the HLA-DR locus (4, 6). Specific predisposing HLA alleles vary based on geography and ethnicity; HLA-DR2 (also known as HLA-DR15) shows the strongest genetic correlation to MS in Caucasian populations whereas other DRs are more strongly predictive in other ethnic groups (19). For instance, HLA-DR4 (DRB1*0401) is frequently associated with an increased risk of

MS in patients of Mediterranean heritage and has been linked to a more severe clinical outcome in patients (17, 19-22). Genes associated with HLA-DR15 include those for transforming growth factor ß (TGF-ß), cytotoxic lymphocyte antigen 4 (CTLA-4), tumor necrosis factor (TNF) genes as well as IL-1-related genes and estrogen receptor (19). Additionally, several HLA-DQ alleles, including DQB1*0602 which is linked to the DR15 haplotype, have been linked to MS (19). Additionally certain HLA-class I and HLA-class II alleles have been shown to be protective for MS (6, 19).

Investigation into the linkage of MHC class II genes with MS has led to several proposed hypotheses. Some have suggested that the polymorphisms of the HLA-DR and –DQ alleles lead to altered binding of peptides and preferential presentation of certain peptide motifs on these HLA molecules (23). It is unclear why certain alleles increase risk for development of MS and others do not but this remains the topic of active investigation.

III. Mouse models of MS

Experimental autoimmune encephalomyelitis (EAE) is an invaluable model of MS as it is also an autoimmune demyelinating disease. Both EAE and MS are characterized by an autoimmune attack of T and B lymphocytes directed toward myelin antigens, specifically epitopes of myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), proteolipid protein (PLP) and myelin associated glycoprotein (MAG). In mice, EAE is characterized by an ascending paralysis, which begins with a loss of tone in the tail, followed by hindlimb weakness and paralysis and eventual weakness or paralysis the forelimbs at which point mice often become moribund. CD4 T

cells are thought to be the key mediators of pathogenesis but roles have also been described for CD8 T cells (24), B cells and anti-myelin antibodies (25, 26).

Several mouse models of EAE have been described using different strains of mice and a variety of myelin antigens for disease induction. Most often, disease is induced with an injection of myelin antigen emulsified in Complete Freund's Adjuvant (CFA) or by the adoptive transfer of myelin-reactive CD4 T cells. When disease is induced by immunization with myelin peptide, CD4 T cells are activated peripherally and begin to express activation markers that facilitate their passage across the blood brain barrier. In the B6 model, MOG peptide (MOG35-55) emulsified in CFA is injected subcutaneously and an intraperitoneal pertussis toxin injection is given at 0 and 48 hrs. Pertussis toxin is not always used in disease induction although it is thought that use of the toxin stimulates CCL2-dependent leukocytes to help permeabilize the blood brain barrier and aid in the entry of lymphocytes into the brain parenchyma (27). Mice become symptomatic roughly two weeks after disease induction with peak disease occurring by day 20 or 21 and then continuously experience symptoms (28). This progression of symptoms resembles the primary progressive disease course seen in human MS. The B6 model has been instrumental in the development of several first line treatments for MS including glatiramer acetate (29), type I interferons (30, 31), and natalizumab (32). Despite the development of these successful treatments, many criticize the EAE model due to the failure to predict the adverse effects of other therapies such as anti-TNF monoclonal antibody as well as IFN-g therapies or the inability to predict other negative outcomes such as the development of progressive multifocal leukoencephalopathy (PML) in, for example, some natalizumab-treated patients. Anti-TNF monoclonal antibody and IFN-g

therapies were shown to ameliorate EAE symptoms while they worsened symptoms in MS patients (33, 34). It is important to note that many of the studies testing therapeutic efficacy were performed in the B6 model of EAE, which demonstrates a disease course observable in only 10% to 20% of MS patients. Therefore it is possible that a more clinically applicable model in which mice experience a relapsing-remitting disease followed by secondary progression would be more useful in the prediction of clinical efficacy of MS therapies.

Relapsing-remitting disease course has been described in several mouse models. First and most well-known is the SJL model in which a PLP epitope (PLP139-151) is used to induce disease (35). Naïve SJL mice have a high precursor frequency of CD4 T cells reactive to PLP139-151 (36). This relative predominance of autoreactive cells is attributed to the antigen-binding properties of the H-2^s MHC class II allele expressed in these mice, and its tendency to allow autoreactive cells to escape negative selection in the thymus (37).

Relapsing remitting disease course has also been described in the nonobese diabetic (NOD) mouse model. These mice are better known for their proclivity to develop autoimmune diabetes, which is thought to be associated with expression of the I-Ag7 class II allele. Interestingly, there is a greater incidence of autoimmune diabetes in female NOD mice, a trend that is observed in both autoimmune diabetes and MS in humans (38). Several groups have demonstrated the relapsing-remitting disease course in these animals by inducing disease with MOG35-55 antigen (39, 40). Biozzi ABH mice also express the I-Ag7 allele and have been shown to experience a relapsing-remitting disease course in response to a distinct epitope of MOG (MOG8-21) peptide (41). Taken together this

implicates a clear role for the I-Ag7 allele in conferring genetic risk for autoimmune responses.

IV. Relapse and Remission: Epitope Spread or Single Determinant Reactivity

Relapsing-remitting disease is a phenomenon that many have attempted to explain. One proposed mechanism is epitope spread in which an autoimmune response is mounted against one CNS antigen, which initiates destruction of the myelin sheath and subsequent presentation of other myelin antigens to CD4 T cells by MHC class II molecules on antigen presenting cells (42). Relapse of symptoms is thus proposed to be due to an immune response directed against a second CNS antigen, which is temporally separated from the initial insult. The novel, "second-wave" antigen response can be the result of several different antigen-processing outcomes.

The second wave response can be directed against a new CNS peptide derived from a different myelin protein. Cross et al has shown in the SJL model that in EAE induced by adoptive transfer of MBP-reactive T cells, that approximately 1/3 of mice develop a proliferative response to PLP (43). These cells were grown on PLP antigen and then adoptively transferred into naïve SJL mice and were able to transfer disease indicating the response to the novel antigen was sufficient to cause symptoms.

The secondary response can also be directed against a new epitope of the same CNS protein or a distinct epitope presented on a different MHC molecule. This has been demonstrated in a (SJLxPL) F1 EAE model in which T cells harvested at disease relapse time points were reactive to antigen epitopes distinct from that with which disease was induced (44, 45). This model was chosen as two distinct epitopes of MBP are

respectively encephalitogenic in SJL mice (H-2^s) and PL mice (H-2^u). It was shown that when EAE was induced with MBP1-37 (H-2^u restricted), cells could be isolated at later time points that were reactive to MBP89-169 presented by H-2^s-expressing APCs (44). This showed not only that a novel response could be generated to a distinct epitope but also to epitopes with differing MHC-class II restriction. Further, inducing peptide-specific tolerance to the alternate antigens is able to prevent relapse of symptoms demonstrating the pathogenicity of this novel response (45).

While most investigations of epitope spread have taken place in the SJL model, little work has been done to examine this phenomenon in the NOD model of EAE which also display a relapsing-remitting disease course that evolves into secondary progressive disease. However, epitope spread has been demonstrated in the NOD model of autoimmune diabetes in which an immune response generated against proinsulin led to a response directed against other pancreatic antigens (46). The I-Ag7 MHC allele has been suggested to be the cause of the propensity towards autoimmune disease in the NOD model in that it renders the mouse unable to maintain self-tolerance after self-peptide/adjuvant immunization (37).The proposed mechanism for this "autoproliferation" is that I-Ag7 binds poorly to self-antigen and allows for the escape of self-reactive cells from negative selection in the thymus (37). The concept that certain MHC class II molecules can predispose to epitope spread has been investigated in other models of EAE.

Epitope spread is widely used as the explanation for relapsing-remitting disease pathologies observed in autoimmune disease, however significant evidence showing that reactivity towards a single determinant can elicit a relapsing-remitting phenotype has

been published. In the Biozzi mouse model of relapsing-remitting demyelinating disease, epitopes of both MOG and PLP are able to elicit relapsing-remitting disease and after immunization, T cell reactivity to epitopes distinct from that with which mice were immunized can be observed (41). Importantly, in this model inhibiting the dominant T cell response directed against inducing antigen is able to ameliorate disease symptoms suggesting the T cell responses directed towards distinct epitopes were not required for eliciting disease symptoms. It was suggested that these responses act to exacerbate disease symptoms while not being able to elicit symptoms independently of the dominant response.

We recently demonstrated in the NOD model of secondary progressive disease that the vast majority of CD4 T cells infiltrating the CNS of mice induced with MOG35-55 were reactive to MOG throughout disease including time points of acute symptoms, remission, relapse and chronic progression (47). Additional studies in TCR transgenic mice showed that a single T cell specificity can elicit relapsing-remitting disease course (48). Similarly, evidence from the SJL model of relapsing-remitting disease showed a predominant T cell response to inducing antigens with no evidence of epitope spreading during disease relapse (49).

Although evidence exists supporting both a role for epitope spread in eliciting disease relapse and a more limited role for reactivity to new antigens, definitive explanations for relapsing-remitting disease remain elusive. There is a clear correlation between MHC alleles and the development of relapsing-remitting disease and reactivity to multiple antigens. Explaining this phenomenon in human patients is further

complicated as MS patients express many HLA alleles that can contribute in different ways to disease pathology.

V. CD4 T Cells in Demyelinating Disease

T cells have long been thought to be the primary mediators of MS and EAE pathogenesis. Early studies identified oligoclonal expansions of T cells in the CSF and blood of MS patients (50, 51) and since then the respective contributions of CD4 and CD8 T cells have been under intense investigation. As MHC class II genes confer the greatest genetic risk for MS development, it is intuitive that CD4 T cells would play a crucial role in disease pathology. More recent work has been directed at elucidating which CD4 T cell subset(s) are responsible for disease and also investigating roles for CD8 T cells in EAE and MS pathogenesis.

V.B. Th1 and Th17 Cells in MS

Early experiments in the animal model of EAE showed that disease could be transferred by CD4 T cells supporting an essential role for these cells in pathogenesis (52). Helper CD4 T cells are divided into several subsets based on the cytokines that induce their differentiation, the transcription factors and programs that get activated, and the cytokines that are produced by differentiated cells. When early studies of MS and EAE were being conducted, only the Th1 and Th2 subsets had been described. Several groups showed that the Th1 subset, characterized by production of IFN-g and the expression of the transcription factor T-bet, was responsible for initiating disease in mouse models of disease (53-55). Adoptive transfer of Th1 cells has been shown to elicit

EAE symptoms while loss of the canonical Th1 transcription factor Tbet was shown to confer resistance to developing EAE (55, 56). Once in the CNS, it is thought that Th1s secrete large amounts of IFN-g that subsequently activate macrophages and microglia and stimulate MHC-class II expression on these cells (53).

The essential role for Th1s in eliciting EAE was called into question when several groups showed that inhibiting this subset exacerbated rather than alleviated disease. Data from the mouse model demonstrated that IFN-g was not required for induction of EAE and that mice deficient in IFN-g developed symptoms of EAE that were accompanied by a large infiltration of lymphocytes and macrophages into the CNS (57). Similarly, mice deficient in IL-12, the key cytokine for Th1 development, were shown to be susceptible to development of EAE (58, 59). In two different mouse strains known to be resistant to developing EAE, disrupting the IFN-g/IFN-gR axis in these mice rendered them susceptible to symptomatic disease (60, 61). The severity of symptoms in mice in which the Th1 signaling axis has been disrupted was often reported as being worse than those in which it was intact even suggesting a potential neuroprotective role for IFN-g in demyelinating disease.

A key study that described IL-23 as essential to EAE pathogenesis shifted the long-held Th1 paradigm as IL-23 is the cytokine responsible for Th17 development (62). Several studies that followed showed the crucial role for Th17 cells in demyelinating disease and demonstrated the presence of these cells in the demyelinated plaques in MS patient brains (63, 64). Studies similar to those conducted while elucidating a role for Th1s demonstrated through IL-17 and IL-17R knockout models that Th17 cells were (also) required to elicit demyelinating disease (65, 66). Evidence that showed adoptively

transferred Th17 cells could not transfer disease suggested a synergistic role for Th1 and Th17 cells (67). An elegant fate-mapping study that tracked IL-17 producing cells demonstrated the inherent plasticity of these cells in that they were able to convert into IFN-g producing cells, an observation that perhaps explains data supporting essential roles for both subtypes (68). Further complicating matters, a third cytokine GM-CSF was described as essential to demyelinating disease development as mice deficient in this cytokine did not develop symptoms (69).

A study that sparked interesting debate over the role Th1 cells play in MS sought to investigate the therapeutic and neuroprotective effects of IFN-g in MS patients. This study was preceded by reports that MS patients were deficient in IFN-g production, which suggested the potential use of this cytokine therapeutically (70, 71). In the trial, 18 MS patients with relapsing-remitting disease were given intravenous IFN-g treatment twice per week for four weeks (34). Throughout the course of the trial, 7 out of 18 patients experienced disease exacerbations and this was significantly more than the number/frequency of exacerbations experienced by these patients over the preceding 2 years (34). Interestingly, patients were evaluated for clinical symptom severity and no significant changes in patients at pre- and post-treatment evaluations were observed. Exacerbation rates and disability scores on 12-20 month follow up were normal. Remarkably, the majority of patients receiving IFN-g treatment experienced fever, a known elicitor/aggravator of MS symptoms, and the nature of exacerbations resembled previously experienced attacks. Indeed, the administration of IFN-g may have elicited "pseudorelapses" due to increased body temperature or the involvement of other cytokines and what was observed and reported as worsening of disease course may truly

have been the exacerbation of pre-existing pathology and not the direct consequence of treatment. This would lead to the conclusion that IFN-g administration may have actually attained therapeutic efficacy if the trial had not been stopped which does not eliminate the possibility that IFN-g production by Th1 cells can be neuroprotective.

V.C. Regulatory T cells

Immunologic self-tolerance is maintained by a variety of cellular subsets and processes. During T cell development in the thymus, after positive selection, it is thought that T cells expressing a TCR that binds too strongly to self-peptide/MHC undergo negative selection however this process does not eliminate all self-reactive T cells. Auto-reactive cells that escape central tolerance must then be controlled in the periphery. Peripheral tolerance is maintained when these autoreactive T cells become anergic or die after encounter with self-antigen. Additionally, a subpopulation of CD4 T cells, regulatory CD4 T cells or Tregs, are able to inhibit the activation and function of autoreactive T cells. While there are many subpopulations and classifications of Tregs, there are two main derivations of this regulatory population. CD4+ Tregs that are generated in the thymus and express the Forkhead box P3 (Foxp3) transcription factor are known as “natural Tregs.” Naïve T cells can also be induced to become Tregs in the periphery and these are known as “inducible Tregs” or “adaptive Tregs” (72).

Tregs have been proposed to suppress responses by modulating the effects of other T cells either by suppression of proliferation or cytokine production by T effectors and both cell-contact-dependent and cytokine-mediated mechanisms have been investigated (73). Tregs have also been suggested to influence antigen-presenting cells (APCs) by reducing their capacity to present antigen and inducing the secretion of

suppressive cytokines or other factors (73). While many subpopulations of Tregs have been identified based on surface expression of regulatory molecules and cytokine secretion profiles, the precise mechanism by which Tregs exert their suppressive function remains the subject of investigation.

Tregs are thought to play a key role in regulating and preventing autoimmune disease as they control and eliminate the self-damaging effects of autoreactive T cells. T cells that were able to suppress harmful responses of other T cells were initially described in the literature as T suppressor cells but the legitimacy of this population was not widely accepted until Sakaguchi and colleagues identified the IL-2 receptor alpha chain (CD25) as a key marker of these cells which he termed regulatory T cells or "Tregs" (74). Further, it was demonstrated that eliminating this CD4+CD25+ cellular population from mice led to systemic autoimmunity (74). Since then, the role Tregs play in controlling autoimmune disease has been explored in several mouse models including EAE.

In early work exploring the role Tregs play in controlling demyelinating disease, CD4 T cells from healthy, normal mice were able to prevent EAE when adoptively transferred into recipient mice (75). It was observed that disease was only prevented if healthy CD4 T cells were transferred before symptom onset implying a portion of these CD4 T cells were able to regulate and prevent EAE onset in these mice. Further work exploring the role of Tregs in EAE have shown that adoptive transfer of Tregs into MOG-immunized mice is able to significantly protect recipients from developing symptoms as well as significantly reduce lymphocytic infiltration and CNS inflammation (76). In a model of acute EAE in which mice develop symptoms and then, without intervention, completely recover from these symptoms, Tregs have been shown to

accumulate in the CNS with a frequency of up to 1 in 3 CNS-infiltrating CD4 T cells (77). Additionally, through CD25+ depletion studies it was demonstrated that these Tregs were responsible for recovery from symptomatic disease as well as the resistance to reinduction of disease characteristic of this acute disease model (77).

Many mechanisms of suppression have been proposed to be essential to the efficient regulatory functions of the Treg population. Cell-contact independent mechanisms have been proposed and involve the secretion of inhibitory or suppressive cytokines or factors while contact-dependent mechanisms that involve binding of inhibitory receptors with their ligands have also been described. In B6 EAE, Tregs have been shown to suppress IFN-g production and proliferation of MOG-reactive Th1 cells in *vitro* (76).Two mechanisms were suggested to account for improvement of symptoms *in* B6 mice including increased expression of adhesion molecules that allowed Tregs to interact with and prevent trafficking of pathogenic CD4 T cells to the CNS (76). Alternatively, an increase in Th2 cytokines has also been noted in Treg recipients implying Tregs are able to skew the CD4 T cell response to a protective phenotype (76). Tregs isolated from symptom recovery time points in acute disease were shown to produce IL-10 and express Treg markers CTLA-4, GITR and $a_E ß7$ in addition to Foxp3 (77).

To further explore the role of Tregs in acute EAE, Foxp3GFP knock-in reporter mice were used with MHC-class II tetramers (I-A^b-MOG) to track antigen-specific Foxp3-expressing Tregs as well as effector CD4 T cells throughout disease (78). This question of whether Tregs suppress in an antigen-directed manner is interesting considering many believe Tregs exert their suppressive function, at least in part, by

bystander suppression, the concept that once the TCR is activated in an antigen-specific manner, Tregs are able to suppress in an antigen-nonspecific way (72). Previous studies in transgenic TCR mouse models demonstrated that natural Tregs efficiently suppress T effector responses when both T cell populations are reactive to the same antigen however Tregs are significantly less capable of suppressing T effectors reactive to a distinct antigen (79). Korn et al addressed this question and found tetramer-positive/MOG-reactive, Foxp3-positive CD4 T cells in peripheral lymph nodes as well as in CNS tissue after immunization with MOG peptide but the expansion of these cells did not prevent the onset of symptoms (78). Interestingly, the highest frequency of T effectors was noted at the peak of acute disease after which the number of these cells in the CNS declined. After accumulation in the CNS, Tregs did not decline in number which substantially changed the ratio of Tregs:Teffectors from 1:13 during acute disease to 1:4 during recovery from disease (78). Once isolated from the CNS, Tregs were able to suppress the proliferative capacity of MOG-reactive cells from naïve MOG TCR-transgenic animals but failed to suppress proliferation of CNS-infiltrating CD4 T effector cells from sick animals (78). This was found to be attributable to the ability CNS-infiltrating T effectors to secrete IL-6 and TNF-a which when added to cultures of CNS-derived Tregs and naïve MOG-reactive effectors, abrogated the ability of Tregs to suppress the proliferation of T effectors (78). It was concluded that these cytokines, as part of the acute inflammatory response, prevented Tregs from adequately controlling the onset of symptoms. This suggests that with the control of the inflammatory environment in the CNS, Tregs would better be able to regulate the CNS-infiltrating CD4 T effectors.

Tregs have also been postulated to play a role in symptom progression throughout relapsing-remitting models of demyelinating disease. In naïve SJL mice, a high frequency of PLP-reactive CD4 T cells can be detected in the peripheral blood, which implies these cells are able to escape negative selection despite their autoreactive character (80). SJL mice treated with a CD25-depleting antibody before disease induction experience accelerated onset and a more severe disease than mice not receiving antibody (81). Interestingly, mice that had been depleted of CD25-expressing Tregs still experienced the first disease remission after acute symptoms however mice proceeded to experience chronic (secondary progressive) symptoms post-Treg depletion characterized by a strong Th1 response and both IFN-g and TNF-a cytokine production (81). It was concluded that Tregs do not play an essential role in primary remission after acute onset of disease but that these cells are critical for the transition into remission following secondary relapses (81).

Further studies in the SJL model found the frequency of Tregs in the peripheral blood, lymph nodes and spleen of EAE-recovered mice to be higher than in both naïve mice and immunized mice in the acute phase of disease (82). These cells were found to be anergic and resistant to proliferative stimuli in vitro but were able to suppress the proliferative capacity of CD4+CD25- cells in suppression assays (82). Additionally depletion of Tregs using anti-CD25 antibody during the recovery phase of disease led to exacerbation of symptoms which was accompanied by an expansion of PLP tetramer-positive cells as well as an enhanced production of IFN-g (82). This study implicated a key role for TGF-ß as the frequency of CD4 T cells expressing TGF-ß increased during disease recovery, and *in vivo* neutralization of TGF-ß halted disease recovery.

Many have attempted to characterize the Treg response in MS patients in an effort to better understand human disease. The current data in the literature regarding the role of Tregs in MS patients is somewhat conflicting with some groups claiming an increased frequency of Tregs in MS patients and others claiming no differences in Treg frequency between MS patients and healthy controls (83, 84). Further complicating the investigation of Tregs in MS are the inherent differences between murine and human Tregs. The specific and certain identification of human Tregs has proved more difficult than the identification of their counterpart in the mouse. First, human Tregs express high levels of CD25 yet $CD25^{hi}$ cells comprise only 1-2% of the circulating CD4 T cell population (85). Further, it has been shown that only these cells that expressed the highest levels of CD25 that are capable of suppressing proliferation of T effectors (85).

The nuclear transcription factor FoxP3 has been the most specific identifying marker of Tregs and both human and murine Tregs express high levels of this transcription factor. However, in human studies, the expression of low levels of FoxP3 in activated CD4 T cells as well as in some CD25-, $CD25^{intermediate}$, and CD8 T cells further complicates the study of Tregs in humans (86). While murine Tregs can be identified by their surface expression of CD4 and CD25 as well as the expression of the FoxP3 transcription factor, human Tregs are more specifically $CD4^{+}CD25^{hi}$FoxP3+ however functional similarities between Tregs of mouse and human origin have been described (85). First, stimulation of human-derived Tregs does not cause them to proliferate but instead causes them to suppress proliferation of and cytokine production by activated CD4+CD25- and CD8 effector T cells in a contact-dependent manner (85, 87). Human-derived Tregs express high levels of HLA-DR, an interesting distinction as murine Tregs

do not express MHC class II molecules (85). Human Tregs express CD45RO but not CD45RA (85, 87), which is characteristic of antigen-experienced cells and consistent more with a memory phenotype. Human Tregs are also known to maintain their regulatory function in the absence of the PD-1/PDL1 and CTLA-4/B7 pathways and their suppressive function has been linked to signal strength via the TCR and is hindered by large amounts of exogenous IL-2 and IL-15 (85, 87). Production of IL-10 has been demonstrated in human Tregs although the suppressive ability of these cells is thought to be independent of IL-10 production (85, 87). These early descriptions of regulatory T cells in the peripheral blood of humans and the fact that they comprised 2-4% of all circulating CD4+ T cells prompted other groups to look further into the function and mechanism of suppression of human regulatory T cells particularly in the context of autoimmune disease.

