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Tracking Myelin Specific CD4 T Cells during Demyelinating Disease Post-Infection

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Tracking Myelin Specific CD4 T Cells during Demyelinating Disease Post-Infection

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Abstract

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By Jennifer M. Cosby

Multiple sclerosis is a debilitating demyelinating autoimmune disease characterized by the recruitment of myelin-specific CD4 T cells into the CNS. The mechanism for the initiation of this disease, however, has yet to be uncovered. Infections have been proposed as triggers that activate myelin-specific CD4 T cells to begin the process of demyelination. In contrast to this paradigm, our studies reveal that infections are able to provide protection from a well-studied model for demyelinating disease (EAE). In one of our described studies, we generated a molecular mimic (Listeria monocytogenes expressing MOG, or Lm-MOG) to determine if infection can trigger disease symptoms. Lm-MOG did not break tolerance to induce EAE and instead was able to protect from EAE by generating a MOG-specific Treg population that most likely is able to limit the expansion of effector CD4 T cells. However, mice that lack MOG-specific tolerance mechanisms (MOG-/- mice) are not able to generate Tregs to MOG and therefore have a large effector T cell expansion. These effector CD4 T cells are then able to initiate disease symptoms. In addition, the Treg generation observed in wild-type mice was antigen specific as Lm-gp66 did not generate Tregs toward the gp66 antigen. Due to these results, it could be suggested that individuals that lack MOG-specific tolerance mechanism may be at risk for demyelinating disease whereas individuals with MOG-specific tolerance mechanisms intact are able to prevent demyelinating disease through Treg generation. We also infected mice with LCMV during EAE disease course to determine the effects of unaltered infections during EAE. Contrary to the paradigm that infection promotes or exacerbates demyelinating disease, LCMV delayed autoimmune disease in animals. The initiators of EAE, CD4 T cells, were less able to traffic to the CNS compared to uninfected controls. The canonical proinflammatory Th17 response was also impaired in the CNS. High affinity myelin-specific CD4 T cells were also lacking in the CNS compared to uninfected controls. Together, these results explore the role that infections may play in protection against autoimmune disease in individuals with intact tolerance mechanisms.

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Table of Contents

Chapter 1: Introduction…….....1

Figure Legends……..…….....25

Figure 1 Schematic of molecular mimicry theory...……...……27

Figure 2 Representation of micropipette adhesion frequency assay....……...…28

Chapter 2: Antigen specific Tregs are selectively expanded to prevent demyelinating disease.....29

Abstract……...…29

Introduction..……....30

Materials and Methods……32

Results…….35

Discussion……...39

Acknowledgements……..43

Figure Legends……44

Supplemental Figure Legends……..48

Figure 1 Infection initiates EAE in genetically susceptible mice.....……...50

Figure 2 Myelin specific CD4 T cell expansion is limited post-infection....……...51

Figure 3 LLO specific CD4 T cells are correlated in MOG-/-, but not C57Bl/6, mice..…...52

Figure 4 Tregs expanded in wild-type mice but not genetically susceptible mice.....……...53

Supplemental Figure 1……..……54

Supplemental Figure 2……..……55

Chapter 3: LCMV Delays EAE by limiting high affinity myelin CD4 T Cell Responses.....56

Abstract……...…56

Introduction..……...57

Materials and Methods……59

Results……62

Discussion……...66

Acknowledgements……..70

Figure Legends……71

Supplemental Figure Legends……..76

Figure 1 EAE is less severe and delayed during LCMV infection…..……...77

Figure 2 LCMV reduced the CD4 T cell response…….……...78

Figure 3 Cytokine profile is altered post-infection…….……...79

Figure 4 Myelin specific CD4 T cells are reduced in the CNS post-infection….……...80

Figure5 High affinity MOG-specific CD4 T cells are absent post-infection.....……81

Supplemental Figure 1…….……82

Chapter 4: Discussion.....…….……83

Figure Legends……88

Figure 1 Model for molecular mimicry induced demyelinating disease.....……...89

Literature Cited…….90

Chapter I

Introduction

Introduction to Multiple Sclerosis

The national multiple sclerosis society estimates 2.3 million people are afflicted with multiple sclerosis (MS) worldwide. MS is an inflammatory demyelinating autoimmune disorder characterized by destruction of the myelin sheath surrounding neurons, axonal loss, and oligodendrocyte death in the CNS. This leads to formation of plaques which have been seen in both white and grey matter (Brownell and Hughes 1962; De Stefano, Matthews et al. 2003). As described in 2003 by Bo et al., there are four widely accepted types of lesions found in MS patients that occupy different areas of the CNS (Bo, Vedeler et al. 2003). Type 1 consists of lesions that extend across both the white and grey matter. Type 2 lesions lie within the cerebral cortex and do not reach the surface of the brain or to subcortical white matter. Type 3 lesions, or subpial legions, consist of the most area occupied by legions. Lastly, type 4 lesions do not reach into the subcortical white matter but extend the full width of the cerebral cortex. Lesions can be active or chronic. Documented by Lucchinetti et al. in 2000, active lesions are infiltrated by macrophages and activated microglia containing intracytoplasmic granules of myelin debris reactive for myelin peptides (Lucchinetti, Bruck et al. 2000). The active lesions were described to follow four different patterns of demyelination with differences in deposition of IgG and complement, infiltration of T cells, macrophages and activated microglia, apoptosis, location, and other factors. These

infiltrating elements contribute to the inflammation and demyelinating process in the plaque. However, chronic lesions are inactive and devoid of infiltrate with evidence of some remyelination and repair of neurons localized to the edges of plaques (Chang, Smith et al. 2008). Chronic lesions can be reactivated with characteristics of acute lesions during times of relapse. This reactivation contributes to further disease progression.