VI. CD8 T cells in demyelinating disease

Many have postulated that CD8 T cells are important in EAE and MS pathogenesis although the nature of their role remains unclear. Studies from the early 1990s began to investigate the nature of T cells isolated from lesions in MS patients. Wucherpfennig et al examined CNS tissue specimens from both live and cadaverous MS patients and demonstrated by RT-PCR analysis of V-alpha and V-beta gene segments a broad, polyclonal TCR repertoire in active MS lesions (88). Using similar techniques, Oksenberg et al were able to demonstrate the presence of CD4 T cells with TCR specific for MBP epitopes in the brains of MS patients (89).

Babbe and colleagues first demonstrated a distinct role for CD8 T cells in MS patient lesions using single cell PCR amplification of TCR-ß gene rearrangements (90). In this study, both CD4 and CD8 T cells were found in perivascular infiltrates and parenchymal tissue but the CNS-infiltrating CD8 T cells were representative of only a few clones while CD4 TCRs were much more heterogeneous (90). Further MS patient studies demonstrated a role for CD8 T cells in the mechanism of action of glatiramer acetate (GA or Copaxone) therapy as CD4 T cells both from healthy individuals and untreated MS patients were able to proliferate to GA whereas CD8 T cells from untreated MS patients proliferated less well than those from healthy individuals (91). Initiation of GA therapy led to a restoration of this CD8 proliferative response to GA in MS patients comparable to that of healthy controls (91). This role for CD8 T cells in the therapeutic mechanism of GA suggested critical involvement of CD8 T cells in MS pathogenesis. Potential mechanisms of this pathogenic role come from studies showing CD8 clones isolated from MS patients are able to lyse MBP-transfected target cells and secrete IFN-g and TNF-a (92). Further evidence shows that independently of costimulation, CD8 T cells isolated from the peripheral blood of SPMS patients were shown to secrete significantly more lymphotoxin than those isolated from healthy controls (93). RRMS patients showed a trend toward higher lymphotoxin secretion that did not reach significance leading to the conclusion that lymphotoxin secretion by CD8 T cells was involved in the transition from RRMS to progressive disease (93).

The argument for the involvement of CD8 T cells in the pathogenesis of MS is often discredited by the fact that the major genetic risk factors for disease are MHC-II genes. Studies of MHC-class I genes have suggested both pathogenic and protective roles

for CD8 T cells in MS. The HLA-A3 allele has been shown to increase susceptibility to MS independently of class II genes (94), while HLA-A2 has been argued to be protective and reduce the relative risk by half (6, 95, 96).

Many studies have demonstrated a protective or suppressive role for CD8 T cells in EAE. Adoptive transfer of CD8 T cells in the B6 model are not able to induce disease and transfer of CD8 T cells prior to MOG-induction effectively attenuates disease symptoms (97, 98). B6 CD8 knockout mice also experience more severe symptoms than wildtype counterparts (97). In an H-2^u acute disease model, depletion of CD8 T cells after recovery from symptoms causes mice to experience symptom relapse while H-2^u-expressing mice that lack CD8 T cells ($CD8^{-/-}$ PL/J) experience a chronic relapsing disease course (99, 100). It is thought that CD8 T cells in this model specifically inhibit MBP-specific CD4 T cells and when transferred are able to prevent disease in immunized mice (101). Similarly in the B6 model, the mechanism of CD8-mediated suppression was shown to be CD28-dependent as CD8+CD28- T cells, and not CD8+CD28+ T cells, are able to suppress IFN-g production by MOG-specific CD4 T cells and this suppression is proposed to be cell-contact dependent and APC-dependent as well (97). Further, it was shown that CD8+CD28- T cells inhibit the upregulation of costimulatory molecules on APCs causing decreased antigen presentation (97).

An alternative mechanism of CD8-mediated suppression is dependent upon secreted factors as a regulatory CD8 T cell population capable of secreting TGF-ß and IFN-g has been identified in both SJL and B6 EAE models (102). Interestingly, these cells do not express CD25 on their surface nor do they express the FoxP3 transcription factor like their CD4 Treg counterparts but they are able to attenuate disease symptoms

upon adoptive transfer and reduce IFN-g production by MOG-specific CD4 T cells while possibly inducing or expanding traditional CD4 Tregs (102). This not only demonstrated a suppressive role for CD8s in EAE but also suggested a potential mechanism using an APC intermediate and CD8-induced reduction of antigen-presenting capability of these cells. It remains unclear whether the mechanism of suppression of CD8 T cells in EAE is cell-contact dependent or reliant on secreted factors. Recently both mechanisms were shown to be important in CD8-mediated suppression of EAE as antigen-specific CD8 T cell proliferative response could be observed in the B6, B10.PL and SJL models of EAE (98). The CD8s were able to produce IFN-y, TNF-a as well as perforin suggesting both cytotoxic and cytokine-mediated suppressive mechanisms. It was additionally shown that APCs isolated from disease-protected mice secreted significantly more IL-10 and significantly less IL-12 than those isolated from controls implying the MOG-specific CD8 T cells may be enacting their suppressive role via modulation of APCs (98). This study supports the suppressive role for CD8 T cells in EAE and offered an antigen-dependent mechanism of APC modulation leading to suppressed CD4 T cell responses.

Although there is substantial work in mouse models supporting a suppressive role for CD8 T cells, evidence also supports a pathogenic role for these cells. Adoptive transfer experiments have demonstrated that MBP-specific CD8 T cells transfer disease independently of CD4 T cells in the C3H model (103). Similarly, in the B6 model MOG-specific CD8 T cells are able to transfer a more severe and sustained disease than immunization with MOG peptide (103, 104). The magnitude of the CD8 response to MOG in B6 mice was further examined using a MOG-specific class I (D^b) tetramer to track CD8 responses throughout the course of disease. MOG-specific CD8 T cells were

found in the brain early in disease with approximately half of CNS-infiltrating CD8 T cells staining positive with tetramer at day 10 post-induction (24). These MOG-specific CD8 T cells persisted in the CNS throughout acute disease and it was later demonstrated to be dependent on MOG-reactive CD4 T cell help as immunization of B6 mice with a truncated CD8 epitope within the MOG35-55 peptide led to less severe symptoms than mice immunized with full-length peptide (105).

Further support for a pathogenic role for CD8 T cells comes from the NOD model of EAE. Mayo et al compared EAE in the susceptible NOD strain to the nonobese resistant (NOR) strain, an I-Ag7 expressing mouse on the NOD background that does not develop autoimmune diabetes. Although NOR mice did not develop EAE they still showed a robust CD4 T cell response to the MOG peptide and a diminished CD8 T cell response compared to their NOD counterparts (40). These data suggest a pivotal role for CD8s in eliciting symptoms in EAE in these two strains.

Recently, Huber et al described an essential role for IL-17-secreting CD8 T cells in the pathogenesis of disease. These "Tc17" cells are thought to aid in the development of the Th17 response as well as stimulate these cells such that they can cross the blood brain barrier and enter the CNS (106). Additionally, Tc17 cells were identified in the CSF of patients in the early stages of MS indicating a role for these cells in human disease.

Frequency of myelin reactive cells in MS patients versus healthy controls.

While the precise pathophysiology of MS remains unclear, a distinct role for CD4 T cells reactive to CNS antigens has been clearly demonstrated. Mouse models show a crucial role for CD4 T cells specific to many myelin antigens in developing disease

symptoms and the severity and phenotype of the disease course is dependent on the strain of mouse and importantly upon their MHC haplotype. Many have attempted to identify the causative myelin antigen by evaluating the frequency of myelin-reactive CD4 T cells in the blood of MS patients and healthy controls. Despite numerous studies over the last two decades, the question of whether MS patients inherently have more myelin-reactive CD4 T cells than healthy controls remains unclear.

Several groups have shown that CD4 T cells isolated from MS patients have an increased functional reactivity to myelin antigens compared to healthy controls (107-110). Using limiting dilution assays, it was shown that MS patients had significantly higher mean frequencies of MBP-reactive CD4 T cells compared to control patients with other neurological diseases while no significant differences in the frequency of cells reactive to PLP or Herpes Simplex virus (HSV) were observed (111).

A similar study investigated the frequency of circulating CD4 T cells reactive to epitopes of PLP and MBP at monthly intervals in RRMS patients and demonstrated that RRMS patients have surges of high frequencies of T cells reactive to PLP184-209 and these surges were significantly more frequent than in healthy controls (107). However these surges correlated to disease activity as measured by gadolinium-enhancing lesions on MRI and clinical relapse in only one of the patients studied (107). Further, although periodic high frequencies of MBP-reactive cells were observed in RRMS patients, these were not significantly more frequent than those observed in healthy controls (107).

Functional studies assessing cytokine production from T cells isolated from peripheral blood sought to determine the frequency of myelin-reactive Th1 cells in a large group of MS patients (RR, PP, and SP disease courses) and healthy volunteers

(112). Both whole MBP protein and whole MOG protein as well as a broad panel of myelin-derived peptides were used to stimulate cells including several epitopes of each MBP, MOG and PLP. The results of this study indicated that although MS patients have a very heterogeneous reactivity to myelin antigens no significant differences were found between healthy controls and MS patients in their ability to respond to myelin-derived peptides, whole MOG or whole MBP with nearly 100% of MS patients and 100% controls tested displaying cytokine production in response to the whole proteins (112). Interestingly, a correlation was found between combined reactivity to both MOG and MBP in MS patients but not in control subjects, which seemed to suggest intermolecular epitope spread could be occurring in MS patients (112).

When investigating the proliferative or functional response of CD4 T cells to a specific epitope, HLA-restriction must be considered. CD4 T cells recognize their cognate antigen in the context of HLA molecules, and the epitopes of myelin proteins that have been reported as encephalitogenic, whether in mice or in humans, are only able to stimulate an immune response when recognized in the context of the appropriate HLA molecule. Studies that assess proliferative or functional readouts of CD4 T cell activity in response to whole protein are only somewhat helpful in that specific peptide epitopes and their HLA restriction must then be determined. Additionally, due to the extensive diversity of HLA-genotypes within the population, it is intuitive that the immune response that provokes and perpetuates MS can be directed to several different epitopes within a variety of myelin proteins. These responses to different antigens can also manifest at different points in time. When no significant differences between myelin-reactive cells are observed between MS patients and healthy controls, some have

suggested that a phenotypic difference accounts for the disparity in pathology (113). Many studies investigating antigen-reactivity in MS patients only characterize this reactivity at a single point in time or a few times over the course of several months. This snapshot of cellular reactivity to myelin antigens is a clear limitation to fully understanding the course of the immune response in MS and the specific antigens that elicit this response. If epitope spread plays a role in the pathogenesis of MS, this would further complicate the results from studies investigating myelin-reactivity of CD4 T cells in MS patients as the timing of responses would drastically affect results.

Recently, the frequency of MOG-reactive cells in the peripheral blood of MS patients was investigated. This focused study examined the reactivity of CD4 T cells to a DR4-restricted epitope of MOG (MOG_{97-109}) using MHC-class II tetramer staining after *in vitro* stimulation (110). In this study, a tetramer with an MHC-variant epitope of MOG97-109 ($MOG97\text{-}109_{107S}$) was used as this epitope binds the DR4 molecule more efficiently than the native MOG epitope (110, 114). The results of this study indicated not only that MHC-class II tetramers could be used to detect MOG-reactive T cells in the peripheral blood of MS patients but also that MS patients have a greater frequency of MOG-reactive CD4 T cells than do HLA-matched healthy controls (110). The frequency of the MOG-reactive cells isolated from MS patients was still low despite *in vitro* stimulation illustrating the inherent handicap in using peripheral blood to inform the mechanisms of an immune response taking place in the CNS. Indeed, many have criticized the use of MHC class II tetramer staining for T cells thought to be at a low frequency in a population or tissue and remain critical of tetramer data obtained after *in vitro* stimulation as this process can alter results (115).

I. DR4 AND MS

Select MHC class II alleles are associated with the strongest genetic risk for MS. HLA-DR2 (DRB1*1501) confers the strongest risk for disease, while HLA-DR4 (DRB1*0401) is frequently associated with MS development particularly in patients with Mediterranean heritage (17, 20-22). With known HLA-risk haplotypes, specific encephalitogenic epitopes of myelin peptides that are presented by these HLA molecules have been under investigation. In the context of HLA-DR4, human B cells have been shown to functionally present an epitope of MOG (MOG97-109)(22).

HLA-DR4-restricted T cell responses to MOG and other myelin epitopes have been investigated in the peripheral blood of MS patients. An epitope of MBP (111-129) was identified to elicit strong CD4 T cell proliferative responses from T cell clones generated from MS patient blood (116). A distinct epitope of MBP (81-99) has been shown to be presented by and have high affinity interactions with both DR4 and DR15 and elicit a diverse repertoire of T cell responses (116, 117). Interestingly, epitopes of MBP have been shown to bind HLA molecules differently as MBP81-99 binds tightly to both DR4 and DR15, while MBP111-129 interacts weakly with DR4 and it has been proposed that this unstable MBP111-129:DR4 complex is the reason that only high affinity T cells can respond to this epitope (116).

T cell reactivity to myelin epitopes in the context of DR4 has also been investigated in HLA-DR4 transgenic mice in which mice express HLA-DRB1*0401 instead of endogenous murine MHC class II molecules. In these mice, an epitope of MOG (MOG97-109) is able to elicit a chronic progressive demyelinating disease and both MOG-specific T cell responses and MBP-specific responses have been shown

demonstrating a role for epitope spread in this model (22, 118). Human B cells presenting MOG97-109 in the context of DR4 are able to elicit proliferative T cell responses from these mice demonstrating the utility these mice hold as a model of human disease (22).

II. DR4 AND CITRULLINE

HLA-DR4 is a genetic risk factor for MS however it also confers an increased risk for rheumatoid arthritis (RA) (119, 120). While diagnosis of RA involves the detection of autoreactive antibodies (rheumatoid factor and anti-citrullinated protein antibodies, ACPAs) the genetic risk association with class II MHC combined with the therapeutic efficacy of drugs targeting T cells supports the significant role of CD4 T cells in RA pathogenesis (121, 122). The use and specificity of ACPAs in RA diagnosis implicates a clear function for citrulline in pathogenesis of disease. Citrulline is an amino acid produced from the post-translational deimination reaction carried out in a calcium-dependent manner by peptidyl arginine deiminase (PAD) enzymes that are upregulated in the context of inflammation. Five isoforms of PAD enzymes have been described with PAD2 and PAD4 being particularly relevant to autoimmune disease as both have shown to be expressed in inflamed joints and also in the CNS and monocytes/granulocytes, respectively (123-125). As a result of the deimination reaction, arginine loses its positive charge and gains 1 Dalton of mass in its conversion to citrulline. The presence of citrulline is also known to make the protein more susceptible to proteolytic digestion, which would perhaps aid in its presentation in the context of MHC (126). The presence of antibodies specific to citrullinated proteins (ACPAs) in RA patients is significantly linked to the HLA-DRB1*0401 haplotype which suggests this molecule is well-suited to present

citrulline-containing peptides and subsequently generate citrulline-specific CD4 T cell responses.

The association of RA and HLA-DR alleles prompted the investigation into the biophysical characteristics of these HLA molecules and the discovery of the "shared susceptibility epitope" contained in DRB1*0401, DRB1*0404 and DRB1*0101 risk alleles. These gene variants all encode a hypervariable region that maps to amino acids 70-74 in the third hypervariable region encoding the anchoring P4 pocket of the HLA molecule (120). The shared epitope variants all encode a combination of amino acids that effectively line this pocket with positively charged amino acids (specifically at position 71) which limits the ability of positively charged peptide ligands to be bound to and presented by these HLA molecules (120, 127, 128). This physical property of the RA-associated HLA risk alleles bears immense significance when considering the ability of these HLA molecules to present citrullinated peptides. Peptides in which an arginine constituent has been converted to citrulline have effectively lost a positive charge with the substitution of a charged amino group with the uncharged carbonyl group of citrulline. Crystallography studies have shown that the electropositive P4 binding pocket within DRB1*0401 is permissive to binding citrullinated epitopes of synovial antigens while their arginine-containing counterparts are not able to be bound and presented by this HLA (129). Amino acids in positions 13 and 71 of the HLA molecule were demonstrated to be crucial to citrulline binding in all epitopes tested (129). Clinical data supporting this hypothesis comes from a large genome-association study in seropositive RA patients in which most of the HLA-DR-associated risk was attributed to 4 amino acid

positions: 11, 13, 71 and 74 the latter three having been identified as citrulline-accommodating polymorphisms within shared epitope HLA molecules (130).

III. CITRULLINE AS BIOMARKER OF DISEASE

Disease biomarkers, or measurable factors indicating disease presence and/or status and severity, are clinically useful for diagnostic purposes as well as the evaluation of therapeutic efficacy. Ideally, a test for a biomarker is both sensitive and specific for the presence of a given disease. Anti-rheumatoid factor antibodies (IgM-RF, antibodies against IgG) have traditionally been used in RA diagnosis and antibodies specific for citrullinated proteins, or ACPAs, have been known for decades to develop in RA patients nearly 5 years prior to disease onset (126). The use of a citrulline-specific immune biomarker as a read out of disease activity is intriguing in that the generation of citrulline-containing peptides is itself the result of an inflammation-induced post-translational modification. The presence of citrulline is therefore a marker of inflammatory activity and an immune response directed against these moieties would be an indication of immune pathology.

Antibody-biomarker assays are simple to perform, however many autoimmune diseases including RA and MS are thought to have a predominant CD4 T cell component to disease pathology. Evidence for a pathogenic role of citrulline-specific T cells in RA has been explored in murine models and in humans. In a humanized mouse model of RA, HLA-DR4-expressing mice immunized with citrullinated fibrinogen epitopes experience an arthritic phenotype while those injected with unmodified fibrinogen do not develop symptoms (131). Lymphocytic infiltrates into joint space as well as citrulline-specific

proliferative responses of CD4 T cells were observed in this model and thought to mediate disease pathology (131). In the collagen-induced model of arthritis, the presence of citrulline was demonstrated to be associated with symptom severity as mice in which PAD enzymes were inhibited experience less severe symptoms than wildtype counterparts along with a decreased antibody response to several autoantigens (132). Upon transfer of citrullinated-fibrinogen-specific CD4 T cells into collagen-induced mice, these cells effectively traffic to the joint synovium, worsen arthritic symptoms and increase IgG2a antibody levels to mouse type II collagen (133).

Citrulline-reactive CD4 T cells have been identified from the peripheral blood of RA patients further implicating their contribution to disease pathogenesis. In several studies, CD4 T cells reactive to citrullinated epitopes of synovial antigens have been identified including citrullinated epitopes of vimentin, collagen, cartilage intermediate layer protein 2, and aggrecan (121, 127, 134, 135). Citrulline-specific proliferative and cytokine-secreting phenotypes have been identified with both Th1 and Th17 phenotypes (121, 127). Tetramer technology has also been used to identify CD4 T cells reactive to citrullinated synovial antigens in peripheral patient blood ex vivo and these were found to have a memory Th1 phenotype (121, 129).

IV. CITRULLINE IN MS/DEMYELINATING DISEASE

Citrulline as a post-translational modification has been studied in the context of MS for over a decade. PAD2 is the hallmark PAD expressed in the CNS and is expressed in oligodendrocytes, microglia and astrocytes particularly in the context of inflammation or hypoxia (124, 136, 137). Initial studies described a naturally occurring isomer of

myelin basic protein (MBP) termed “C8” that differs from the most common MBP isomer “C1” in that 6 out of 19 arginine residues in C8 are replaced with citrulline which results in the loss of 6 positive charges (138-140). The presence of citrullinated MBP has been measured in MS patient brains and was determined to be much higher than in healthy controls or patients with other neurodegenerative diseases such as Parkinson’s, Alzheimer’s or amyotropic lateral sclerosis (139). This isomer possesses the same physical properties as immature or developing myelin and it was determined the brains of MS patients contained similar proportions of citrullinated MBP as brains of 3-4 year old children (139).

Once an increased proportion of citrullinated MBP had been demonstrated in MS patient brains the question of the encephalitogenicity of this modified peptide became the focus of many studies**.** Evidence demonstrating the presence of citrullinated MBP in the CNS of EAE mice at the onset of MBP-induced symptoms and the growth in number and size of these areas with the worsening of symptoms suggested a pathological correlation between the presence of citrulline in the CNS and the severity of disease (141). Citrullinated MBP was used to induce demyelinating disease in various rodent models and was found to be encephalitogenic in Lewis rats and PL/J mice (142, 143). These studies showed a decrease in severity of disease induced with citrullinated MBP compared to those induced with native MBP (142). Further, CD4 T cells specific to citrullinated-MBP were isolated that preferentially reacted to citrullinated over unmodified epitopes of MBP (142, 143). The immunodominant epitope of MBP in Lewis rats (72-85) contains an arginine in position 76 which functions as a TCR contact residue (144). In the SJL mouse model in which PLP is used to induce EAE, a citrullinated-MBP

specific antibody response could be detected suggesting citrulline-mediated pathology may not solely be mediated by T cells (145).

CD4 T cell specificity for citrullinated myelin proteins has also been explored in MS patients. T cell reactivity to both MBP and citrullinated MBP has been shown in the peripheral blood of MS patients who, compared to healthy controls, demonstrated a greater number of citrullinated-MBP reactive CD4 T cells and a greater functional sensitivity to the citrullinated epitope (146). The citrulline-specific CD4 T cell response was further characterized as having a Th1 phenotype and was more robust than the response specific to non-citrullinated MBP epitopes (147).

With growing evidence for a role for citrullinated MBP in MS, the question of whether these post-translational modifications could be found in other myelin antigens with proven encephalitogenicity remained to be answered. Mice deficient in PAD2 have shown no significant difference in MOG-induced disease course suggesting a limited role for citrulline in eliciting symptoms and progression of disease (148). A study using the encephalitogenic epitope of MOG (MOG35-55) investigated the generation of citrullinated-MOG-specific CD4 T cell responses in B6 EAE and identified 2 arginine residues within the core epitope, both TCR contact residues at P2 and P7, that had the potential to be converted to citrulline (149). This study found that, when citrullinated at P2, the MOG peptide was able to elicit a larger T cell response than unmodified MOG peptide, which was suggested to be due to the lack of citrulline in the thymus during selection (149). However, the CD4 T cells specific to the citrullinated epitope were not sufficient in eliciting demyelinating symptoms and cells that could effectively recognize native MOG in the uninflamed CNS were required to initiate disease symptoms (149).

This is intuitive as a robust T cell response specific for citrullinated MOG would not find its target citrullinated-myelin epitope in the CNS without prior inflammation and induction of PAD enzymes. Interestingly, within the MOG_{35-55} epitope, there are 3 possible citrullinated epitopes that can be made, one with citrulline at position 41 (P2), one at position 46 (P7) and a third epitope with modifications at both sites. This study found that while MOGCit41 acted as a strong agonist, MOGcit46 was a weak agonist and citrulline modifications at both position 41 and 46 resulted in a null peptide (149). These findings demonstrate that the location of the citrulline residue within the core epitope presented to the T cell is crucial in the response the modified peptide is able to elicit and that the presence of citrulline within myelin-derived proteins can evoke a range of T cell responses that could have any number of effects on disease progression.

Further evidence supporting a role for citrullinated MOG in disease pathogenesis comes from recent work in nonhuman primates. In this study, rhesus monkeys were immunized with transformed B cells pulsed with MOG, citrullinated-MOG (citMOG) a CMV-derived peptide known to mimic MOG and observed for symptoms of disease (150). Monkeys receiving cells pulsed with MOG or the CMV-derived peptide also received active immunization of MOG peptide emulsified in Incomplete Freund's Adjuvant while mice receiving citMOG-pulsed B cells did not. Interestingly, all monkeys receiving citMOG-pulsed B cells developed inflammatory lesions in the CNS resembling those of MS patients with distinct perivascular infiltrates of macrophages, T and B cells (150). While this effect was observed in some monkeys receiving the other immunization protocols it was most remarkable in monkeys receiving citMOG-pulsed B cells

implicating citrullinated epitopes of MOG are encephalitogenic in nonhuman primates (150).

V. PAD INHIBITORS

The proposed pathogenic role citrulline-reactive T and B cells play in RA and MS motivated the investigation into therapeutic inhibitors of the PAD enzymes known to convert arginine to citrulline. PAD enzymes have been known contribute to pathology in recovery from ischemic injury, making their inhibitors even more clinically valuable (151, 152). Five PAD enzymes are expressed in mammalian tissues with PAD2 and PAD4 being the most prominent in the CNS (153). Specifically PAD2 is expressed largely in the brain and also skeletal muscle and spleen while PAD4 is expressed in neutrophils and monocytes (153). PAD enzymes are upregulated in the context of inflammation of both ischemic and autoimmune etiologies and also in cancer (154). Cl-amidine has been identified as a pan-PAD inhibitor and has shown efficacy in mouse models of RA and autoimmune colitis (132, 155). F-amidine, similar to Cl-amidine in that both are haloacetamidines, is also a potent inhibitor of PAD4 and both small molecules inhibit PAD as they structurally mimic the enzyme's substrate (156).

As PAD enzymatic function is dependent on calcium, inhibitors such as ruthenium red have been designed to specifically target the calcium-bound form of the enzyme (157). As of yet, PAD inhibitors have not be assessed for clinical efficacy in inflammatory autoimmune diseases such as RA or MS, however as small molecule inhibitors continue to show efficacy in mouse models this is a promising next step in therapeutic avenues for these disease.

Given our interest in demyelinating disease the HLA-DR4 model provides a clinically relevant model for demyelinating disease in which inflammation induced modification of myelin epitopes occurs and CD4 T cell reactivity to this epitope can be tracked. As HLA-DR4 is associated with a host of autoimmune diseases and the citrulline modification seems to be a unique trait of HLA-DR4, this model uniquely allows for the investigation of the role of citrulline in the progression of autoimmune demyelinating disease.

CHAPTER I.

TITLE:

Progression of relapsing-remitting demyelinating disease does not require increased TCR affinity or epitope spread

Contributing Author: Lindsay Edwards, PhD

FOOTNOTES:

Abbreviations used in this article: MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; EAE experimental autoimmune encephalomyelitis; pMHC, peptide-MHC; 2D, two-dimensional; 3D, three-dimensional; SPR, surface plasmon resonance; AUC, area under the curve; Treg, regulatory T cell.

ABSTRACT:

In this study, we investigate the basis of T cell recognition of myelin that governs the progression from acute symptoms into disease remission, relapse and chronic progression in a secondary progressive model of demyelinating disease. Until now, the frequency and affinity of myelin-reactive CD4 T cells that elicit relapsing-remitting disease have not been quantified. The micropipette adhesion frequency assay was used to obtain a sensitive and physiologically relevant two-dimensional measurement of frequency and TCR affinity for myelin, as the inherent low affinity does not allow the use of specific-peptide:MHC-II tetramers for this purpose. We found the highest affinity and frequency of polyclonal myelin oligodendrocyte glycoprotein (MOG)-reactive cells infiltrate the CNS during acute disease, while affinities during remission, relapse and chronic disease are not significantly different from each other. Frequency analysis revealed that the vast majority of CNS-infiltrating CD4 T cells are MOG-reactive at all time points demonstrating epitope spread is not a predominant factor for disease progression. Further, time points at which mice were symptomatic were characterized by an infiltration of Th17 cells in the CNS while symptom remission showed an enrichment of cells producing IFN-γ. Also, the ratio of regulatory T cells to $Foxp3^-$ CD4 T cells was significantly higher in the CNS at remission than during acute disease. The results of this study indicate that a high frequency of T cells specific for a single myelin antigen, rather than increased TCR affinity or epitope spread, govern the transition from acute symptoms through remission, relapse and chronic disease states.

INTRODUCTION:

Multiple sclerosis (MS) is an autoimmune disease characterized by demyelination, inflammation and gliosis of the central nervous system (CNS). The greatest genetic risk factor for developing MS is in HLA-class II genes and the disease is predominantly mediated by a CD4 T cell response. Experimental autoimmune encephalomyelitis (EAE) in the nonobese diabetic (NOD) mouse is a clinically relevant model of MS due to the fact that these mice develop relapsing-remitting symptoms that become chronic, similar to the disease course experienced by approximately 80% of MS patients(1). Additionally, the NOD model is ideal to study as these mice have an MHC-class II-associated proclivity to autoimmune disease and are well-studied for their tendencies to develop autoimmune diabetes (46, 158) as well as inducible autoimmune thyroiditis (159) and systemic lupus erythematosus (160).

Although relapsing-remitting disease is the most common disease course experienced by MS patients, the factors that govern the progression from symptomatic disease through remission and relapse remain unclear. One explanation for symptom relapse is epitope spread, which is the concept that the damage that occurs during acute disease releases CNS antigens that prime a second T cell response to a distinct epitope that elicits symptom relapse (39, 161). Proliferation and cytokine data from various mouse models suggest that responses to a secondary myelin antigen can occur, yet it is unclear whether these responses are strong enough to independently elicit symptomatic disease(41, 44, 45, 162, 163). Others have attributed the resolution of symptoms observed in relapsing-remitting disease to the activity of regulatory T cells (Tregs) (164). Using the micropipette adhesion frequency assay we quantify the frequency of MOG-reactive T

cells throughout disease course and the affinity of T cell receptor (TCR) for myelin:MHC-II for the first time in a secondary progressive model of MS(28, 165, 166). Our laboratory has demonstrated that the micropipette assay more sensitively measures the frequency of myelin-reactive cells and defines the binding kinetics as compared to MHC class-II tetramer staining of CD4 T cells isolated from B6 mice and human patients (28, 165). This is in large part because it interrogates the affinity of single TCR:peptide-MHC (pMHC) receptor ligand interactions and does not rely on tetramer driven avidity for the detection of MOG-reactive cells (167). MHC class-II tetramers are limited in their ability to detect antigen-reactive cells that are present at a low frequency in a population and also those CD4 T cells with moderate to lower affinities for antigen (28, 115). The micropipette-based technology also outperforms surface plasmon resonance (SPR) or 3D technologies for analysis of polyclonal T cell populations, especially in correlation with pathogenicity or functional output of T cells (168-171). From a practical point of view, the micropipette assay allows us to interrogate TCR affinities in a polyclonal response immediately *ex vivo* whereas SPR and other methods may require the generation and analysis of individual T cell clones. In addition, the micropipette assay has been shown to more sensitively measure affinities below the affinity threshold for SPR measurement and that these affinities can be more finely and definitively resolved (168). Thus the micropipette adhesion frequency assay uniquely allows one to better assess the entire T cell response ex vivo in autoimmune diseases. In this study, we found that the CNS-infiltrating, polyclonal MOG-specific CD4 T cells with the highest affinity occurred during acute disease at onset of symptoms. No significant differences in T cell affinity exist between remission, relapse and chronic time points. Moreover, the high frequency

of MOG-reactive T cells limits the impact of epitope spread as a means for disease progression as the majority of CNS-infiltrating cells were MOG-specific at every time point examined demonstrating that relapsing-remitting disease can be elicited by T cells of a single myelin specificity.

MATERIALS AND METHODS

Mice.

NOD mice were purchased from The Jackson Laboratory and Taconic facilities. Mice were housed and bred in the Emory University Department of Animal Resources facility and used in accordance with the Institutional Animal Care and Use Committee-approved protocols. Female mice were used in all EAE experiments while male mice were used for lymph node priming. Mice were used for experiments at 8-11 weeks of age.

EAE Induction.

EAE was induced with a single (d0) subcutaneous (s.c.) injection in the hind flank of 300 μg MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA containing 5 mg/ml heat-inactivated *Mycobacterium tuberculosis* (Difco). MOG_{35-55} peptide and truncated peptides within MOG_{35-55} (Figure 2A and B) were synthesized on a Prelude Peptide Synthesizer (Protein Technologies, Inc.). Mice also received 250 -300 ng of pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Disease severity was assessed using the following scoring rubric: 0, no disease; 0.5 weak tail; 1, flaccid tail, 2, hind limb weakness/poor grip; 3, hind limb paralysis; 4, hind limb paralysis and forelimb weakness; 5, moribund/death.

CNS mononuclear cell isolation.

Mice were euthanized with CO_2 and perfused through the left ventricle with PBS. The brain and spinal cord were harvested from each animal and homogenized through a 100-μm filter. Mononuclear cells were isolated using a Percoll (Sigma-Aldrich) gradient, washed and counted using a hemocytometer. Cells were then prepared for flow cytometric analysis or use in the micropipette adhesion frequency assay.

Cells and reagents.

Culture medium utilized in intracellular cytokine staining and cell culture consisted of RPMI 1640 medium (Mediatech) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Mediatech), 100 μg/ml gentamicin (Mediatech), and 2 x 10^{-5} M 2-ME (Sigma-Aldrich). MOG-specific T cells were generated by footpad and tail-base priming of male mice with 100 μg of MOG_{35-55} emulsified in CFA containing 1mg/ml heat-killed *M. tuberculosis*. Inguinal, lumbar and popliteal lymph nodes were harvested 14 days after priming and stimulated in culture with 1 μM of MOG peptide.

Tetramer staining.

MOG_{42-55}-IA^{g7}, hen egg lysozyme $(HEL)_{11-25}$-IA^{g7}, $OVA_{141-160}$-IA^{g7}, and human $CLIP_{87-101}$-IA^{g7} monomers and tetramers were provided by the National Institute of Health Tetramer Core Facility at Emory University. Mononuclear cells were stained with tetramer immediately post- Percoll gradient isolation. Live cells were recovered from lymphocyte culture by subjecting cells to a Ficoll gradient (Mediatech) and then stained with 4μg/ml tetramer in culture medium for 4h at 37˚C. Cells were then stained with anti-CD4-FITC (RM4-5, BD Pharmingen), anti-CD11b-PerCP (M1/70, BD Pharmingen), anti-CD11c-PerCP (HL3, BD Pharmingen), anti-CD19-PerCP (1D3, BD Pharmingen), anti-CD44-PE-Cy7 (IM7, Biolegend), and anti-CD8a-V450 (53-6.1, Tonbo Biosciences) for 30 minutes on ice. Flow cytometry was performed on either a BD Biosciences FACSVerse or LSRII flow cytometer. Data were analyzed using FloJo software (Tree Star).

Intracellular cytokine staining.

Mononuclear cells isolated from the CNS and spleen of immunized animals were stimulated for 4.5 hours with 20 nM PMA (Fisher Biotech) and 1 μM ionomycin (Sigma-Aldrich) at 37˚C in the presence of 10 μg/ml of brefeldin A (BFA; MP Biomedicals). Lymphocytes harvested from lymph nodes grown on MOG were stimulated with 10 μM peptide for 5 hours, with BFA included for 4.5 hours. Cells were stained extracellularly with anti-CD4-FITC, anti-CD8a-V450, anti-CD11b-PerCP, anti-CD11c-PerCP, and anti-CD19-PerCP for 30 minutes on ice, fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) for EAE experiments and the Fix&Perm Cell Permeabilization Kit (Life Technologies) for lymph node cultures. Both kits were used according to manufacturer's protocol. Cells were then stained for intracellular cytokines using anti-GMCSF-PE (MP1-XT22, Biolegend), anti-IL-17A-allophycocyanin (eBio17B7, eBioscience), anti-IFN-γ- allophycocyanin-Cy7 (XMG1.2, BD Pharmingen), and anti-TNFα-PE-Cy7 (MP6-XT22, Biolegend) for 30 minutes on ice.

Foxp3 Stain

Cells isolated from the CNS and spleen of immunized animals were stained extracellularly with anti-CD4-FITC, anti-CD8-V450, anti-CD11b-PerCP, anti-CD11c-PerCP, and anti-CD19-PerCP for 30 minutes on ice. Cells were fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer set (eBioscience) per manufacturer's instructions and then stained intracellularly using anti-Foxp3-PE (FJK-16s, eBioscience) for 30 minutes on ice.

Micropipette adhesion frequency assay.

A detailed description of the micropipette adhesion frequency assay has been published elsewhere (28, 168, 171). Briefly, human red blood cells were biotinylated using Biotin-

X-NHS (EMD4 Biosciences). Cells were again coated with streptavidin (Thermo Scientific) and biotinylated peptide:MHC-II monomers. Mononuclear cells were isolated from brains and spinal cord of MOG-induced mice and CD4 T cells were isolated from the pooled samples by CD4-positive magnetic separation using MACS L3T4 CD4-positive selecting beads (Miltenyi Biotec). A single pMHC-coated RBC and a T cell were aspirated onto opposing micropipettes in a cell chamber mounted on the stage of an inverted light microscope. Using micromanipulators, cells were aligned against each other and a computer-controlled piezoelectric actuator brought the T cell into contact with a stationary pMHC-coated RBC. Cells remained in contact for 2 seconds and upon retraction a pMHCII:TCR binding event was visualized as an elongation of the ultrasoft RBC membrane. In the absence of a binding event, the RBC membrane instantly reverts to its spherical shape. Cells were brought into contact 50 times with the same contact time and area (A_c) and an adhesion frequency (P_a) was calculated. Quantification of surface pMHC and TCRß densities was determined by flow cytometry and BD QuantiBRITE PE Beads for standardization (BD Biosciences). Surface densities as well as adhesion frequency were then used to calculate two-dimensional affinity using the following equation: $A_cK_a = -\ln[1-P_a(\infty)\}/m_r m_l$ where m_r and m_l represent TCR and pMHC surface densities, respectively. Geometric mean affinities are reported ± SEM.

Statistical analysis.

Statistical analyses were performed using GraphPad Prism 6 software (Software for Science). Significance was calculated using one-way ANOVA and Student's t-tests.

RESULTS

NOD mice develop a relapsing-remitting EAE disease course

To characterize relapsing-remitting demyelinating disease, NOD mice were assessed for weight and clinical score daily after induction to track disease progression. With the described induction protocol, mice become acutely sick with an incidence of 78.4% (Table 1) and experience symptom onset as early as 11 days post-induction (Figure 1A). Acute disease was defined as a score of 1 or greater for at least 2 days. In experiments with mice originating from Jackson Laboratories, acute disease occurred within the first 20 days post-induction. Interestingly, acute disease occurs later post-induction in mice procured from Taconic facilities. To ensure these differing timelines did not affect results, clinical scores were plotted against time and areas under the curve (AUCs) were calculated. AUCs were calculated to standardize and quantify cumulative disease experienced by each mouse used in experiments. The day at which mice experienced acute symptoms, relapse and chronic progression varied from mouse to mouse and variation was more pronounced when comparing mice sourced from different vendors. To confirm the accuracy of our comparisons of mice from different experiments isolated at the same point in disease, AUCs were calculated to ensure the similarity of both duration of disease and symptom severity. Mice from both facilities experience acute disease followed by entrance into remission, symptom relapse and chronic progression. Expectedly, AUCs for acute and remission time points did not vary significantly (6.59±0.55 and 9.63±0.79 respectively) whereas mean AUC increased for both relapse (22.32±2.99) and chronic time points (39.03±3.80). For each time point, AUCs did not vary significantly between experiments regardless of the facility from which the mice

were procured (Figure 1C). Of the mice that became acutely sick, 100% of these mice then entered a period of symptom remission, defined as a score drop to ≤1 with at least a 1-point delta. Throughout disease, weight was inversely related to clinical score as mice lost weight with symptom onset and regained weight as symptoms remitted (Figure 1B). Mice that went on to experience relapse of symptoms again dropped weight, which validates the scoring rubric. Among mice that became acutely sick, relapse was experienced with an incidence of 55.6% and was defined as an increase in disease score by ≥1 grade for 2 or more days while the incidence of chronic progression was 88.9% and was defined as symptoms experienced after remission that last for 7-14 days. Mice that did not develop acute symptoms were sacrificed and not used in experiments (but are included in acute incidence calculations). Of note is that NOD mice are prone to develop autoimmune diabetes with an incidence of 85% in females by 30 weeks for Jackson mice and 60% in 6-month-old females from Taconic facilities. Blood glucose levels were monitored throughout experiments and mice that developed diabetes (nonfasting blood glucose of 250mg/dl or greater) were sacrificed and not included in analysis. We found that 12.0% of mice used in these experiments became diabetic post-induction and were thus excluded from analysis as it is unknown what affects autoimmune diabetes has on the immune response in demyelinating disease.

MOG_{42-55} is the encephalitogenic IA^{g7}-restricted epitope

The 21 amino acid long MOG_{35-55} peptide could contain several possible T cell epitopes and MHC binding registers. In order to determine the IA^{g7}-restricted MOG epitope, lymph nodes were primed in vivo with MOG_{35-55} in CFA and enriched for MOG-specific

cells by culturing on MOG_{35-55} peptide. Seven peptides truncated from the C- and N-termini of MOG_{35-55} were synthesized and used to stimulate cytokine production to determine the core pathogenic epitope(s) presented by IA^{g7}. Of the fragments tested, MOG_{42-55} was the only epitope able to elicit a significant production of IFN-γ and IL-2 by MOG-primed T cells. The 42-55 peptide was selected as the epitope for monomer and tetramer synthesis by the NIH Tetramer Core Facility.

To verify functionality of the tetramer, mice were primed with MOG_{35-55} emulsified in CFA, draining lymph nodes were harvested and stimulated with MOG_{35-55} peptide in culture. After antigen-specific enrichment roughly 7% of cells were found to be MOG-specific by tetramer staining (Figure 2C and D). Interestingly, cells isolated from the CNS of mice acutely sick with MOG-induced EAE did not stain with MOG-IA^{g7} significantly above background control IA^{g7}-tetramers (Figure 3C). Importantly, intracellular cytokine stain assays on MOG-primed lymphocytes in culture were able to detect two-fold more MOG-reactive cells than MOG-IA^{g7} tetramers indicating, as we have seen in the B6 model of MOG-induced EAE, that MHC class II tetramers fail to identify most antigen-reactive T cells (28).

CD4 T cell affinity for MOG is greatest during acute phase of disease

To investigate the frequency and affinity of MOG-reactive CD4 T cells infiltrating the CNS during progression from acute disease through symptom remission, relapse and chronic progression, the micropipette assay was chosen over MHC class II tetramers because tetramer technology uses avidity-based interactions that miss the known contribution of lower affinity T cells to autoimmunity. Further, in our own preliminary

experiments using IA^{g7}-tetramers, no MOG-specific CD4 T cells were identified in CNS isolates from acute disease time points (Figure 3C). For the micropipette assay, CD4 T cells were isolated from the CNS of mice sacrificed at acute, remission, relapse and chronic time points and loaded into a microscope chamber along with red blood cells (RBCs) coated with peptide-loaded MHC molecules of different surface densities. RBCs serve as biosensors in the micropipette assay allowing for visualization of a single TCR-pMHC binding event. The micropipette assay is preferable for assessing the frequency and affinity of CD4 T cells for antigen in that both TCR and pMHC in this assay are cell-anchored thus providing a biologically relevant assessment of binding events. In the assay, an adhesion frequency is calculated after 50 contacts between cells and from this adhesion frequency as well as quantified surface densities of both TCR and pMHC, the affinity can be calculated (168). In these experiments, MOG_{42-55}-IA^{g7}-coated RBCs were used as the experimental antigen and monomers presenting irrelevant peptide epitopes of HEL or hCLIP were used as negative controls. At the acute disease time point, none of the CD4 T cells bound to RBCs loaded with control antigens indicating the affinity for these antigens were below the level of detection of the micropipette assay ($<1 \times 10^{-7}$ μm^4) (data not shown).

CD4 T cells in the CNS during acute disease have a significantly higher mean affinity for MOG than at remission or chronic time points ($2.34 \times 10^{-5} \pm 1.04 \times 10^{-5}$ for acute, $8.65 \times 10^{-6} \pm 1.01 \times 10^{-6}$ for remission, $1.30 \times 10^{-5} \pm 2.95 \times 10^{-6}$ for relapse, and $1.11 \times 10^{-5} \pm 4.40 \times 10^{-6}$ for chronic; Figure 3). Further the affinities for all time points adhere to a Gaussian distribution (Figure 3A, C) with r^2 values>0.830 for remission, relapse and chronic time points. The lowest r^2 value of 0.673 was observed for the curve fitted to

affinities from acute disease, which is expected as the greatest range of affinities was observed at this time point. The breadth of affinities is best highlighted on inspection of the distributions and shows 10-fold greater spread during acute disease compared to disease remission, 5-fold greater than during relapse and twice as great as during chronic disease. Of note no significant difference exists between the affinities measured during remission, relapse and chronic time points.

MOG-reactive T cells predominate in CNS at all time points

The frequency of myelin-reactive T cells in the CNS is a driving force for disease (24, 28, 65, 172). In addition to the 2D affinity, micropipette analysis gives the most sensitive measure currently available as to how frequent a given CD4 T cell specificity occurs in the immune repertoire. Our lab has previously shown that in the B6 model of EAE, nearly 10% of CNS-infiltrating CD4 T cells are MOG-specific by IA^b-tetramer staining whereas more than 65% show specificity for MOG in the micropipette assay(28). In this study in NOD mice, at all time points the majority of the CD4 T cells in the CNS were reactive to MOG (Figure 3D). This number is starkly different when compared to ex vivo MOG_{42-55}/IA^{g7}- tetramer staining (87% vs. <1% of CNS-infiltrating cells at acute time points are MOG-reactive by micropipette and tetramer respectively). Of note the same monomer is used for both techniques. Our findings in this study suggest IA^{g7}-tetramers are even more inefficient than IA^b-tetramers at detecting MOG-specific CD4 T cells from the CNS and that the micropipette assay is the only reliable way to identify these cells.

Although tetramer negative, the frequency of high affinity cells was quantified throughout disease using the geometric mean of cells isolated during acute disease (2.34 x 10^{-5} μm^4) as the threshold for definition of higher affinity cells. Higher affinity cells were only detected during symptomatic disease time points and their frequency in the CNS declined throughout disease. Despite not being high affinity, nearly 84% of the CNS-infiltrating CD4 T cells in the CNS remain reactive to MOG during disease remission. The presence of CD4 T cells with higher affinities for MOG during symptomatic disease indicates that symptomatic disease is predominantly, if not exclusively, initiated and maintained by MOG-reactive cells. Additionally, this preponderance of MOG specific cells limits any role for epitope spread as nearly all T cells are specific for MOG.

IL-17-producing CD4 T cells infiltrate CNS during symptomatic disease

Cytokine production profiles were investigated to examine the functional differences of CD4 T cells isolated from acute, remission, relapse and chronic disease time points. Specifically, CD4 T cells in the CNS were examined for their ability to produce IFN-γ and IL-17. Pathogenic roles have been demonstrated for both Th1 and Th17 cells in EAE and more recent work has investigated the nature of IFN-γ/IL-17-double producing cells, or Th1-like Th17 cells. When looking at CD4 T cells that produce IL-17 in the absence of IFN-γ production, we found significantly lower frequencies of these cells at disease remission compared to disease relapse and chronic progression time points (Figure 4B). Moreover, when comparing the frequency of IL-17-producing CD4 T cells in the CNS to those in the spleen, we found a significant 10-fold enrichment in the CNS at acute and a

6-fold enrichment at relapse time points ($p=0.004$ and $p=0.012$, respectively with two-tailed paired Student's t-test) and a 4-fold enrichment of IL-17- producing CD4 T cells in the CNS during chronic disease ($p<0.0001$). The frequency of IL-17-producing CD4 T cells was not significantly greater in the CNS compared to the spleen during symptom remission indicating Th17 cells play a role in symptomatic disease.

We also investigated the role of CD4 T cells that produce IFN-γ in the absence of IL-17 (Th1 cells) during relapsing-remitting disease (Figure 4C). We found these cells were most frequent in the CNS at acute and remission time points with diminishing frequencies at relapse and chronic time points. This downward trend of IFN-γ production over the course of disease supports the idea of its neuroprotective role and that the loss of IFN-γ in chronicity allows for symptom-eliciting mechanisms to dominate the response. A 4-5-fold increase in frequencies of Th1 cells was observed in the CNS compared to the spleens of mice during acute disease ($p=0.0002$) and disease remission ($p=0.0005$) whereas no difference in frequencies were observed during relapse and chronic time points.

We next looked at CD4 T cells producing both IFN-γ and IL-17 and found the frequency of these cells in the CNS to be highest at disease remission and significantly higher than those found during relapse or chronic disease (Figure 4D). When comparing the frequency of IL-17/ IFN-γ-producers in the CNS to that observed in the spleen we found a significant enrichment in the CNS for acute ($p=0.0005$) and remission ($p=0.0003$) time points and no significant difference at relapse and chronic time points. The implication from these data is that IFN-γ producers and IL-17/ IFN-γ-double-producers begin to enter the CNS during acute disease and the increased production of IFN-γ, especially with the

exodus or death of Th17 cells, has an overall protective effect during disease remission. Further, it is possible that the absence of IFN-γ production later in disease facilitates the development of symptomatic disease during relapse and chronic time points.

Increased Treg frequency at symptom remission.

To further investigate the cellular factors involved in the transition from acute disease to symptom remission, we analyzed regulatory T cells in the spleen and CNS of NOD mice induced with MOG peptide. At the remission time point, significantly more Tregs were found in the CNS than were found during acute disease (Figure 5). Interestingly, an enrichment of Tregs in the CNS was observed as these cells were nearly two-times more frequent in the CNS than in the spleens of remitting mice (26.06 ± 2.154% versus 11.47 ± 0.6541% respectively).

We next evaluated the ratio of Tregs ($CD4^+Foxp3^+CD25^+$) to non-Treg CD4 T cells ($CD4^+Foxp3^-$) during acute disease and symptom remission. We found that in the CNS, this ratio significantly increases at remission time points compared to acute disease (Figure 5C) while the ratio was not significantly different between the two time points in the spleen. This further supports the hypothesis that an increased frequency of Tregs in the CNS contributes to symptom remission in a relapsing-remitting disease model (173) and that this effect is most pronounced in the CNS.

DISCUSSION:

In this study, we have investigated the phenotype of CD4 T cells from acute disease, through symptom remission, relapse and chronic progression in the NOD model of secondary progressive demyelinating disease. We used the micropipette adhesion frequency assay to quantify the affinity of these cells because it allows for the detection of MOG-reactive cells below the avidity threshold of detection of MHC-class II tetramers (28, 115, 174). Several studies have demonstrated the increased sensitivity of 2D affinity measurements as compared to 3D (SPR) data with regard to biologically meaningful measures (168-170, 175). The micropipette assay measures affinity of TCR for pMHC in a cell-membrane-anchored, physiologically relevant context and steric restrictions imposed by the cell membrane have been suggested to be the reason for the lack of correlation of 3D measurements with functional output (169, 170). In this study, we quantify the affinities of CNS-infiltrating CD4 T cells ex vivo throughout the course of relapsing-remitting disease. While SPR requires the generation of T cell clones making *ex vivo* measurements difficult, micropipette allows for ex vivo measurement of a polyclonal T cell response and thus this technique is essential to enumerate the frequency and affinity of the entire CD4 T cell response in autoimmune demyelinating disease (28, 176, 177).

Although a direct correlation exists between responding T cell affinity and extent of immune response (178, 179) we instead observed the highest affinity CD4 T cells infiltrate the CNS during the initiation/acute phase of disease. IA^{g7}-tetramers, as an avidity-based detection method, failed to detect MOG-specific CD4 T cells in the CNS of sick mice while the micropipette assay as well as intracellular cytokine data demonstrate

MOG-reactive cells are present in the CNS at all time points of disease. The affinities measured during acute disease were significantly higher than those measured during remission or chronic progression however they did not differ significantly from those isolated at symptom relapse. This suggests a similar pathology behind the resolving symptoms that occur during acute and relapsing disease and a role for higher affinity T cells in eliciting symptoms. Moreover it suggests a role for lower affinity MOG-reactive cells, perhaps with distinct phenotypic profiles, in eliciting chronic symptoms. The affinities for the polyclonal response to MOG measured throughout the course of demyelinating disease measured here are similar to those measured in the B6 model and also to affinities for MBP measured in CD4 clones derived from MS patients (28, 165). Recently, it was shown in the NOD model of autoimmune diabetes that both high and low 2D-affinity insulin-specific CD4 T cells were able to cause insulitis but higher affinity cells were more susceptible to tolerogenic mechanisms (180). In our study, roughly 30% of CD4 T cells infiltrating the CNS during acute disease are of higher affinity and are not present in the CNS during symptom remission. This observation can be explained by the death or regulation of these higher affinity cells upon disease remission, which is supported in our model by the increased frequency of Tregs in the CNS during remission. Higher affinity cells are present again in the CNS at later time points of relapse and chronic disease. This observation suggests a second-wave of MOG-reactive cells are acting in the CNS during later time points of symptomatic disease. Whether these cells are recruited to the CNS from the periphery or are endogenously primed in the CNS during early stages of disease remains unclear and warrants further investigation. Others have demonstrated a second wave response to inducing antigen in

the relapsing-remitting SJL mouse model and suggested this was due to release of neuroantigen during the primary response (162). CNS-derived CD4 T cells have been investigated in other models of EAE and have been shown to be resistant to regulation by Tregs (78) which suggests an important pathogenic role for this second wave T cell response.

In this study, the majority of CNS-infiltrating CD4 T cells remained reactive to MOG at all time points measured throughout disease. The frequency of MOG-reactive CD4 T cells in the CNS was highest during acute disease (87.1%) indicating at most 13% of CNS-infiltrating CD4 T cells are reactive to antigens other than MOG_{42-55}. This is similar to the results seen in the B6 model of chronic EAE in which 65% of CNS-infiltrating CD4 T cells were determined to be MOG-reactive (28). Throughout disease course in the NOD model, the frequency of MOG-reactive cells does not fall below 65% indicating, as in the B6 model in which epitope spread does not occur, that the response throughout disease is directed predominantly to the inducing MOG epitope. Even if the entire T cell population in the CNS not detected as MOG-reactive by micropipette was reactive to a single CNS antigen it would still comprise at best a minor component of the response. Further we expect that many of the remaining cells that did not show affinity for MOG in the micropipette assay to actually be MOG-reactive but below our assays detection limit ($\sim 10^{-7} \mu m^4$) in a 2 second analysis time frame. For example, it has been shown in the 2D2 MOG-reactive TCR transgenic model that CD4 T cells compensate for low, undetectable 2D affinity in the micropipette assay by increasing the time of antigen recognition (166). Thus, almost all CD4 T cells in the CNS are likely MOG-reactive in the NOD and B6 models.

The predominance of MOG-specific cells would seem to discredit the notion that epitope spread is exclusively responsible for disease relapse and chronic progression. Epitope spread has been suggested to be the cause of relapse in other relapsing-remitting models of EAE (43-45) and both initiation and progression of epitope spread are strongly dependent upon MHC class II haplotype in animal models and patients (39, 118, 172, 181, 182). Further, intra- and intermolecular epitope spread have been demonstrated in the context of IA^{g7} in the autoimmune diabetes model in NOD mice (46, 158). Although other peptides have been identified as encephalitogenic in IA^{g7}-expressing mice, EAE experiments in Biozzi (IA^{g7}+) mice also demonstrated that the T cell response to the priming epitope was dominant throughout a relapsing EAE course as opposed to alternate CNS antigens (41). Additional studies in the NOD model will determine whether any non-MOG reactive CD4 T cells emerge during disease course but their low frequency make it unlikely that this population would contribute substantially to disease pathology. In addition to quantifying affinity and frequency of MOG-reactive cells, the cytokine profile of CD4 T cells was assessed throughout disease. Although early publications of EAE models demonstrated that IFN-γ-producing Th1 cells elicit disease, data also demonstrate a neuroprotective role for IFN-γ (54, 57, 60, 67, 183-185). Clear roles have also been demonstrated for IL-17-producing Th17 cells in eliciting EAE (65, 186, 187). In this study, we show that IL-17-producing CD4 T cells are enriched in the CNS during periods of symptomatic disease while disease remission is accompanied by an increase in IFN-γ-producing cells as well as regulatory T cells. Interestingly, the increase in frequency of IL-17-producing cells accompanying relapse and chronicity coincides with the observation of a second wave of higher affinity cells in the CNS at these time points.

While we did not formally address the differences in affinity among CD4 T cells subtypes, ongoing studies are investigating whether Th17 cells have higher affinity for MOG than Th1 cells. Of further interest is the possibility that a population of IL-17-producing cells in acute disease seems to gain the ability to produce IFN-γ which may neutralize the pathologic effect of the Th17 cells (Figure 4B and D). The plasticity of Th17 cells and their ability to convert in vivo to IFN-γ producers has been documented in both mice and humans (188) however protective effects of this double-cytokine producing population have not been published. Moreover, we demonstrate a decline in frequency of IFN-γ producing cells in the CNS throughout disease course with the lowest frequency observed during chronic disease. We suggest that the absence of IFN-γ-producing cells during chronic disease renders the CNS vulnerable to pathogenic processes and allows for the establishment and maintenance of chronic symptoms. Although this study shows that, in the CNS, IL-17 production correlates with symptom relapse and chronicity, it suggests a neuroprotective effect of IFN-γ during disease remission.

We also observe an enrichment of regulatory T cells in the CNS during disease remission and a significantly higher ratio of Tregs to non-Treg-CD4 T cells compared to acute time points. An essential role for Tregs in mediating the recovery from symptomatic EAE has been demonstrated by several groups (77, 189). We believe Tregs play an important role in promoting remission however these regulatory mechanisms are clearly overcome upon entry into relapse. This could be explained by the observation that Tregs are less effective at regulating the response of Th17 cells compared to Th1 cells (190). If Tregs are not able to effectively regulate this pathogenic population, the symptoms experienced during

relapse and chronic progression can be explained by the reinfiltration or *in situ* priming of Th17 cells in the CNS. Additionally, Korn et al found that Foxp3+ Tregs isolated from the CNS were able to suppress naïve MOG-specific CD4 T cells but were unable to suppress CNS-infiltrating T cells from MOG-induced animals (78). They attributed this observation to the ability of CNS-infiltrating Teffs to secrete IL-6 and TNF-α which abrogated the ability of Tregs to suppress Teffector responses (78). This functional exhaustion of Tregs might accompany the transition from remission to relapse and chronicity in the NOD model although further study is needed to answer this question.

In summary, this study demonstrates that CD4 T cell affinity for MOG is highest during acute disease and that the majority of CNS-infiltrating CD4 T cells remain MOG-reactive throughout disease. Additionally, symptomatic time points of relapse and chronic disease are accompanied by a small population of higher affinity MOG-reactive cells infiltrating the CNS supporting the argument that a second wave of MOG-reactive cells is responsible for relapse and disease progression. Since the vast majority of CD4 T cells in the CNS are reactive to MOG this severely limits any role epitope spread could play in disease progression which challenges the widely held belief that relapsing-remitting disease is elicited by epitope spread. The reinfiltration of higher affinity cells at symptomatic time points of relapse and chronicity coincides with higher frequencies of Th17 cells in the CNS. It is clear that with the changing affinity and cytokine profiles of CNS-infiltrating MOG-reactive CD4 T cells throughout secondary progressive disease, the evolution of symptoms is governed by a balance of many pathogenic and regulatory/protective processes. One great challenge that is faced by those attempting to design antigen-specific therapies is choosing the antigen (and its HLA-restriction) to

which they wish the patient to be tolerized. This is particularly challenging in that many CNS antigen specific clones can be grown out of both patient and healthy donor blood so identifying which antigen-specific T cells are actually pathogenic seems daunting. Our data would suggest that there may in fact be one (or few) CD4 T cell specificity that is responsible for eliciting relapse of disease. Further, the concept of epitope spread maybe somewhat limited. Of interest, ongoing antigen-specific therapy trials involved administration of autologous PBMCs chemically coupled with only seven myelin peptides (191), which resulted in a decrease in antigen-specific T cell responses. We propose tolerogenic efforts for relapsing-remitting autoimmune disease may be successful if focused to a single or limited number of epitopes.

Figure 1:

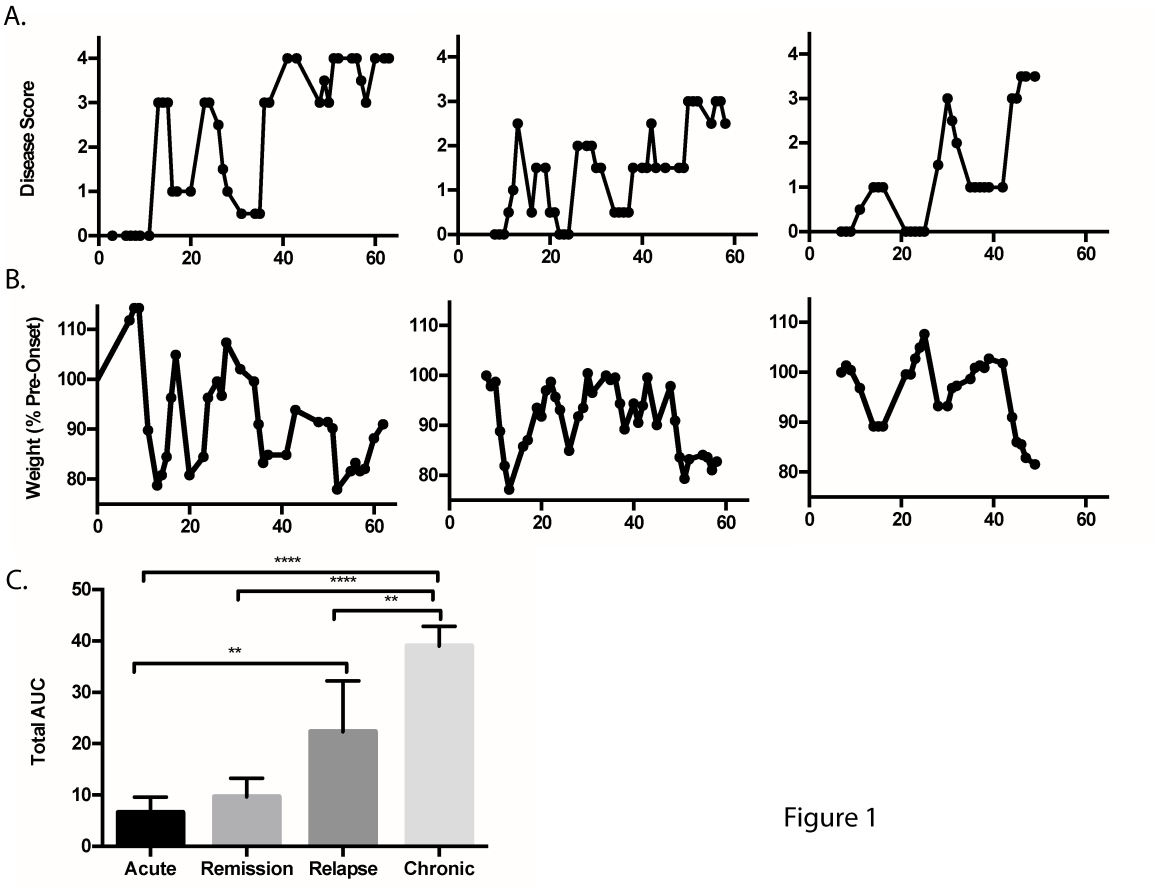


Figure 1

TABLE I.

Table I. Incidence and disease course of secondary progressive MOG-induced EAE in NOD mice.

Expt	Incidence Acute Disease	Mean Day of Acute Disease	Max. Acute Score	Incidence of Relapse	Mean Day of Relapse	Max. Relapse Score	Incidence of Chronicity	Mean Day of Chronicity	Max. Chronic Score
1	5/6	11.0	3.5						
2	9/9	11.3	3.0						
3	5/8	11.0	2.0						
4	3/5	12.0	2.5						
5	4/4	11.5	3.0						
6	4/4	10.8	3.0						
7	3/3	13.0	2.5	0/1	----	----	0/1	----	----
8	10/10	13.2	2.5						
9	5/5	13.8	3.0						
10	2/4	12.0	3.0						
11	8/14	23.3	2.0	2/3	60.0	1.0	3/3	94.7	5.0
12	3/4	13.3	1.5	2/3	40.5	3.0	3/3	69.0	3.5
13	4/5	13.5	2.5	3/4	30.0	3.5			
14	4/5	14.0	2.5	3/4	36.0	4.0	3/3	69.3	3.5
15	4/4	12.5	3.0	3/4	35.0	3.0	3/3	62.7	3.5
16	1/2	12.0	2.5	1/1	28.0	2.5			
17	7/7	12.1	2.5	4/7	24.5	3.0	7/7	46.6	4.0
18	8/10	12.8	2.5	4/8	36.3	3.0	7/8	50.7	3.0
19	7/10	50.6	2.5	0/7	----	----	7/7	77.4	3.5
20	1/2	14.0	1.5	1/1	36.0	1.0	1/1	62.0	3.5
21	1/4	33.0	1.5	0/1	----	----	1/1	56.0	3.0
22	2/3	14.0	2.5	2/2	67.5	2.5	2/2	108	3.0
23	3/3	13.0	1.5	2/3	26.0	3.5	1/2	107	3.5
24	2/4	17.0	2.5	2/2	36.5	4	1/1	104	2.5
25	4/4	13.0	3.0	1/4	44.0	2.5	1/3	45.0	3.5

Figure 2:

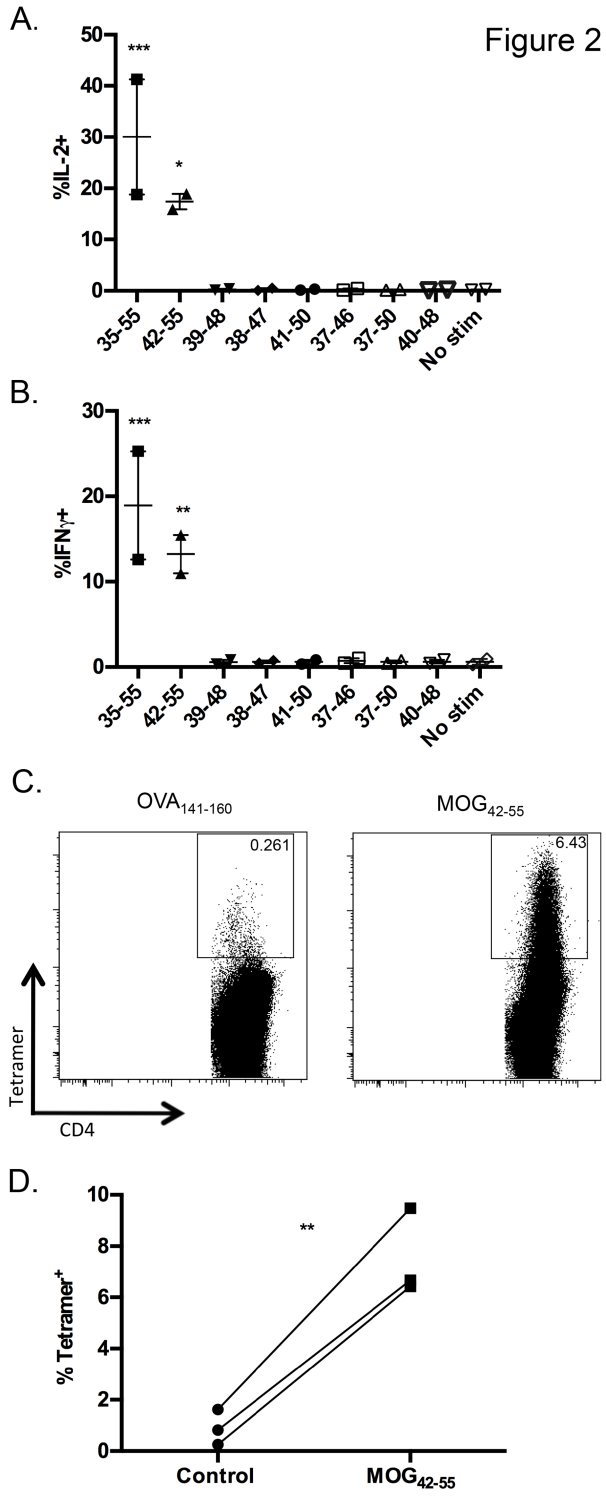
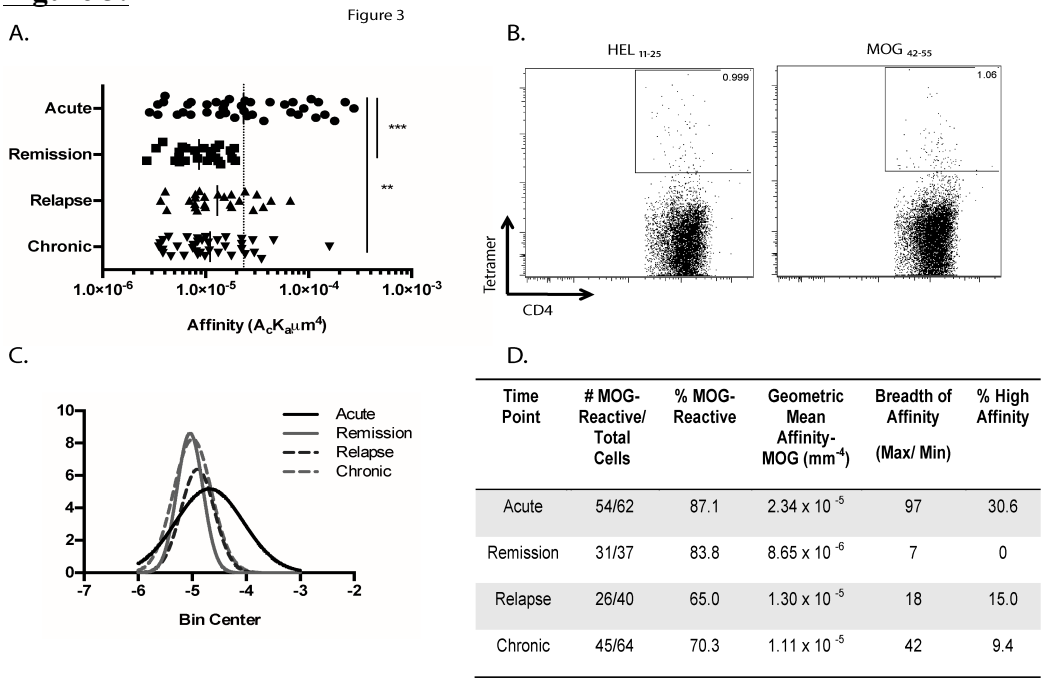

A.
Figure 2
%IL-2+
***
*
35-55
42-55
39-48
38-47
41-50
37-46
37-50
40-48
No stim
B.
%IFNγ+
***
**
C.
OVA141-160
MOG42-55
0.261
6.43
Tetramer
CD4
D.
% Tetramer+
**
Control
MOG42-55

Figure 3:



Time Point	# MOG-Reactive/ Total Cells	% MOG-Reactive	Geometric Mean Affinity-MOG (mm^{-4})	Breadth of Affinity (Max/ Min)	% High Affinity
Acute	54/62	87.1	2.34×10^{-5}	97	30.6
Remission	31/37	83.8	8.65×10^{-6}	7	0
Relapse	26/40	65.0	1.30×10^{-5}	18	15.0
Chronic	45/64	70.3	1.11×10^{-5}	42	9.4

Figure 4:

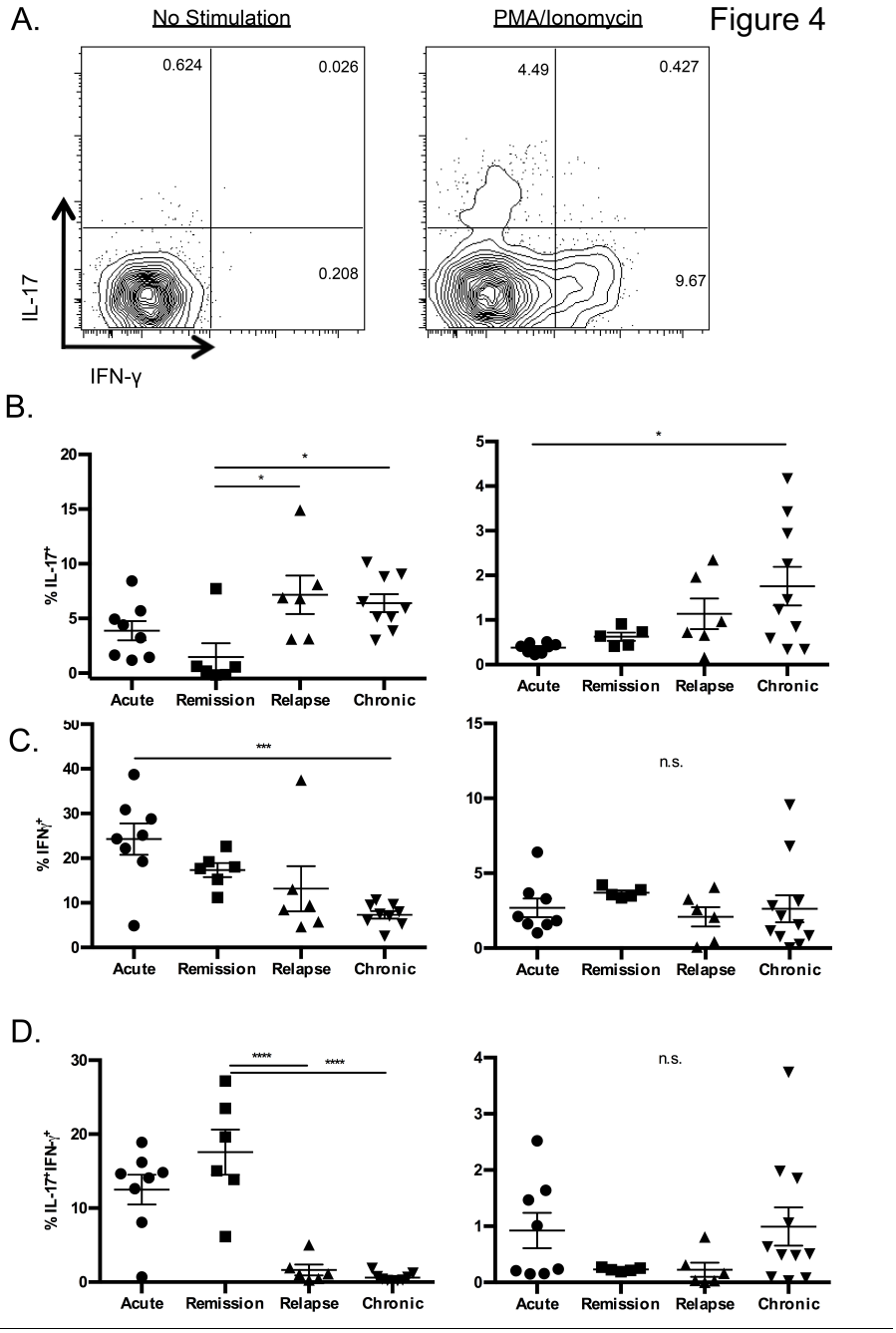

A.
No Stimulation
PMA/Ionomycin
Figure 4
0.624
0.026
0.208
4.49
0.427
9.67
IL-17
IFN-γ
B.
% IL-17+
Acute
Remission
Relapse
Chronic
C.
% IFNγ+
n.s.
D.
% IL-17+IFN-γ+
n.s.

Figure 5:

A.

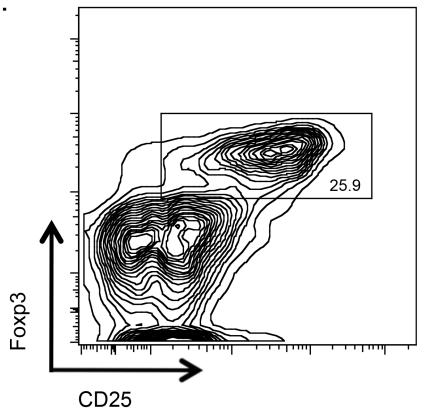
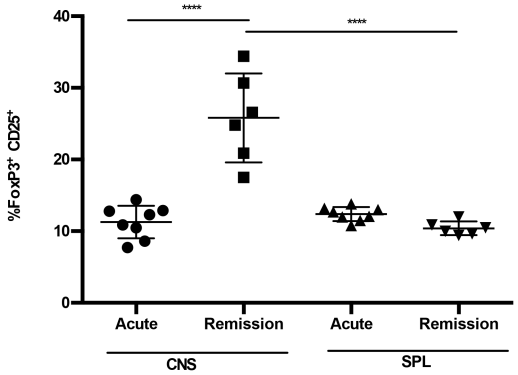


Figure 5

B.

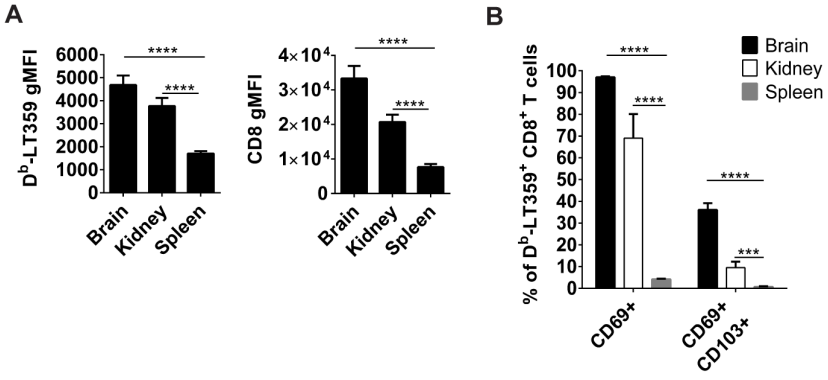


C.

Tregs:non-Tregs

Tissue	Acute	Remission
CNS	1 : 7.5	1 : 2.8
SPL	1:7	1: 6.5

Supplemental Figure 1.



Supplemental Figure 1. Phenotypic analysis of D^b-LT359$^+$ CD8$^+$ T cells from brain, kidney, and spleen at 30 dpi showing (E) mean ± SD of the gMFI of D^b-LT359 (left) and CD8 (right) and (F) frequency of cells expressing CD69 and CD103. Data are representative of 2 independent experiments of 4-5 mice/experiment. ***, $P < 0.0005$; ****, $P < 0.0001$.

FIGURE LEGENDS:

Figure 1: Secondary progressive disease course in NOD mice with MOG-induced disease

EAE was induced with a single s.c injection of 300μg of MOG_{35-55} emulsified in CFA. Mice received 250-300 ng of pertussis toxin i.p on days 0 and 2. Representative plots of clinical score (A) and weight loss presented as a percentage of pre-symptom onset weight (B) are shown for 3 mice from 3 independent experiments. Each plot shows the progression of clinical score and accompanying weight loss from a single mouse. (C) Total area under the curve of clinical score plotted against time was calculated for each mouse used in experiments. Mean AUC ± SEM is plotted for each time point. One-way ANOVA was performed for statistical significance with **** indicating $p<0.0001$.

Table I. Incidence and disease course of secondary progressive MOG-induced EAE in NOD mice.

Incidence of disease as well as mean day of onset of acute, relapsing and chronic symptoms in EAE experiments are summarized. Mice from Taconic and Jackson Laboratories as well as Emory-bred mice originating from both facilities are included. Maximum score for each time point within each experiment is reported. Mice that developed diabetes (blood glucose >250mg/dl) were excluded from experiments and all incidence calculations. Mice that did not become acutely sick were sacrificed and not used in experiments (or incidence calculations for relapse and chronic disease) but were included in calculation of acute incidence. Incidence of chronicity was calculated from all mice that developed acute symptoms regardless of whether or not a relapse in symptoms was observed. After mice were sacrificed for use in experiments they were not included in further incidence calculations. For example, experiments 1 through 10 involved the characterization of mice experiencing acute disease or symptom remission and thus no incidence of relapse or chronicity was calculated on these mice (with the exception of one surviving mouse in experiment #7).

Figure 2: MOG42-55 is the immunogenic IA^{g7}-restricted epitope within MOG35-55

A and B) MOG-primed lymph node cells from male mice were activated in culture on MOG_{35-55} for 10 days. Live cells were stimulated with indicated peptide epitopes for 5 hours. BFA was added for the latter 4.5 hours of this incubation. Cells were stained for flow cytometry and gated on lymphocytes (identified by FSC/SSC) and CD4+ cells. Statistical significance was determined using Sidak's multiple comparison test and one-way ANOVA. In A) significance of staining as compared to no stimulation control is indicated with *** and * corresponding with p values of 0.0007 and 0.02 respectively. In B) significance is indicated with *** and ** corresponding with p values of 0.0006 and 0.007 respectively.

C) Lymph node cells from 4-5 male mice were activated in culture on MOG_{35-55}. Live cells were stained with MOG-IA^{g7} tetramer and OVA-IA^{g7} tetramer was used as a negative control. Representative flow cytometry data plots shown are gated on lymphocytes as identified by FSC/SSC, and CD4-positive, CD11b/CD11c/CD19-negative gates. D) Summary tetramer staining data from 3 experiments of 4-5 male mice each. Each pair of data points represents control and MOG tetramer staining from a single experiment. Statistical significance was determined with a one-tailed, paired Student's t-test ($p=0.0044$).

Figure 3. CNS-infiltrating CD4 T cell affinity for MOG is greatest during acute disease.

CNS-infiltrating lymphocytes were isolated from the brain and spinal cord of MOG-induced mice. CD4 T cells were isolated by magnetic purification and the micropipette adhesion frequency assay was performed. A and B) Effective 2D affinities for MOG_{42-55} (shown) were calculated from adhesion frequency as well as pMHC and TCR surface density. Each individual data point represents the affinity of a single CD4 T cell. Each experiment was performed on the pooled CNS-infiltrating CD4 T cells from 2-10 mice and were representative of 4, 2, 2 and 3 independent experiments for acute, remission, relapse and chronic time points respectively. Bars represent the geometric mean affinity of the population. The dotted line is drawn at the geometric mean affinity for CD4 T cells isolated during acute disease (2.34×10^{-5} μm^4). Statistical significance determined with one-way ANOVA with *** indicating $p<0.001$ and ** indicating $0.001<p<0.01$. B) Frequency distributions of the log of affinities for all time points examined. All distributions adhere to a Gaussian distribution with R-square values of 0.6730 (acute), 0.9214 (remission), 0.8683 (relapse) and 0.8324 (chronic). C) Representative flow cytometry data of tetramer stain of mononuclear cells isolated from CNS with MOG-IA^{g7} tetramer and HEL-IA^{g7} tetramer as a negative control. Data shown is from the acute disease time point. Plots shown are gated on lymphocytes (FSC/SSC) and CD4-positive, CD11b/CD11c/CD19-negative gates. Data is representative of 2 experiments, n=2-3 mice each. D) Summary of micropipette experiments performed including number and frequency of MOG-reactive cells and geometric mean affinities for MOG. Cells were defined as antigen-reactive with a binding frequency above 0.12. Binding frequencies of

1.0 were tested on RBCs with a lower pMHC density to obtain an adhesion frequency between 0.12 and 1.0 (affinity cannot be calculated from a 1.0 adhesion frequency). Percentage of high affinity cells were calculated based on the percentage of MOG-reactive cells that bound with an affinity greater than the geometric mean of cells measured during acute disease (2.34×10^{-5} μm^4).

Figure 4. IL-17 production by CD4 T cells coincides with symptomatic disease while IFN-γ production declines throughout disease.

Mononuclear cells were isolated from brain and spinal cord samples from MOG-induced mice and processed for intracellular cytokine staining. Cytokine-producing cells were identified by flow cytometry, (A) representative plot of cytokine data and gating strategy from a CNS isolate from the chronic disease time point. Plots shown are gated on lymphocytes (FSC/SSC) and CD4-positive and CD11b/CD11c/CD19-negative cells. (B-D) Summaries of the data of cytokine production in the CNS (left panels) and spleen (right panels) are plotted displaying the mean frequency of cytokine producing cells as a percentage of CD4+ cells with error bars showing SEM. Summary data for IL-17 producing CD4 T cells (B) as well as IFN-γ (C) and IL-17/IFN-γ double-producers (D) are shown. Each data point is representative of a sample from one mouse, the data is representative of the following number of mice: 8, 5, 6, 11; and independent experiments: 2, 2, 5, 9; for acute, remission, relapse and chronic time points. Significance among all time points within each tissue (CNS or spleen) was analyzed with one-way ANOVA with Tukey's multiple comparison test. Significance is shown with * indicating $0.01<p<0.05$, **:$0.001<p<0.01$, and ***: $p<0.001$.

Figure 5. Tregs are more frequent in the CNS at disease remission than during acute disease.

Regulatory T cells (Tregs) were identified in the CNS and spleens of MOG-induced mice as $CD25^{+}Foxp3^{+}$ CD4 T cells by flow cytometry. (A) A representative flow plot showing the identification of Tregs from the spleen of a mouse experiencing symptom remission is shown. Cells shown have been gated on lymphocytes (FSC/SSC) and CD4-positive, CD11b/CD11c/CD19-negative cells. B) The frequency of Tregs in the CNS and spleen are shown as a percentage of CD4+ T cells. Bars are plotted at the mean frequency ± SEM. Acute time point data were collected from 8 mice from 3 independent experiments. Remission data are representative of 6 mice in 2 independent experiments. One-way ANOVA was used to determine significance, **** indicates $p<0.0001$. C) Ratios of the number of infiltrating Tregs to the number of non-Tregs ($CD4^{+}Foxp3^{-}$) in the CNS and spleen are shown.

CHAPTER 2:

TITLE: HLA-DR4 Presentation of Citrullinated Modified MOG Epitope Drives Demyelinating Disease

ABSTRACT

Autoimmune disease has been suggested to progress through the recognition of new or modified epitopes. Deimination of arginine to form citrulline within connective tissue epitopes can occur as a result of inflammation in rheumatoid arthritis, and T cell reactivity to citrullinated epitopes has been demonstrated particularly in patients expressing HLA-DR4. As the HLA-DR4 gene is also associated with increased risk for multiple sclerosis, we make use of the humanized HLA-DR4 mouse model to demonstrate that the progressive demyelinating disease in these mice in response to myelin oligodendrocyte glycoprotein (MOG) immunization involves not only a T cell response specific for MOG but also a substantial, measurable population of cells that are increasingly in reactive to citrullinated MOG (citMOG). The micropipette adhesion frequency assay was used to obtain a sensitive, physiologically relevant measurement of frequency and TCR affinity for MOG as well as citMOG as these T cells are not detectable by tetramer staining. We examined CNS-infiltrating CD4 T cells by the micropipette assay and found a substantial portion of CD4 T cells were cross-reactive to both MOG and citMOG. Interestingly, CD4 T cells that were only reactive to MOG decreased in frequency while those that were reactive only to citMOG increased throughout the course of disease and were exclusively detected at symptomatic time points. HLA-DR4 tetramers did not accurately distinguish between cells that were reactive to MOG and those that were cross-reactive to both epitopes. Further, we confirmed the functional reactivity of these cells and found that both MOG and citrullinated MOG upregulate the transcription factors IRF4 and Nur77 in CNS-infiltrating CD4 T cells. Despite this, immunization with citrullinated MOG does not

induce demyelinating disease. These results are the first demonstration of T cell reactivity to citrullinated-MOG epitopes in the context of HLA-DR4 demyelinating disease and the first demonstration of cross-reactivity to native and citrullinated epitopes of MOG. Further, reactivity to the citrullinated neoepitope has great implications for use as a biomarker of disease and as a target in antigen-specific immunomodulatory therapies.

INTRODUCTION

Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation and demyelination of the central nervous system (CNS). HLA class II genes confer the greatest genetic risk for MS supporting the role for a CD4 T cell mediated disease pathology (6). HLA-DR4 (DRB1*0401) is associated with an increased risk for MS and is known to present an epitope of myelin oligodendrocyte glycoprotein (MOG) epitope that has been shown to be encephalitogenic in mice that express HLA-DRB1*0401 instead of murine class II genes (118). CD4 T cell reactivity to this MOG_{97-109} epitope has also been demonstrated in the peripheral blood of MS patients (110). In MOG-induced disease in DRB1*0401-expressing mice, reactivity to novel myelin epitopes including MBP and PLP has been suggested to occur, thus demonstrating epitope spread within the DR4 demyelinating disease model (118).

The amino acid citrulline is the product of a post-translational deimination of arginine catalyzed by peptidyl arginine deiminase (PAD) enzymes that are upregulated during inflammation (153). Citrulline has been studied in the context of MS for over a decade with initial studies finding an increased proportion of citrullinated MBP in MS patient brains compared to healthy control brains (139). In mice with experimental autoimmune encephalomyelitis (EAE), the presence of citrullinated MBP in the brain and spinal cord coordinates with the onset of symptoms and intensifies with symptom severity (141). Citrullinated MBP has been demonstrated to be encephalitogenic in both mice and rats and T cell reactivity to citrullinated MBP has been demonstrated in MS patient peripheral blood (142, 143, 146). Investigations into the presence or relevance of a citrullinated MOG epitope have shown no difference in MOG-induced disease in B6

mice deficient in PAD2, and therefore unable to generate citrulline, compared to wildtype mice (148). Interestingly, other studies in the B6 model have identified 2 different citrullinated altered peptide ligands (APL) within the MOG35-55 epitope that elicit T cell responses (149).

Both T and B cell reactivity to citrulline have been demonstrated in rheumatoid arthritis (RA) patients and mouse models. RA is an autoimmune disease characterized by inflammation and destruction of the joint synovium and, like MS, risk of developing this disease is associated with HLA-DR4 haplotype. Patients with RA, particularly those expressing HLA-DR4, are known to develop anti-citrullinated protein antibodies (ACPAs) and recent studies have shown citrulline-specific CD4 T cell responses in RA patients (121, 127, 134).

Given the strong evidence linking HLA-DR4 and citrulline within the RA model and the increased risk for MS conferred by the HLA-DR4 gene, the goal of the present study was to examine a role for citrulline within the context of MOG-induced demyelinating disease in HLA-DR4-expressing mice. As $MOG_{97\text{-}109}$ contains an arginine residue at position 101, we use the micropipette adhesion frequency assay to determine the frequency and two-dimensional (2D) affinity of responding CD4 T cells specific for MOG, citrullinated MOG, and cells that are cross-reactive to both antigens. The micropipette assay is unique in that it provides a sensitive and physiologically relevant measurement of TCR affinity of CD4 T cells that cannot be identified by MHC class II tetramers (28, 47). The micropipette assay also allows for the interrogation of a single T cell for its affinity for multiple antigens uniquely allowing for the quantification of affinity of cells cross-reactive to both MOG and citMOG. We found that, by

micropipette, a cross-reactive population of CD4 T cells can be identified in mice induced with MOG_{97-109} before the onset of symptoms and this population increases through the development of chronic symptoms. HLA-DR4 tetramers grossly underestimate the frequency of this population in the CNS and the periphery. A population of CD4 T cells reactive only to citMOG develops during acute symptoms and is only observed during symptomatic stages of disease suggesting its role in eliciting or exacerbating symptoms. Further, both citMOG and MOG are able to signal through the T cell receptor as downstream targets IRF4 and Nur77 are upregulated in response to both peptides. These findings have great implications for a role for citMOG-specific CD4 T cells in exacerbating disease and suggest that the detection of citMOG-reactive CD4 T cells could be useful as a biomarker of disease severity and/or therapeutic efficacy.

MATERIALS AND METHODS

Mice

HLA-DR4 transgenic mice (B6.129S2-H2-Ab1^{tm1Gru} Tg (HLA-DRA/H2-Ea, HLA-DRB1*0401/H2-Eb)1Kito) were purchased from Taconic facilities. Mice were bred and housed in the Emory University Division of Animal Resources facilities and were used in accordance with protocols approved by the Institutional Animal Care and Use Committee. Male and female mice aged 6-8 weeks were used in experiments as both experienced similar disease incidence and severity.

EAE Induction

EAE was induced with two injections of 200µg MOG97-109 (TCFFRDHSYQEEA) peptide emulsified in CFA containing 5 mg/ml heat-killed *Mycobacterium tuberculosis* (Difco) at days 0 and 7. Mice also received injections of 250 ng of Pertussis toxin (List Biological Laboratories) i.p on days 0 and 2. The same induction protocol was used for experiments containing MOG97-109Cit101 (TCFF-Cit-DHSYQEEA). All peptides were synthesized on a Prelude Peptide Synthesizer (Protein Technologies). Disease severity was assessed using the following scoring rubric: 0, no disease; 1, tail atony or stiffness following atonic tail; 1.5, single hindlimb weakness/poor grip; 2, symmetrical hindlimb weakness/poor grip; 3, hindlimb paralysis; hindlimb paralysis with some forelimb paralysis; 5, moribund.

CNS Mononuclear Cell Isolation

Mice were euthanized with CO_2 and perfused through the left ventricle with PBS. Brain, spinal cord, cervical lymph nodes and spleen were harvested from each animal and homogenized using a 100-µm-cell strainer. Mononuclear cells were isolated using a

Percoll (Sigma-Aldrich) gradient, and were prepared for magnetic separation or cell-staining for micropipette and flow cytometry experiments, respectively.

Cells and Reagents

Media used in tetramer staining, cell stimulation and cell culture consisted of RPMI 1640 medium (Mediatech) supplemented with 10% FBS (HyClone), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Mediatech), 100 μg/ml gentamycin (Mediatech), and 2 x 10^{-5} M 2-ME (Sigma Aldrich). MOG- specific and CitMOG- specific lymphocytes were generated from by footpad and tail base priming with 100 μg MOG_{97-109} or $MOG_{97-109Cit101}$ emulsified in CFA containing 1 mg/ml heat-killed *M. tuberculosis*. Inguinal, lumbar, and popliteal lymph nodes were harvested and homogenized 12-14 days post-priming, and were analyzed *ex vivo* by tetramer staining and micropipette analysis.

Adoptive Transfer Experiments

Lymphocytes isolated from lymph node priming experiments stimulated in culture for 4-5 days on inducing peptide. Post-stimulation, live cells were purified with Lymphocyte Separation Medium (Corning Cellgro) and were transferred into naïve HLA-DR4 mice. Each naive mouse received 5-6 x 10^6 cells by tail vein injection. One day post-transfer, recipient mice were given a single injection of 200 μg of inducing peptide (MOG or citMOG) emulsified in Incomplete Freund's Adjuvant, along with 250 ng Pertussis toxin i.p (days 1 and 3 post-transfer). Mice were monitored for disease symptoms.

Tetramer Staining

MOG_{97-109}-DRB1*0401, $MOG_{97-109Cit101}$-DRB1*0401, and human $CLIP_{87-101}$-DRB1*0401 tetramers were generously provided by The National Institutes of Health

Tetramer Core Facility at Emory University (Atlanta, GA). Mononuclear cells isolated from the CNS were stained immediately post-Percoll gradient separation. Cells isolated from lymph nodes as well as splenocytes were stained post-homogenization. Cells were stained with 8 mg/ml tetramer in complete media for 4 hours at 37˚ C. Cells were then stained with anti-CD4 FITC (RM4-5; BD Pharmingen), anti-CD11b PerCP (M1/70; BD Pharmingen), anti-CD11c PerCP (HL3; BD Pharmingen), anti-CD19 PerCP (1D3; BD Pharmingen), anti-CD44 PE-Cy7 (IM7; Biolegend), and anti-CD8a V450 (53-6.1; Tonbo Biosciences) for 15 minutes at room temperature in the dark. Flow Cytometry was performed on a LSRII Flow Cytometer (BD Biosciences) and all data were analyzed using FloJo software (TreeStar).

Transcription Factor Analysis

Mononuclear cells isolated from the CNS as well as homogenized lymphocytes and splenocytes were stimulated in culture media for 6 h with 50 μM of peptide at 37˚C. Isotype control wells were stimulated with 50 μM MOG peptide. Cells were stained extracellularly with anti-CD4 FITC, anti-CD11b PerCP, anti-CD11c PerCP, anti-CD19 PerCP for 15 minutes at room temperature in the dark. Cells were fixed and permeabilized using FoxP3/Transcription Factor Staining Buffer set (eBioscience), per manufacturer's instructions. Cells were then stained for intracellular factors using anti-IRF4 eFluor 450 (3E4; eBioscience) and anti-Nur77 PE (12.14; eBioscience) or anti-FoxP3 PE (FJK-16s, eBioscience) for 30 minutes at 4˚ C. Mouse IgG1 K isotype controls in PE and eFluor 450 (both clone P3.6.2.8.1; eBioscience) were used for all IRF4 and Nur77 experiments.

Micropipette Adhesion Frequency Assay

A detailed description of the micropipette adhesion frequency assay has been published (28, 168, 171). Briefly, human red blood cells (RBCs) were biotinylated using Biotin-X-NHS (EMD4 Biosciences), coated in streptavidin (Thermo Scientific) and then coated with biotinylated peptide:HLA class II monomers. Mononuclear cells were obtained from the brain and spinal cord of MOG-immunized mice and CD4 T cells were isolated by CD4-postiive magnetic separation using MACS L3T4 selecting beads (Miltenyi Biotec). CD4 T cells were similarly isolated by magnetic purification from homogenized lymph nodes and spleen. RBCs were coated with DRB1*0401 monomers presenting MOG97-109, MOG97-109cit101, and hCLIP87-101. A single pHLA-coated RBC and a CD4 T cell were aspirated onto opposing micropipettes in a glass chamber mounted on the stage of an inverted light microscope. Cells were aligned and then brought into contact using a computer-controlled piezoelectric actuator that maintained constant contact time (2s) and area. Upon retraction of the T cell from the stationary RBC, a binding even was visualized as elongation/distension of the ultrasoft RBC membrane. When no binding occurs, the RBC retains its spherical shape and these nonbinding events are recorded. Cells were brought into contact 30 times and an adhesion frequency (Pa) was calculated. Cells that bind pHLA with an adhesion frequency of 0.1 or greater are considered to be specific to that pHLA. Frequency of antigen-specific T cells is determined by the number of T cells with an adhesion frequency > 0.1 divided by the total number of cells assessed. T cells are screened for antigen specificity using an RBC coated with a high density of pHLA monomer and if the T cell binds with every contact a lower density RBC is used to determine affinity. Quantification of surface densities of pMHC and TCRß was

determined by flow cytometry using BD QuantiBRITE PE beads for standardization (BD Biosciences). Surface densities as well as adhesion frequency were used to calculate two-dimensional affinity of each cell using the following equation: $A_cK_a = -\ln[1 - P_a(\infty)]/m_r m_l$ where m_r and m_l represent TCR and pMHC surface densities, respectively. Geometric mean affinities are reported ± SEM.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6 Software (Software for Science). Significance was calculated using one-way ANOVA and Student's t-tests. P-values are reported.

RESULTS

MOG97-109 induces chronic demyelinating disease in DR4 mice while citrullinated MOG does not

A previously identified encephalitogenic epitope (MOG_{97-109}) was used to induce demyelinating disease in male and female mice expressing HLA-DRB1*0401 (HLA-DR4) in place of endogenous murine MHC class II genes. This epitope of MOG contains a critical arginine in position 101, which, based on published data, has been postulated to be in the TCR contact position P3 in the 9 amino acid core (MOG_{99-107}) (Figure 1A). This arginine is susceptible to a deimination reaction that is catalyzed by PAD enzymes that are upregulated in the context of inflammation (Figure 1B). The deimination of arginine yields the amino acid citrulline, which is slightly larger (1 Dalton) and less positively charged than arginine. While MOG_{97-109} has been shown by others to be encephalitogenic (118), we sought to elucidate whether the citrullinated MOG epitope was also encephalitogenic and whether a citrullinated MOG (citMOG) specific CD4 T cell response could be generated upon immunization. HLA-DR4- expressing mice were immunized with MOG and citMOG epitopes emulsified in Complete Freund's Adjuvant and assessed daily for symptoms of demyelinating disease. Mice induced with MOG_{97-109} experienced symptoms with an incidence of 79.0% (49/62 mice) with symptoms beginning on average at day 23 while mice induced with the citrullinated epitope of MOG did not experience disease symptoms (followed to at least 60 days post-induction) (Figure 1C). This data indicates that a citMOG-specific CD4 T cell response that is generated from demyelinating disease induction protocols is not sufficient in and of itself to elicit disease symptoms.

MOG and CitMOG Reactivity Detected in CNS throughout disease

As the citrullinated MOG epitope was not able to elicit disease symptoms on its own, the question remained whether or not a citMOG-specific CD4 T cell response is generated during MOG-induced disease. In order to determine the antigen-specificity of cells contributing to demyelinating disease, CD4 T cells were isolated from the CNS at pre-symptomatic, acute and chronic time points of MOG-induced disease. The pre-symptomatic mice were sacrificed 10 days post-induction before symptoms appeared while acute mice were analyzed within 7 days of symptom onset and chronic mice were evaluated after at least 14 days of symptoms. Antigen-specificity of CD4 T cells was determined using the micropipette adhesion frequency assay. A detailed description of the micropipette adhesion frequency can be found elsewhere (28, 47). Briefly, human red blood cells (RBCs) were coated with various surface densities of peptide:HLA (pHLA) monomers using biotin-streptavidin interactions. A T cell of interest aspirated on one micropipette was brought into contact with a RBC coated with either MOG:HLA-DR4, CitMOG:HLA-DR4 or human CLIP:HLA-DR4. Upon retraction of the T cell, a TCR:pHLA binding event was visualized as distention of the RBC membrane whereas when no binding occurs the RBC membrane remains spherical. After 30 touches, an adhesion frequency is calculated and is used to determine the 2D affinity of TCR for pHLA. T cells are initially interrogated with RBCs coated with a high-density of pHLA. If the T cell expresses high affinity TCRs, the TCRs will bind the RBC on every contact and the adhesion frequency will be 1. At this point, an RBC coated with a lower surface density of pHLA is used to determine 2D affinity.

CD4 T cells isolated from the CNS of pre-symptomatic, acutely and chronically symptomatic mice were evaluated for their reactivity towards MOG, citMOG and hCLIP (Figure 2 A-C). The micropipette adhesion frequency assay uniquely allows for the quantification of the affinity of a single T cell for multiple antigens as the same T cell can be interrogated with multiple RBCs coated with different antigens. In mice induced with MOG_{97-109}, 59 CD4 T cells isolated from the brain and spinal cord at the pre-symptomatic time point were assessed for reactivity to MOG and citMOG (Figure 2A). We found 36 out of 59 CD4 T cells in the brain (61.0%) to be cross-reactive to both MOG and citMOG whereas 13 cells were reactive to MOG alone (23.3%) and only one cell was reactive to citMOG alone (1.7%). Cells that were cross-reactive exhibited a significantly higher affinity for MOG than those that were only reactive to MOG ($p=0.0008$) (Figure 2B).

For analysis of acutely symptomatic mice, 133 CNS-infiltrating CD4 T cells were evaluated by the micropipette 39 of which were determined to be cross-reactive to MOG and citMOG (29.3%), 32 were reactive to citMOG alone (24.1%) and 16 were reactive only to MOG (12.0%). Again it was observed that cells that were cross-reactive had significantly higher mean affinities than cells that were only reactive to either peptide ($p=0.039$ for MOG and $p<0.0001$ for citMOG). For CD4 T cells isolated from the CNS of chronically symptomatic mice, 39 out of 71 cells analyzed were cross-reactive to MOG and citMOG (54.9%) while 13 cells (18.3%) were reactive to only citMOG and no cells were detected that were reactive to MOG alone. Again cross-reactive cells possessed a higher affinity for citMOG than did CD4 T cells only reactive to citMOG ($p=0.049$).

In order to evaluate the significance of the citMOG-reactive and cross-reactive cells to disease pathology, peripheral CD4 T cells isolated from the spleen of chronically symptomatic mice were evaluated for reactivity to MOG and citMOG by the micropipette adhesion frequency assay. Of 100 cells analyzed, only 7 cells (7.0%) were found to be cross-reactive to both MOG and citMOG, which demonstrates a nearly 8-fold enrichment of these cross-reactive cells in the CNS during chronic disease (Figure 2G). These cross-reactive populations were not found to have significantly higher affinities for antigen than their monoreactive counterparts, a distinct difference from cross-reactive cells evaluated at all time points in the CNS (Figure 2H). Additionally, 22 (22.0%) CD4 T cells in the spleen were reactive to citMOG alone and 12 (12.0%) were reactive to MOG alone indicating cells reactive only to MOG can still be found in the periphery although not detected in the CNS during chronic symptoms.

Interestingly, for all time points cross-reactive cells in the CNS possessed a higher TCR affinity for individual antigens than did cells reactive to only one antigen. Important to note is that of the single-specificity populations, the MOG-specific population dominates by frequency during the pre-symptomatic time point whereas at the acute time point a greater frequency of citMOG-reactive cells is observed. As the frequency of Tregs at both time points is comparable in each tissue (Figure 2I), we do not believe the changing reactivity of CD4 T cells in the brain is due to an influx or efflux of Tregs. By the time chronic symptoms are reached, no population reactive strictly to MOG can be detected demonstrating the evolution of a citrulline-specific response that correlates with the presence of symptoms. Further demonstrating this is the gradual increase in geometric

mean affinity for citMOG among cells reactive only to citMOG from pre-symptomatic through chronic time points.

Cross-Reactive CD4 T cells Trend Towards Higher Affinity for MOG

Given the prevalence of CD4 T cells in the CNS that recognize both MOG and citMOG the properties of this population were further evaluated. 2D affinities of cross-reactive T cells for each antigen were compared to each other and evaluated on a cell-by-cell basis (Figure 3 A-C). For all time points, there existed mixed populations of cells with some cells seeing MOG with a higher affinity than citMOG and some that possessed a higher affinity for citMOG. Among the CNS-infiltrating cross-reactive CD4 T cells, no significant difference between 2D affinities for MOG and citMOG was observed at any time point examined. Interestingly, the splenic cross-reactive cells from chronic disease trended toward higher affinity for citMOG although this did not reach significance ($p=0.09$) (Figure 3D).

To examine the affinities of cross-reactive cells as a population, the logarithm of the 2D affinity of a single cell for MOG and CitMOG were plotted on X and Y axes respectively and linear regression was performed. Of all CNS-infiltrating CD4 populations, the affinities measured during acute disease showed the least linear correlation between affinities for MOG and CitMOG ($r^2= 0.1438$) (Figure 3F). Interestingly, the affinities of CNS-infiltrating cross-reactive cells analyzed at pre-symptomatic and chronic time points were similar in their degree of linear correlation ($r^2= 0.4467$ and 0.4375, for pre-symptomatic and chronic, respectively) (Figure 3 E and G). For both populations, affinities for MOG trended higher than affinities for citMOG

on a cell-by-cell basis as evidenced by the slope of both lines being <1 (slopes=0.6112 and 0.5376 for pre-symptomatic and chronic, respectively). By contrast, the cross-reactive cells isolated from the spleens of chronically symptomatic mice possessed affinities for MOG and citMOG that were the most linearly related out of all populations examined (Figure 3H, r^2=0.5056). Further, this correlation favored stronger affinities for citMOG on a cell-by-cell basis (slope=1.25) indicating cross-reactive populations that traffic to the CNS may differ phenotypically than those that remain in the periphery.

HLA-DRB1*0401 Tetramer Staining Detects MOG and Cross-Reactive Cells Although Does Not Reflect the Diversity of Response in CNS

To further investigate the CD4 T cell reactivity to MOG and citMOG, HLA-DR4 tetramers were used to detect myelin-reactive populations in the CNS, cervical lymph nodes and spleen. While MHC class II tetramers have been demonstrated to not effectively detect self-reactive CD4 T cells in a population (28, 47, 115) we attempted their use, as they are high throughput and have been used in the context of HLA-DR4+ MS patients (110). PE-labeled MOG_{97-109}:HLA-DR4 and APC-labeled $MOG_{97-109cit101}$:HLA-DR4 tetramers were used to identify populations that were reactive to citMOG or MOG alone and populations that were cross-reactive (Figure 4A). No CD4 T cells reactive only to citMOG were able to be identified in the CNS at acute or chronic time points by tetramer staining (Figure 4B). Interestingly, CD4 T cells in the CNS stained significantly with MOG-tetramer at both acute and chronic time points showing 9.548% and 15.53% MOG-reactive above background for acute and chronic, respectively (Figure 4C, Table 1). Significant populations of cross-reactive cells stained with both

tetramers at both acute and chronic time points but neither population amounted to more than 1% of CNS-infiltrating T cells (0.3698% and 0.5850% above background for acute and chronic, respectively) (Figure 4D). Strikingly, tetramer data would indicate an enrichment of MOG-specific cells with progression from acute to chronic disease whereas micropipette data indicate a decline in MOG-reactive cells but an enrichment in the frequency of cross-reactive cells. Further, the tetramer assay identified significant populations of cross-reactive cells at both acute and chronic time points as did the micropipette however the frequency of the populations identified by tetramer staining was a fraction of those measured with the micropipette adhesion frequency assay. This indicates that while the tetramer assay is likely accurately identifying CD4 T cells reactive to MOG it fails to adequately detect cross-reactive populations likely due to a problem identifying citMOG-reactive cells.

Tetramer staining was also performed on the cervical lymph nodes and spleens of mice with acute and chronic symptoms. Similar trends were observed in that no population of CD4 T cells reactive only to citMOG was detected at any time point in either tissue (Figure 4 E-H). Similar to the tetramer results in the CNS, populations of cells reactive to MOG alone and cross-reactive to both MOG and citMOG were identified in the cervical lymph nodes and spleen at both time points although only achieved significance during chronic symptoms. Of note is that significant MOG-reactive and cross-reactive populations were identified in the spleen of chronically symptomatic mice whereas the micropipette assay showed a citMOG-dominant response with smaller cross-reactive and MOG-reactive populations.

CD4 T Cells Upregulate IRF4 and Nur77 in Response to MOG and CitMOG at Acute and Chronic Time Points

In order to determine the functional relevance of the MOG and citMOG-reactive cells to disease pathology, the expression of transcription factor IRF4 was examined in CD4 T cells isolated from the CNS, cervical lymph nodes and spleen at acute and chronic time points. IRF4 is a transcription factor that is upregulated upon TCR stimulation and is downstream of NFAT signaling (192). IRF4 has also been shown to be a crucial controller of IL-17 and IL-21 production and thus a key mediator of Th17 pathology (192). CD4 T cells isolated from the CNS, cervical lymph nodes and spleens of acutely and chronically symptomatic mice were analyzed for IRF4 expression after stimulation with MOG or citMOG peptides for 6 hours *in vitro* (Figure 5A). At acute and chronic time points, CD4 T cells isolated from all tissues significantly upregulated IRF4 expression in response to MOG and citMOG peptide stimulation (Figure 5B-D). Further, the gMFI of MOG-stimulated CD4 T cells isolated from the all tissues at both acute and chronic time points trended significantly higher than that of the citMOG stimulated cells (Figure 5B). The upregulation of IRF4 in response to both peptides in CNS-infiltrating CD4 T cells at both acute and chronic time points supports the findings obtained by the micropipette in that cross-reactive CD4 T cells predominate in the CNS throughout symptomatic disease (Figure 5B).

IRF4 upregulation in MOG-stimulated cells was significantly higher than that of citMOG-stimulated cells in all tissues at both time points (Figure 5B-D). Taken together these observations support the micropipette data indicating an enrichment of cross-

reactive CD4 T cells in the CNS and further suggest the different phenotypic profiles of cross-reactive cells in the CNS compared to those in the periphery.

To further understand the differences in CD4 T cell reactivity to MOG and citMOG the expression of Nur77 was analyzed as Nur77 is an immediate early gene upregulated by TCR signaling and is used as a readout of TCR signal strength (193). In the CNS at the acute point, Nur77 expression was induced in CD4 T cells stimulated with MOG and those stimulated with citMOG whereas at the chronic time point Nur77 was only significantly upregulated in cells stimulated with MOG (Figure 6B). At the chronic time point, CNS-infiltrating CD4 T cells stimulated with citMOG upregulated Nur77 but this did not reach statistical significance ($p=0.0575$). These data support the findings obtained with the micropipette assay and IRF4 staining as they identify reactivity to both MOG and citMOG at symptomatic time points of disease implying a role for cross-reactive CD4 T cells in eliciting symptoms. Interestingly, no significant upregulation of Nur77 was observed during acute symptoms in the periphery while CD4 T cells isolated from both cervical lymph nodes and spleen at chronic time points demonstrated a significant increase in Nur77 expression in response to both peptides (Figure 6 C & D). These observations confirm the enrichment of both MOG- and citMOG-specific CD4 T cells in the CNS during acute disease and the enrichment of citMOG-specific cells at the chronic time points while also demonstrating an evolution of functional CD4 T cell reactivity to both antigens with disease progression.

Peripherally primed MOG-reactive lymphocytes generate more cross-reactivity and are able to elicit demyelinating disease while CitMOG-primed cells do not

Lymph node priming with either MOG or citMOG peptide was performed in order to determine the differences in MOG and citMOG affinities and functional reactivities outside of the context of demyelinating disease. *Ex-vivo* MOG and CitMOG primed lymph nodes were evaluated by tetramer staining (Figure 7A, D) and no population stained significantly with HLA-DR4 tetramers. Interestingly, the population that trended towards being best-identfied by tetramer was the cross-reactive population of CD4 T cells although still underestimated by frequency in comparison to the micropipette assay (Figure 7A and D). To elucidate TCR reactivity to MOG and citMOG after lymph node priming, the micropipette adhesion frequency assay was performed on ex vivo CD4 lymphocytes purified from MOG-primed lymph nodes (Figure B-C). Out of 107 CD4 T cells interrogated for affinities for MOG and citMOG, 19 cells (17.8%) were cross-reactive to both MOG and citMOG while 13 cells (12.1%) were reactive only to MOG and 3 cells (2.8%) were reactive only to citMOG (Figure 7C). CitMOG-primed CD4 lymphocytes were also analyzed by micropipette and out of 70 cells analyzed, 16 cells (22.9%) were reactive only citMOG while only 6 cells (8.6%) were cross-reactive to both antigens and no cells were oberved to be reactive to MOG alone (Figure 7 E and F). As seen in peripheral CD4 T cells during chronic disease, the cross-reactive cells did not possess significantly higher affinity for antigen than did the cells only reactive to that antigen.

To determine the functional reactivity of these primed lymphocytes, cells isolated from lymph nodes were stimulated in vitro with the priming peptide for 72-96 hours and adoptively transferred into HLA-DR4^{+} recipient mice. After receiving a modified induction injection (peptide in IFA, pertussis at 0 and 48 hours), mice receiving MOG-

reactive CD4 T cells did develop disease symptoms, the incidence and severity of which were not as robust as for mice that had been induced with MOG peptide and CFA (Figure 7G). Mice that received citMOG-primed cells did not develop disease symptoms (followed 60+ days). These observations indicate that citMOG-reactive CD4 T cells are not capable of causing disease independently of a robust cross-reactive population.

DISCUSSION

The present study aims to determine the roles for citrullinated-MOG and native MOG epitopes when presented by HLA-DRB1*0401 (HLA-DR4) molecules in the context of MOG-induced demyelinating disease. Disease induction with native MOG was able to induce robust progressive demyelinating disease while induction with citMOG elicited no symptoms. We found that while both MOG-specific and cross-reactive cells were detectable by both HLA-DR4 tetramers and by the micropipette adhesion frequency assay in MOG-induced demyelinating disease, the latter proved to be a more sensitive technique for measuring the frequency of citMOG-reactive and cross-reactive cells. We observed a significant cross-reactive CD4 T cell population in the CNS at all time points while the population reactive to MOG alone decreased over the course of disease and the frequency of citMOG-reactive cells increased throughout disease and was notably only detectable at symptomatic time points. Within the HLA-DR4 model, we propose that in response to MOG-induced disease, MOG-specific and cross-reactive T cells generated in the periphery traffick to the CNS and initiate inflammation and demyelination which in turn upregulated the expression of PAD enzymes. These enzymes convert arginine residues within myelin into citrulline and a new citMOG-specific CD4 T cell response is primed. These citMOG-specific T cells are delayed in their appearance as PAD enzymes must be induced and generate citrulline before this response can be primed and expanded. As such, the citMOG-specific T cell response is only observable during symptomatic time points of disease and we believe these cells function to exacerbate disease symptoms and contribute to disease progression.

The HLA-DR4 tetramers misidentified the CNS-infiltrating cells by 1) failing to detect citMOG-reactive cells, 2) underestimating the frequency of cross-reactive cells, and 3) over-estimating the frequency of MOG-reactive cells. The underidentification of citMOG-reactive CD4 T cells can be explained by the observation that at all time points, cross-reactive cells posessed a higher affinity for citMOG than cells that were only reactive to citMOG. Tetramers are known to preferentially identify high affinity cells within a population and the cells reactive only to citMOG may be below the threshold of tetramer-based detection (28, 47, 115). Tetramer stains performed on CD4 T cells from the CNS at acute and chronic time points underestimated the frequency of cross-reactive cells by 30- to 50-fold and seemed to over-report cells that were reactive only to MOG. The excessive identification of MOG-reactive CD4 T cells in the CNS by tetramer is explained by the observation that on a cell-by-cell basis, cross-reactive CD4 T cells were inclined to have higher affinities for MOG than for citMOG and it can be concluded that the tetramer-reported MOG-reactive cells are likely cross-reactive cells that did not appropriately stain with citMOG tetramer. CitMOG tetramers made with the PE-Cy7 fluorophore, more comprable in size to PE than APC, showed no improved identification of citMOG-reactive or cross-reactive cells (data not shown).

Citrullinated MOG peptide was not able to induce disease in DR4-expressing mice and interestingly citMOG-specific T cells were not able to adoptively transfer disease. Similar findings have been reported in the context of citrullinated APLs of MOG_{35-55} in B6 EAE in that these citrullinated epitopes were able to generate more robust proliferative resopnses from CD4 T cells than native MOG-peptide however citrulline-specific cells were not able to elicit demyelinating disease on their own (149).

Similarly, in our study we detect a substantial frequency of cells reactive to citrulline during acute and chronic time points even outnumbering cells reactive only to MOG. These robust T cell responses to citrullinated epitopes are thought to occur due to the absence of this post-translationally modified amino acid product in the thymus during thymic selection therefore allowing for the expansion of CD4 T cells specific to citrullinated peptides(149). Similarly, the inability of citMOG-specific CD4 T cells to elicit demyelinating disease on their own is intuitive as there should be no citrulline-containing myelin targets in the CNS in the absence of inflammation and subsequent expression of PAD enzymes. In the B6 model, the mild EAE symptoms observed in response to citMOG peptide priming were attributable to the substantial amount of cross-reactivity observed when priming with the citrullinated APL (149). In our HLA-DR4 model, citMOG peptide priming is not able to generate a significant cross-reactive population and thus is not able to initiate demyelination in the CNS. We postulate that citMOG-specific CD4 T cells in DR4 demyelinating disease serve to exacerbate symptoms and contribute to disease progression. We believe the role of these cells is dependent upon HLA-DR4 as citMOG has been demonstrated to elicit MS-like pathology in rhesus monkeys with distinct MHC expression (150).

The correlation between frequency of citMOG-specific CD4 T cells in the CNS and symptom severity is of great interest in the value these cells hold as a biomarker for disease. Due to the fact that citrulline is generated in the context of inflammation, cells specific to citrullinated myelin epitopes should not be detectable outside of the context of inflammatory CNS disease. In our HLA-DR4 model, CD4 T cells reactive to citMOG alone are nearly undetectable at pre-symptomatic disease time points while these cells

number roughly a quarter of the CNS-infiltrating CD4 T lymphocytes during acute and chronic time points. This highlights the delayed kinetics of cit-specific CD4 T cell responses allowing for the generation of citrulline by PAD enzyme activity. Interestingly, citMOG-specific cells are also observable in the periphery at the chronic time point which adds value to the use of these cells as a biomarker.

Lymphocytes perpiherally primed with MOG or citMOG also showed very distinct affinity profiles with MOG-priming generating mostly cross-reactive and MOG-reactive cells while citMOG-priming was less efficient at producing cross-reactive cells. Lymphocytes primed with MOG were able to elicit symptoms upon adoptive transfer while those primed with citMOG were not supporting the crucial need for either cross-reactive or MOG-reactive cells in the development of CNS inflammation and disease pathology. The inability of citMOG-specific lymphocytes to transfer disease contrasts starkly with RA models in which priming with citrullinated peptides and transfer of cit-specific CD4 T cells are both able to elicit arthritis symptoms (131, 133). In HLA-DR4-expressing mice, immunization with citrullinated synovial antigen peptides are able to elicit disease while native peptides are not (131). The differences between the RA models and the demyelinating disease model studied herein may be explained by the differences in the way these peptides are presented by HLA-DR4. The MOG_{97-109} peptide contains arginine/citrulline at position 101, P3 within the core epitope, and functions as a TCR contact residue (110) while citrulline residues within synovial antigens have been demonstrated to sit within the electropositive P4 binding pocket of HLA-DR4 and lead to a more stable presentation of these peptides compared to their arginine-containing counterparts (129).

Interestingly, preliminary micropipette data on CNS-infiltrating CD4 T cells isolated from symptomatic mice receiving MOG-primed lymphocytes by adoptive transfer show a predominance of citMOG-reactive cells with more than twice the frequency of either MOG-reactive or cross-reactive cells (data not shown). Moreover, recipient mice developed less severe symptoms than classically induced mice which supports either a less pathological phenotypic response from these cells or perhaps the CD4 T cells measured were regulatory T cells specific for citMOG.

This study presents the HLA-DR4 model of demyelinating disease that holds immense clinical value as these mice develop a T cell response to an inflammation-induced neoantigen, citrullinated MOG, similar to those observed in other HLA-DR4 autoimmune diseases. The concept of epitope spread to a neoantigen within demyelinating disease is intriguing as, in the case of citrulline, this epitope does not exist outside of the context of inflammation. Further, our data suggests that pharmaceutical interventions to prevent the conversion of arginine to citrulline and thus prevent or attenuate the citrulline-specific CD4 T cell response could hold great use as a therpaeutic for HLA-DR4+ MS patients.

Figure 1:

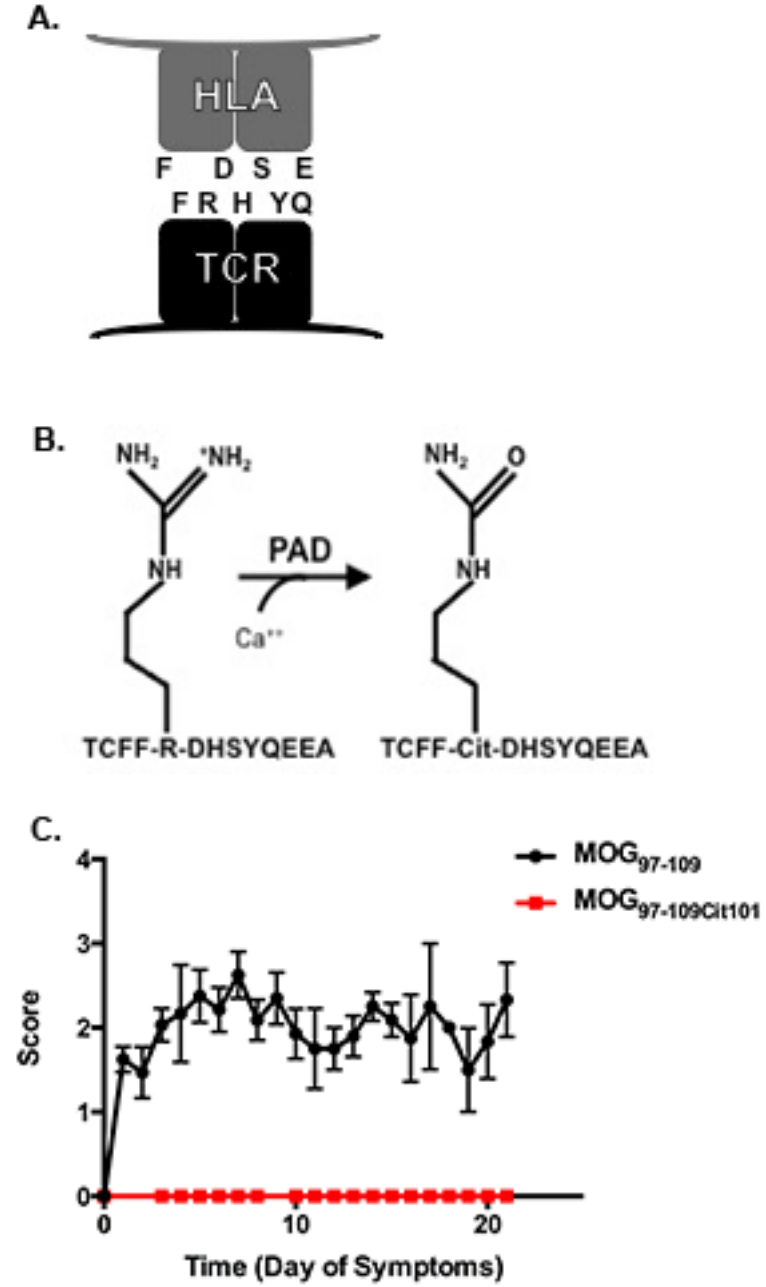

A.
HLA
F D S E
F R H YQ
TCR
B.
NH2 NH2
NH
PAD
Ca++
NH2 O
NH
TCFF-R-DHSYQEEA
TCFF-Cit-DHSYQEEA
C.
MOG97-109
MOG97-109Cit101
Score
0
1
2
3
4
0
10
20
Time (Day of Symptoms)

Figure 2:

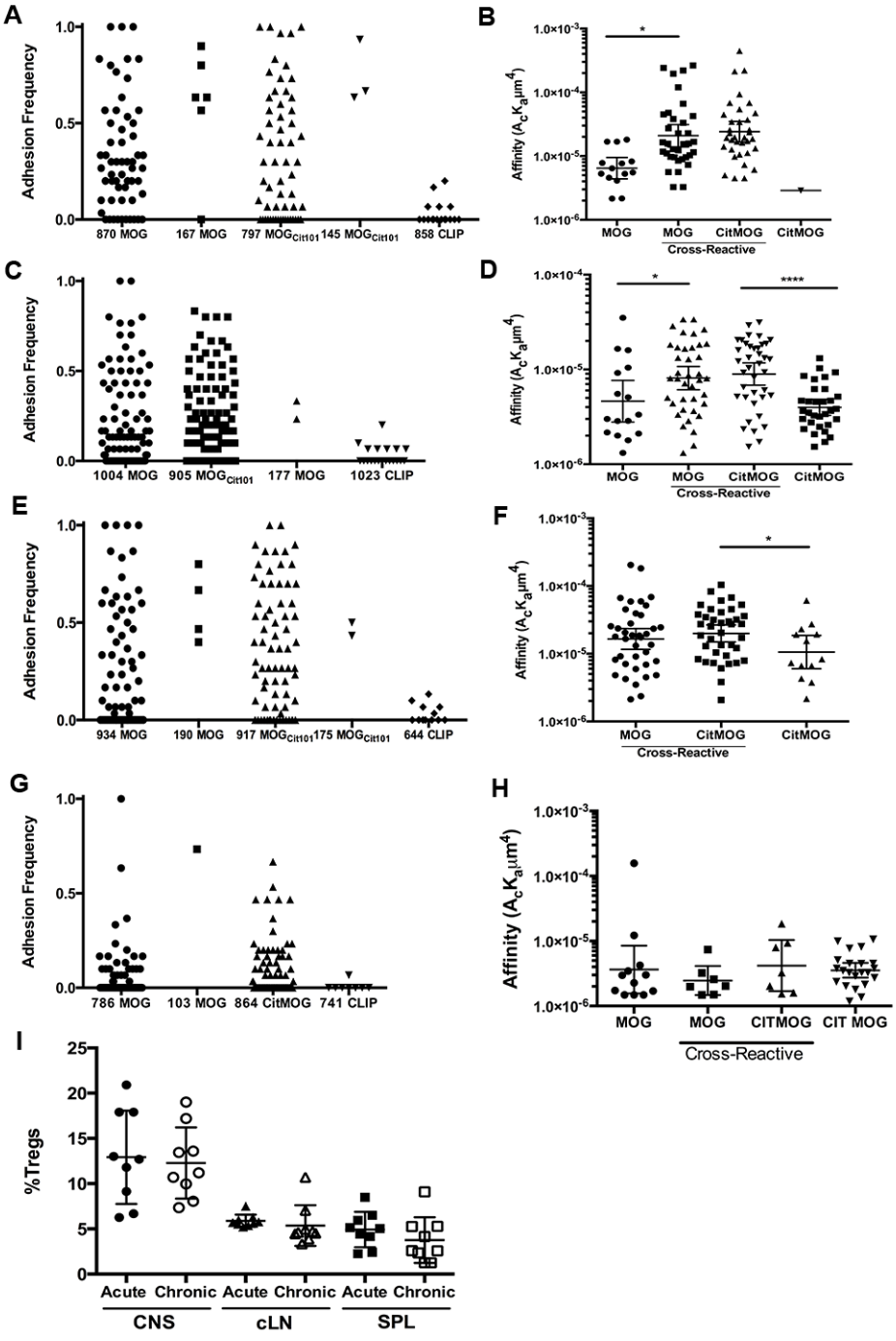

A
Adhesion Frequency
1.0
0.5
0.0
870 MOG
167 MOG
797 MOGCit101
145 MOGCit101
858 CLIP
B
Affinity (AcKaμm4)
1.0×10-3
1.0×10-4
1.0×10-5
1.0×10-6
*
MOG
MOG
CitMOG
CitMOG
Cross-Reactive
C
Adhesion Frequency
1004 MOG
905 MOGCit101
177 MOG
1023 CLIP
D
Affinity (AcKaμm4)
*
****
MOG
MOG
CitMOG
CitMOG
Cross-Reactive
E
Adhesion Frequency
934 MOG
190 MOG
917 MOGCit101
175 MOGCit101
644 CLIP
F
Affinity (AcKaμm4)
*
MOG
CitMOG
CitMOG
Cross-Reactive
G
Adhesion Frequency
786 MOG
103 MOG
864 CitMOG
741 CLIP
H
Affinity (AcKaμm4)
MOG
MOG
CITMOG
CIT MOG
Cross-Reactive
I
%Tregs
25
20
15
10
5
0
Acute
Chronic
Acute
Chronic
Acute
Chronic
CNS
cLN
SPL

Table 1. Tetramer Staining Throughout MOG-Induced Disease.

		CitMOG	MOG	Cross-Reactive
Acute	CNS	0.1465 ± 0.3788	9.548 ± 1.501	0.3698± 0.1127
	cLN	0.4570 ± 0.6361	4.717 ± 1.611	1.473 ± 0.306
	SPL	0.7785 ± 0.4587	3.269 ±1.385	2.007 ± 0.727
Chronic	CNS	-0.5920 ± 0.2758	15.53 ± 3.30	0.5850 ± 0.1484
	cLN	0.0460 ±0.1097	11.12 ± 2.539	0.8010 ± 0.2376
	SPL	0.9299 ± 1.338	10.02 ± 2.918	0.6388 ± 0.2322

Figure 3:

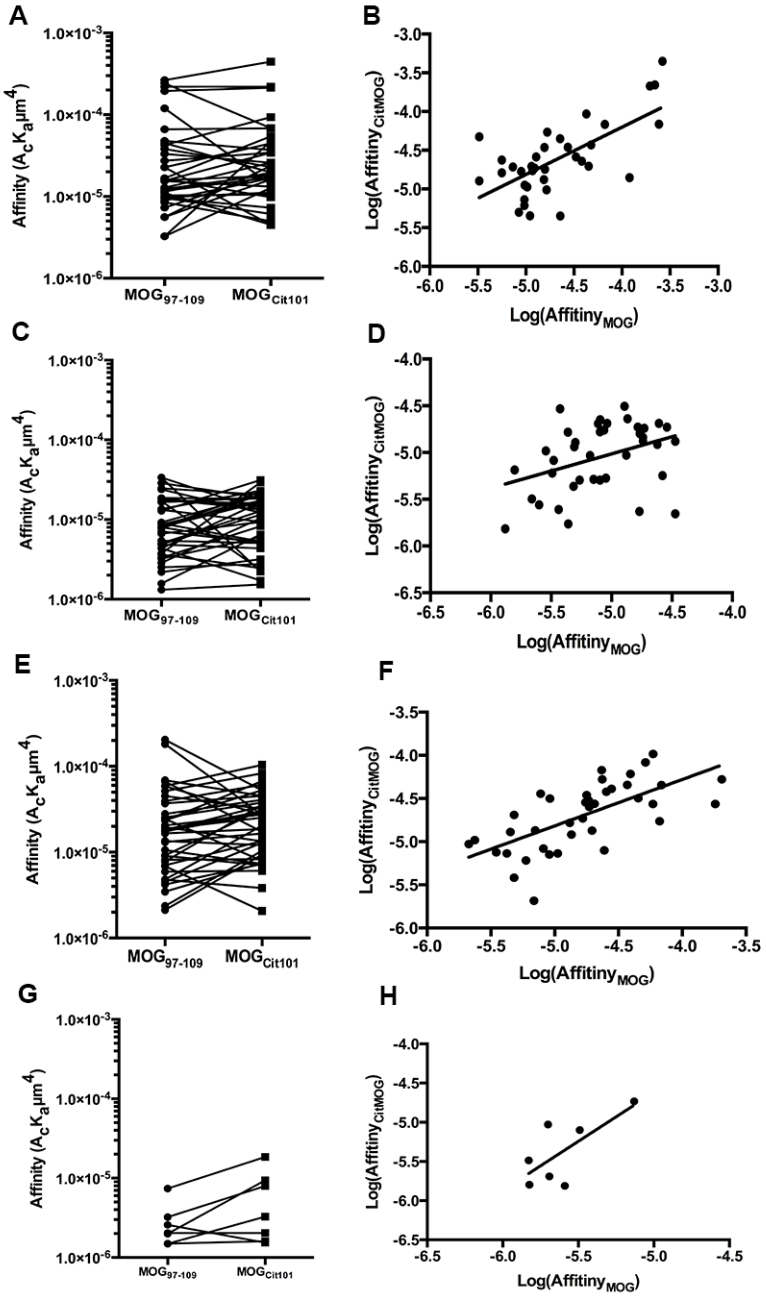

A
B
C
D
E
F
G
H
Affinity ($A_cK_a\mu m^4$)
MOG_{97-109}
MOG_{Cit101}
Log(Affitiny$_{CitMOG}$)
Log(Affitiny$_{MOG}$)

Figure 4:

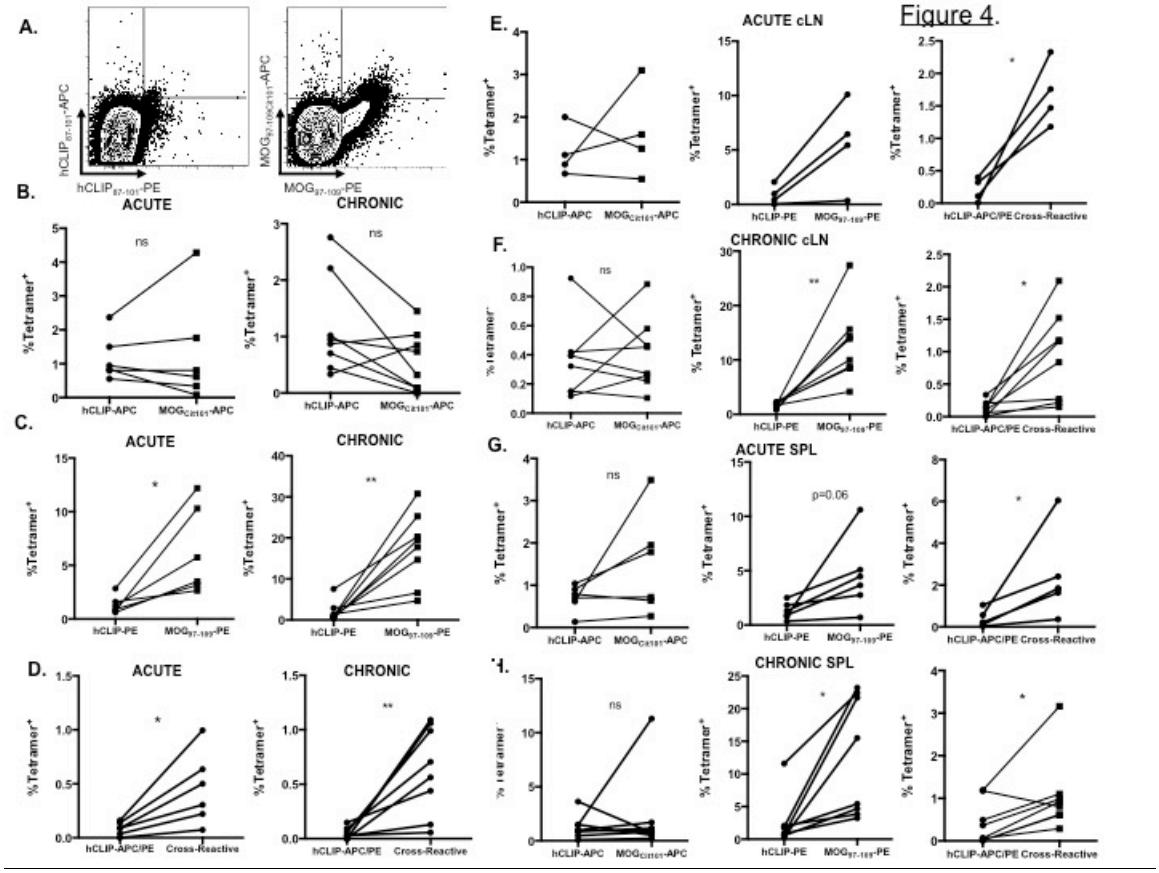


Figure 4.
A.
B.
ACUTE
CHRONIC
C.
ACUTE
CHRONIC
D.
ACUTE
CHRONIC
E.
ACUTE cLN
F.
CHRONIC cLN
G.
ACUTE SPL
CHRONIC SPL
ns
p=0.06
hCLIP-APC
hCLIP-PE
hCLIP-APC/PE Cross-Reactive
% Tetramer+

Figure 5:

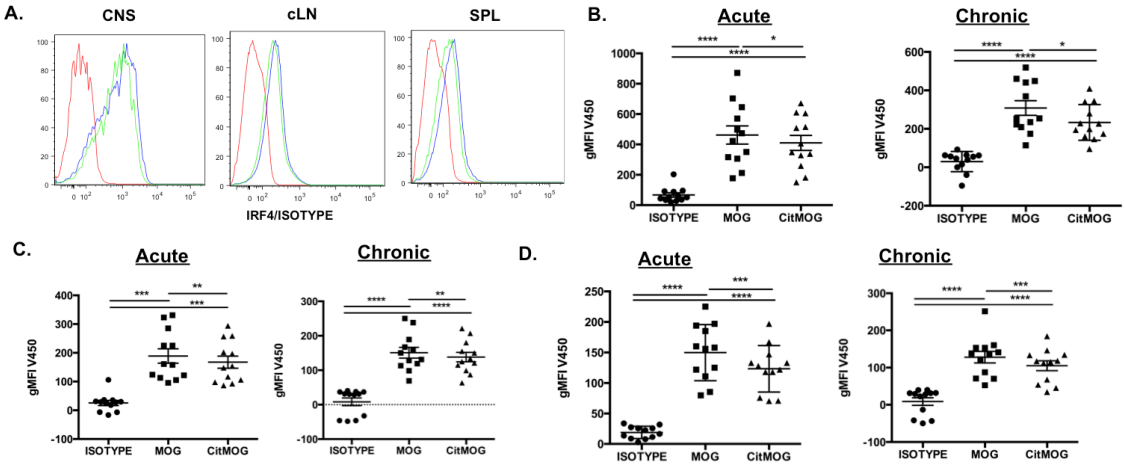

A.
CNS
cLN
SPL
IRF4/ISOTYPE
B.
Acute
Chronic
gMFI V450
ISOTYPE
MOG
CitMOG
C.
Acute
Chronic
D.
Acute
Chronic

Figure 6:

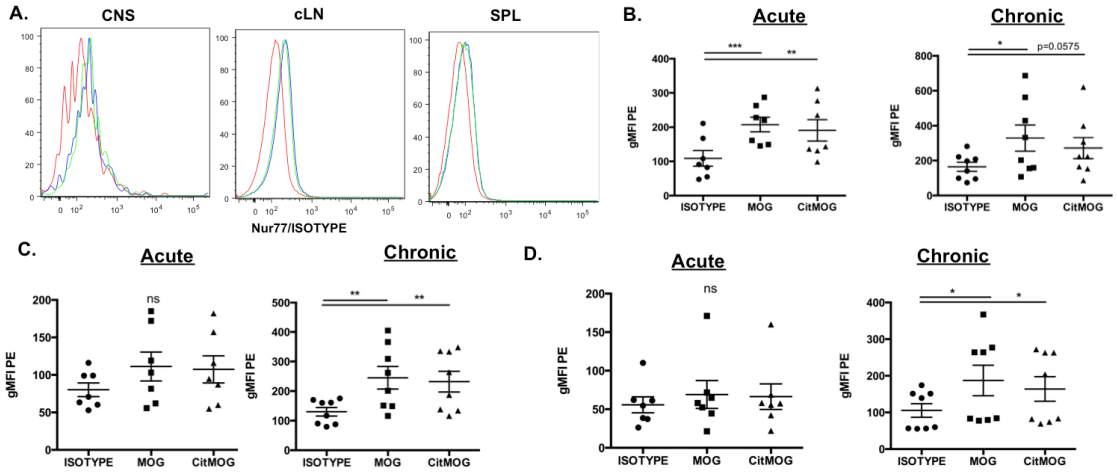

A.
CNS
cLN
SPL
Nur77/ISOTYPE
B.
Acute
Chronic
gMFI PE
ISOTYPE
MOG
CitMOG
p=0.0575
C.
Acute
Chronic
ns
D.
Acute
Chronic
ns

Figure 7:

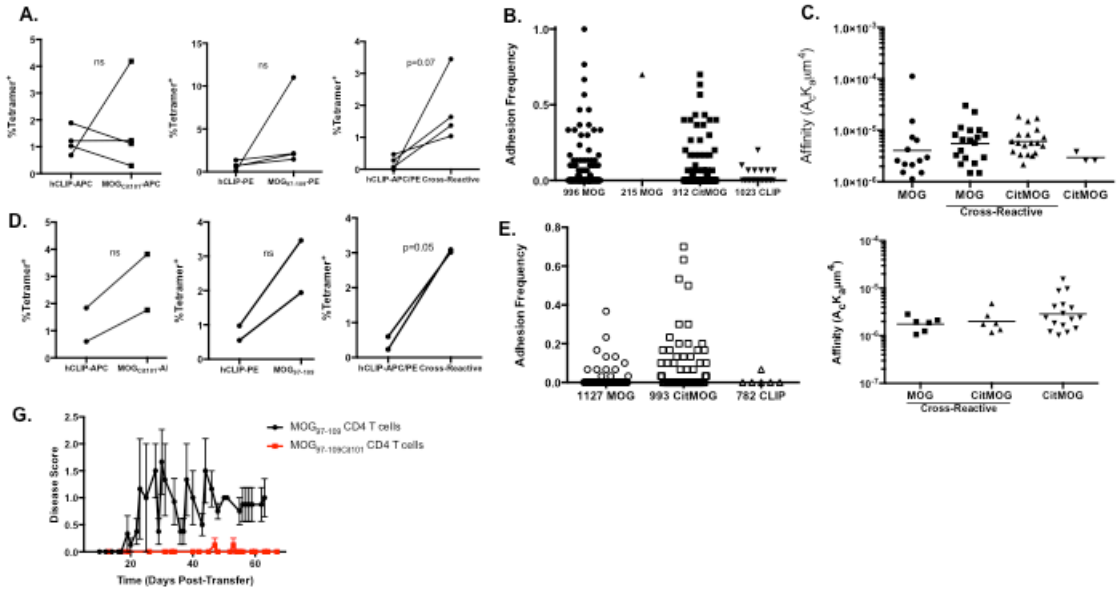

A.
B.
C.
D.
E.
G.
ns
p=0.07
p=0.05
%Tetramer+
Adhesion Frequency
996 MOG
215 MOG
912 CitMOG
1023 CLIP
1127 MOG
993 CitMOG
782 CLIP
MOG
CitMOG
Cross-Reactive
Disease Score
Time (Days Post-Transfer)
MOG97-109 CD4 T cells
MOG97-109Cit101 CD4 T cells

FIGURE LEGENDS

Figure 1. MOG97-109 induces demyelinating disease while citrullinated MOG does not.

A) Proposed epitope mapping of MOG97-109 (core 99-107) within the TCR and DRB1*0401 binding clefts. Arginine in position 101 is a T cell contact residue. B) Deimination reaction converting arginine to citrulline catalyzed by peptidyl arginine deiminase (PAD) enzyme in calcium-dependent manner. Arginine loses its net positive charge. C) Male and female DRB1*0401-expressing mice were induced with MOG peptide or citMOG peptide emulsified in CFA. Scores are plotted against time with the day 1 on the X-axis representing first day of symptoms. 32 female and 30 male mice were used for MOG-induction and 5 female and 6 male mice were used for citMOG-induction. Mean day of onset for MOG-induced mice was 22.09 ± 1.38. 46/62 MOG-induced mice developed symptoms while 0/11 citMOG-induced mice developed disease.

Figure 2. CD4 T cells reactive strictly to CitMOG only present at symptomatic time points.

A-F) Mononuclear cells were isolated from the CNS of pre-symptomatic (A-B), acutely symptomatic (C-D) and chronically symptomatic (E-D) mice and CD4 T cells were purified by magnetic separation. The micropipette adhesion frequency assay was performed and adhesion frequencies (A, C, E) are reported with the mean peptide:HLA surface density reported on the X-axis. (B, D, F) 2D affinities are reported for MOG antigen alone, citMOG alone and affinities for each antigen are reported for cross-reactive cells. Each dot represents a single cell, except for the cross-reactive populations in which a single cell is displayed as two dots- one affinity for MOG and one for citMOG. Each graph represents combined data from 2-4 independent experiments of 3-5 mice. E) Adhesion frequencies and F) 2D affinities are plotted for CD4 T cells purified from the spleen of chronically infected mice. Data have been pooled from 3 independent experiments (one mouse per experiment).

Figure 3. Cross-reactive CD4 T cells in the CNS trend toward a higher affinity for MOG.

A-C) 2D affinities are plotted for cross-reactive CD4 T cells isolated from the CNS for A) presymptomatic, B) acute, and C) chronic time points. Singe cell affinities for MOG and citMOG are connected by a line. D) 2D affinities of CD4 T cells isolated from the spleen are plotted for both MOG and citMOG. A-D) t tests were performed and no significant differences were observed. E-G) The logarithm of the 2D affinity of a single cross-reactive CD4 T cell for MOG is plotted against its affinity for citMOG for all cross-reactive cells isolated from the CNS at E) pre-symptomatic, F) acute and G) chronic time points. H) Affinities are similarly plotted for the spleen. Linear regression was performed with slopes measuring 0.6112, 0.2239, and 0.5376 for pre-symptomatic, acute and chronic CNS, respectively and 1.25 for chronic spleen. R-square values measured 0.4467, 0.1438, and 0.5376 for pre-symptomatic, acute and chronic CNS and 0.5056 for chronic spleen.

Figure 4. **DRB1*0401 tetramer stain does not identify CD4 T cells reactive only to CitMOG**.

Mononuclear cells isolated from the CNS and lymphocytes isolated from the cervical lymph nodes (cLN) and spleen (SPL) were stained with 8 μg/ml of DR4 tetramers of either MOG_{97-109}-PE and $MOG_{97-109Cit101}$-APC or $hCLIP_{87-101}$ in both -PE and -APC. A) Sample plots are shown from the cLN gated on lymphocytes by FSC/SSC, $CD4^{+}CD11b^{-}CD11c^{-}CD19^{-}$, and finally on CD44hi cells. B-D) Tetramer staining of cells isolated from the CNS are shown for acute and chronic time points for cells staining positively for B) only MOG97-109Cit101 C) only MOG97-109 and D) both MOG tetramers. E-H) Tetramer stain data summarized for cells isolated from E) acute and F) chronic cLN and G) acute and H) chronic SPL. Student's t tests were performed and statistical significance is indicated with *: $p<0.05$, **: $p<0.01$, ***: $p< 0.001$ and ns: no significance.

Figure 5. IRF4 is upregulated in CD4 T cells in response to both MOG and CitMOG.

Mononuclear cells isolated from the CNS and cells isolated from the cervical lymph node and spleen were stimulated in vitro with 50 μM MOG97-109 or MOG97-109Cit101 for 6 hours. Cells were surface stained, fixed and permeabilized and evaluated for IRF4 expression. A) Sample plots from the CNS, cLN and SPL gated on lymphocytes by FSC/SSC and $CD4^{+}CD11b^{-}CD11c^{-}CD19^{-}$. Histograms shown are for IRF4-V450 stimulated with MOG (blue), citMOG (green), for MOG-stimulated cells stained with the isotype control (red). B-D) Geometric MFIs are reported for acute and chronic time points in the B) CNS, C) cLN, and D) SPL. One-way ANOVA was performed and significance is reported or indicated with *: $p<0.05$, **: $p<0.01$, ***: $p< 0.001$, ****: $p<0.0001$ and ns: no significance.

Figure 6. Nur77 is upregulated in CD4 T cells in the CNS in response to MOG and CitMOG.

Mononuclear cells from the CNS and cells from the cervical lymph node (cLN) and spleen (SPL) were stimulated in vitro with 50 μM MOG97-109 or MOG97-109Cit101 for 6 hours. Cells were surface stained, fixed and permeabilized and evaluated for Nur77 expression. A) Sample plots from the CNS, cLN and SPL gated on lymphocytes by FSC/SSC and $CD4^{+}CD11b^{-}CD11c^{-}CD19^{-}$. Histograms shown are for Nur77-PE stimulated with MOG (blue), citMOG (green), for MOG-stimulated cells stained with the isotype control (red). B-D) Geometric MFIs are reported for acute and chronic time points in the B) CNS, C) cLN, and D) SPL. One-way ANOVA was performed and significance is reported or indicated with *: $p<0.05$, **: $p<0.01$, and ns: no significance.

Figure 7. Only MOG-primed CD4 T lymphocytes are able to transfer disease.

A-C) Lymph nodes were primed with MOG peptide in CFA and harvested 10-14 days later. A) *Ex vivo* tetramer staining was performed on MOG-primed lymphocytes with DR4 tetramers: $MOG_{97\text{-}109}$-PE, $MOG_{97\text{-}109Cit101}$-APC, and $hCLIP_{87\text{-}101}$ PE and APC. B-C) CD4 T cells were purified by magnetic separation for ex vivo analysis by micropipette and B) 107 cells were interrogated and adhesion frequencies are shown. C) 2D affinities for CD4 T cells reactive only to MOG, only to citMOG and cross-reactive cells are shown. Data are representative of 3 independent experiments of 3-5 mice each. D-F) Lymph nodes primed with citMOG peptide in CFA were harvested 10-14 days post-priming and D) cells were stained with tetramer ex vivo. E-F) The micropipette adhesion frequency assay was performed and E) adhesion frequencies of 70 cells were obtained. F) 2D affinities for cells specific for antigen are shown. Data are representative of 2 independent experiments of 3-5 mice each. G) Lymph nodes primed with either MOG or citMOG were harvested and cultured on 1 μM of the peptide with which they were primed for 72-96 hours. Live cells were collected and adoptively transferred into naïve animals followed a day later (d1) by immunization of inducing peptide emulsified in IFA and pertussis toxin injections at d1 and d3. Symptoms were evaluated daily in mice receiving transferred cells. Student's t-tests were performed; ns: not significant.

DISCUSSION AND CONCLUSIONS

In the studies described herein, we explore CD4 T cell affinities for myelin antigen in the context of two clinically relevant mouse models of demyelinating disease. To accomplish this, the micropipette adhesion frequency assay was used to obtain frequency and 2D affinity measurements of myelin reactive T cells. Previous work from our laboratory demonstrated this assay is a more sensitive means of measuring frequency and affinity of myelin-reactive T cells compared to MHC class II tetramers in the context of demyelinating disease (28, 165). MHC class II tetramers are known to preferentially identify CD4 T cells with a high affinity for antigen and are not accurate at identifying cells present at a low frequency in a population or cells with a lower affinity for antigen (28, 115). Affinities obtained from the micropipette assay correlate better with functional output of T cells compared to affinity data obtained from surface plasmon resonance (SPR) assays and it has been suggested that this is due to the fact that the micropipette assay accounts for steric restrictions imposed by the plasma membrane as in a physiological setting (169, 170).

As MHC class II genes confer the strongest risk for the development of MS, we chose to study two mouse models with MHC class II genes that increase their proclivity to develop autoimmune disease, specifically demyelinating disease (4, 6). In the first study, the nonobese diabetic (NOD) mouse model of demyelinating disease was used to investigate CD4 T cell affinities for inducing antigen throughout the course of relapsing-remitting disease course that evolves into a secondary progressive phenotype. These mice were chosen as the MHC class II molecule (IAg7) they express renders them susceptible to other autoimmune diseases, most notably Type I diabetes but also systemic lupus

erythematosus and autoimmune thyroiditis (46, 158-160). We found that throughout the course of acute disease, remission, symptom relapse and chronic symptom development the vast majority of CNS-infiltrating CD4 T cells were reactive to inducing MOG35-55 epitope limiting the potential contribution of CD4 T cells reactive to other myelin epitopes, or epitope spread. This is a novel finding in that relapsing-remitting disease pathologies have often been explained by the phenomenon of epitope spread (44, 45). In the case of the NOD model, we instead found that the phenotype of CNS-infiltrating CD4 T cells changed throughout disease with an increased number of IL-17-producing cells found during symptomatic phases of disease and a decrease in IFN-g production over time.

The finding that the CD4 T cell response in a relapsing-remitting model remains dominant to a single myelin epitope is promising with regards to MS patient care. It has long been thought that relapsing-remitting disease is due to the sequential development of multiple clones of myelin-specific T cells with different clones becoming activated at different times. Many studies have attempted to quantify myelin-specific T cells in the peripheral blood or cerebrospinal fluid of MS patients and, when comparing reactivity to that observed in healthy controls, the results have been inconclusive with some studies finding significantly more myelin-reactive cells in MS patients and others finding myelin-reactive cells in both populations (107, 110, 112, 113). A potential explanation for this, anchored in epitope spread dogma, has been that detecting myelin-reactive cells in a patient with ongoing disease can be akin to “hitting a moving target” with the myelin reactivity profile of CD4 T cells evolving as disease progresses. The findings from our study show that relapsing-remitting disease can occur with only dominant myelin-epitope

driving disease progression. This has great potential impact for developing antigen specific therapeutics in such cases as the T cell response would not be a continually moving target. In addition, identification of the single target self-myelin antigen would provide a biomarker reflecting disease severity or efficacy of the therapeutic intervention in MS patients. The greatest challenge in implementing the detection of myelin-reactive T cells as a biomarker of disease is the need to determine which myelin peptides to use and the HLA restriction of those peptides. Further, this data suggests that developing a therapy that targets myelin-specific T cells may be more practical than once thought. Antigen-specific therapy trials in which administration of autologous PBMCs chemically coupled with only seven myelin peptides have resulted in decreased myelin-specific responses in MS patients (191). We believe tolerogenic efforts for therapeutics in relapsing-remitting autoimmune disease may be successful if focused to a single or limited number of epitopes and could be more efficiently targeted. As many HLA genes confer increased risk for MS, there are myriad possibilities of which peptide:HLA combination could be dominant in eliciting disease pathology. These studies also require HLA-typing MS patients before assessing for reactivity, which increases the time it takes to get results.

In addition to assessing frequency and affinity of myelin-specific T cells in the secondary progressive NOD model of demyelinating disease, we also evaluated Tregs in the CNS and found an increase in the frequency of Tregs during disease remission. Interestingly, further evaluation of Tregs throughout disease showed a persistent increase in the CNS at both relapse and chronic time points (unpublished data). These cells were evaluated for their ability to produce IL-10 and TNF-a and no significant differences

between remission, relapse and chronic time points were observed suggesting Tregs isolated at remission have no functional advantage compared to those isolated at later symptomatic time points. Interestingly, the surface expression of PD-1 was significantly higher on Tregs isolated at the remission time point compared to their non-Treg CD4+ counterparts but this difference was not observed for relapse or chronic time points. PD-1 expression on Tregs has been linked to the suppression of both CD4 and CD8 proliferative responses, which may suggest that the Tregs at later time points in this model are losing their ability to suppress effector CD4 T cell activation (194, 195). Tregs have also been shown to be less effective at suppressing Th17 cells compared to Th1 cells which may also explain this functional ineffectiveness as a greater proportion of CNS-infiltrating CD4 T cells at later time points secrete IL-17 (190). We also observed an increase in the absolute number of CD8 T cells in the CNS at disease remission compared to relapse and chronic time points (unpublished data) which may point to a regulatory role for these cells which has been confirmed in other models (97-100). Future work in this model will involve the investigation of the affinity of CNS-infiltrating Tregs for myelin and how this compares to Teffector affinities. We will also look into the suppressive ability of these cells throughout disease to further explain why, despite a stable frequency within the CNS, these cells fail to suppress the pathologic myelin-specific CD4 response.

In the second study, CD4 T cell affinities for myelin were explored in a humanized mouse model of demyelinating disease in which mice express HLA DRB1*0401 (HLA-DR4) instead of murine class II genes. As HLA-DR4 is associated with an increased risk of MS, we were interested in evaluating the frequency and affinity

of CD4 T cells specific for both MOG_{97-109} and the citrullinated version of this peptide, in which arginine has been converted to citrulline as the result of an inflammation-induced post-translational modification. Citrulline was of great interest to us particularly in the context of DR4-expressing mice as both B and T cell reactivity to citrullinated synovial antigens have been demonstrated in mouse models of RA as well as DR4+ RA patients (121, 127, 129). Indeed, in mouse models of RA immunization with citrullinated synovial antigens and adoptive transfer of citrulline-specific CD4 T cells are found to be more arthritogenic than those specific to native proteins (131, 133). This observation starkly contrasts with the data presented here in which immunization with citrullinated MOG does not elicit symptoms of demyelinating disease whereas immunization with native MOG peptide does. One potential explanation for this lies in the observation that the citrulline residue of citrullinated synovial antigens lies in the P4 pocket of the HLA-DR4 binding cleft and is thus an MHC contact site (129). The electropositive P4 pocket is thought to form a stronger interaction with the neutral citrulline residue than its positively-charged arginine precursor. Importantly, with citrulline as an MHC contact residue the T cell contact residues of citrullinated synovial antigens would not differ from their native counterparts. Further, improved binding of citrulline to the DR4 molecule may affect the priming of the T cell response as others have shown the affinity with which peptide binds to MHC can alter responding T cell phenotype and identification (110, 196).

In our study, we sought to quantify CD4 T cell reactivity to MOG and citrullinated MOG peptides throughout the course of MOG-induced demyelinating disease. In the DR4 mice, immunization with native MOG peptide was able to produce

demyelinating disease while immunization with the citrullinated peptide was not, an observation distinct from the RA models in which transfer of citrulline-reactivity can elicit symptoms (131, 133). For MOG-induced disease, we used the micropipette adhesion frequency assay and found a cross-reactive population of CD4 T cells in the CNS before the development of symptoms that persisted in the CNS through acute and chronic disease time points. Cells reactive only to MOG were found at early time points of disease but were not detectable in the CNS of chronically symptomatic mice. Interestingly, we found a population reactive only to citrullinated MOG at symptomatic time points of disease (acute and chronic) in the CNS and in the periphery.

The generation of CD4 T cells reactive only to citrullinated MOG and not to native MOG holds value as a candidate for a biomarker of disease status as these cells were only detectable at symptomatic time points of disease. Although readily detectable by the micropipette adhesion frequency assay, these cells were not identified by HLA-DR4 tetramers. Tetramer staining revealed a substantial population of MOG-reactive cells, a small but significant population of cross-reactive cells and no cells reactive to citrulline alone all data that do not align with micropipette-determined frequencies. These findings suggest that tetramer-based technology would not be the ideal modality for use in this biomarker assay. Citrullinated peptides are an interesting candidate for biomarker use as citrulline is not a translated amino acid but is the result of an inflammation-induced post-translational modification. This also makes citrullinated peptides and the T cell responses that develop to target those antigens an achievable target of therapeutics. PAD enzymes are upregulated during inflammation and are able to convert arginine into citrulline essentially making citrulline itself a marker of inflammatory pathology. As

citrulline is presumed to not be expressed in the thymus, there should be no positive or negative selecting signals specific to citrulline. A T cell response specific to citrullinated myelin should therefore be a sensitive and specific marker of demyelinating disease. Our findings suggest that citMOG-specific CD4 T cells exacerbate disease symptoms and are responsible for progression of disease. As such, the use of PAD inhibitors within this model should inhibit or attenuate the citMOG-specific CD4 T cell response and thus alleviate symptoms, which have great implications for the use of these pharmaceuticals to treat MS patients.

Our goals for the HLA-DR4 demyelinating disease project were initially to validate our reagents for use in human patients. Our laboratory in collaboration with Dr. William Tyor obtained 8 MS patient peripheral blood samples for the purpose of evaluating antigen-specificity of CD4 T cells using HLA class II tetramers and the micropipette adhesion frequency assay. As myelin reactive cells are thought to occur at a low frequency in the peripheral blood (110, 115), we stimulated CD4 T cells with peptide and IL-2 before evaluating with tetramers and the micropipette assay. Interestingly, of the 8 patients evaluated, not a single patient expressed DRB1*0401 and thus we were not able to evaluate the peripheral reactivity to the MOG antigens explored in our mouse model. For the purpose of this study we also examined reactivity to DRB1*1501-restricted epitopes of MBP and found 4 of our patients expressed this allele, known to confer the strongest risk for development of MS (6). In these preliminary experiments, DR15-restricted CD4 T cell reactivity was detectable post-enrichment by the micropipette adhesion frequency assay. Although the majority of our patients expressed

DRB1*1501, to our surprise we observed an even higher frequency of patients expressing DQB1*0602 (75%), an allele that is a constituent of the HLA-DR15 haplotype.

As several studies have explored the encephalitogenicity of various myelin peptides in the context of DQB1*0602 (197, 198), we obtained mice expressing this HLA molecule and have since been evaluating encephalitogenic potential of various myelin epitopes. Although others have been able to elicit demyelinating disease in these animals, our efforts at immunizing with DQ6-restricted epitopes of MBP and PLP have not generated symptomatic disease. Interestingly, we have been able to identify a myelin-specific proliferative CD4 T cell response in these animals implying that these responses may contribute to disease pathology although not robust enough to elicit it independently.

CD4 T cell reactivity to citrulline and its correlation with symptom status has prompted our lab to investigate the specific role citrulline plays in demyelinating disease and if it is essential to disease progression. Data presented here show that both cross-reactive CD4 T cells and cells reactive only to citrullinated MOG are present at symptomatic time points of disease. Similarly, adoptive transfer of CD4 T cell deficient in MOG-reactive and cross-reactive CD4 T cells does not elicit disease symptoms. MOG-primed lymphocytes were stimulated in culture and adoptively transferred into naïve animals. *Ex vivo*, a substantial portion of MOG-primed lymphocytes was reactive to MOG alone and cross-reactive while negligible amounts were reactive to citMOG. Upon transfer, mice did not become as sick as those immunized with peptide in CFA. Interestingly, preliminary data showed that the CNS-infiltrating cells of mice that became symptomatic post-adoptive transfer consisted mainly of citMOG-reactive cells with slightly smaller populations cross-reactive or reactive to MOG alone, indicating the

transfer of cross-reactive and MOG-reactive cells elicited enough inflammation to induce PAD enzymes and citrullination of MOG within the CNS and subsequent priming of a citMOG-specific T cell response. Although it is clear the presence of citMOG-reactive CD4 T cells in the CNS is an indication of pathogenic processes, we would hypothesize these cells contribute to rather than drive disease pathology. Therefore, we are currently breeding mice deficient in PAD4 to DR4-expressing mice and are working to acquire mice deficient in PAD2 for the same purpose. Further, we have obtained a pan-PAD small molecule inhibitor and pilot experiments testing the effects of PAD inhibition on MOG-induced EAE are underway. We would predict that inhibition of PAD would reduce or eliminate the citrulline-specific CD4 T cell response and lessen the contribution of this population to demyelination and therefore lessen symptom severity. These results could have further implications for the use of PAD inhibitors as a therapeutic intervention in MS patients.

The studies presented here characterize two distinct clinically-relevant mouse models of demyelinating disease. Mouse models of EAE have long been challenged as being unrepresentative of human disease. The controlled environment and genetics of mouse models are much easier to manipulate than the genetically diverse population of MS patients with myriad environmental exposures and experiences that might affect data garnered from these studies. The models discussed here better approximate the human condition as demyelinating disease in NOD mice takes a relapsing-remitting turned secondary progressive disease course that resembles that experienced by the majority of MS patients. The immunological responses that govern the onset of symptoms, entrance into remission and progression through relapse and chronic disease are more easily

evaluated and the timing of these phases easier to predict in mice compared to patients and it is intuitive that similar cellular mediators would effect these processes in both species. In the HLA-DR4 model of progressive demyelinating disease, the myelin-specific CD4 T cell response generated in these mice is more "human-like" as these cells were selected on human MHC class II molecules. As HLA-DR4 is a genetic risk factor for MS, the T cell response generated to myelin antigens in the context of HLA-DR4 better approximates the MS patient condition than mice expressing murine MHC class II molecules. The link between HLA-DR4 and citrulline has been widely explored in both mouse models and human patients in the context of RA and although demyelinating disease has been explored in DR4 mice, citrulline reactivity has not been evaluated to our knowledge.

The goal of this study was to investigate CD4 T cell reactivity to myelin antigens within clinically relevant mouse models of demyelinating disease that mimic the disease experienced by MS patients. To this end, we focused on models in which MHC class II alleles that predispose to autoimmunity are expressed. We discovered two significant findings that potentially have great impact on the understanding of MS and potential targets for therapeutic intervention in MS patients. First, using the NOD model of relapsing-remitting turned secondary progressive disease we found that this disease phenotype can occur in the absence of epitope spread. Then in the HLA-DR4 model, we found that CD4 T cell reactivity to inflammation-induced neoantigens can occur and drive disease progression serving not only as a biomarker but also as a therapeutic target for patients expressing HLA-DR4.

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