Breakdown of the blood brain barrier (BBB) has been implicated in the pathogenesis of multiple sclerosis. MRI results of patients have shown that breakdown of the BBB occurs after initial lesions have formed but before new lesions arrive, suggesting its role in early pathogenesis of the disease and its role in inflammation (Kermode, Thompson et al. 1990). Increased levels of circulating ICAM-1 and TNF-α in the cerebrospinal fluid (CSF) of MS patients have been observed in conjunction with loss of BBB integrity (Sharief, Noori et al. 1993). As an adhesion molecule, ICAM-1 is important for T lymphocyte attachment to endothelial cells, suggesting a potential route of entry into the CNS before, during, and after BBB breakdown. Presence of TNF-α in the CSF provides further evidence of the inflammatory nature of MS pathogenesis. Tight junctions are compromised mostly in acute plaques of MS patients contributing to BBB leakage (Kirk, Plumb et al. 2003). This likely provides an avenue by which infiltrating inflammatory cells can enter plaques and contribute to disease.

MS is diagnosed based on the McDonald criteria in 2001 (McDonald, Compston et al. 2001) and updated in 2010 (Polman, Reingold et al. 2011). The McDonald criteria discussed guidelines for MS diagnosis, as well as clarified terms used to describe MS pathology. The most important criteria for diagnosis of MS are the accumulation of evidence through both time and space of typical MS lesions. Historical accounts of symptoms and radiological/laboratory investigations (MRI of brain or spinal cord lesions, CSF analysis, and

visual evoke potentials (VEP)) can both be used to add to a clinical diagnosis; however, the diagnosis cannot be made with these alone. The term "attack" was also defined to include only those symptoms that occur for at least 24 hours, and separate attacks must be at least 30 days apart to reduce ambiguity. The McDonald criteria provides a template for what data need to be collected in order to make a diagnosis with the clinical presentation seen. The results are either "MS" or "not MS". However, a diagnosis of "possible MS" can be made if a patient with clinical symptoms has not yet been evaluated or if evaluation is incomplete.

A wide range of symptoms and severity have been observed in MS patients which include sensory, visual, motor, and psychological effects. Fatigue is clearly documented as a symptom of MS (Krupp, LaRocca et al. 1989; Fisk, Pontefract et al. 1994). It is the most common symptom that occurs in MS patients, affecting up to 80% of reported cases. Both acute and chronic pain have been observed in MS patients, with 55% of patients surveyed reporting pain in one study (Moulin, Foley et al. 1988). Consistent with these findings, another study reported 53% of patients with incidences of pain (Archibald, McGrath et al. 1994). Through the use of conventional and magnetization transfer magnetic resonance imaging techniques, vision issues in MS patients have been explored and explained by decreased volumes and magnetization transfer ratio (MTR) values of optic nerves (ONs) in patients with MS compared to healthy controls, but similar volumes and MTRs compared to Leber hereditary optic neuropathy (LHON) patients (Inglese, Ghezzi et al. 2002). Difficulty walking has been linked to MS patients. Those with lower limb pyramidal dysfunction had reduced speed, stride length, and prolonged double limb support compared to MS patients without lower limb pyramidal dysfunction and controls; furthermore, MS patients overall had alterations in the timing of ankle muscle activity and ankle motion compared to controls (Martin, Phillips et al. 2006). In addition to these motor symptoms, urinary dysfunction has

been described in patients with lower limb pyramidal dysfunction (Awad, Gajewski et al. 1984; Betts, D'Mellow et al. 1993). Depression, a psychological condition, has been shown to be elevated in MS patients compared to not only healthy controls, but also patients with other neurological symptoms (Patten, Beck et al. 2003). These and other symptoms, such as numbness, weakness, dizziness, and spasticity, are not mutually exclusive and often present at the same time. The quality of life has been reported to be lower in patients with MS compared to healthy controls due to the effect these symptoms have on the patients (Benedict, Wahlig et al. 2005).

There are four widely recognized disease course patterns based on an international survey to provide consensus in MS terminology pertaining to definitions of disease course (Lublin and Reingold 1996). Typical disease courses include relapsing-remitting MS (RRMS), primary-progressive MS (PPMS), secondary-progressive MS (SPMS), and relapsing-progressive MS (RPMS). RRMS is the most common with 85% MS patients diagnosed with RRMS. The disease course is characterized with exacerbations (relapses) of disease followed by partial or complete improvement (remissions). PPMS accounts for about 10% of MS cases where disease steadily worsens with no distinct relapses or remissions. SPMS follows the RRMS disease pattern initially; however, there is second stage in disease course with a gradual worsening of symptoms like PPMS. The second stage of disease course may or may not have relapses. PRMS includes elements of relapses and remissions occurring at the same time as a steady progression of disease. In 2013, revisions were made to adopt two new definitions of MS disease course: clinically isolated syndrome (CIS) and radiologically isolate syndrome (RIS) (Lublin, Reingold et al. 2014). These two disease courses were introduced to accommodate those who have initial signs of MS, such as inflammation and demyelination, but have yet to meet the criteria of exhibiting clinical symptoms over time.

Models for Demyelinating Disease

Mouse models have been utilized to study the basic biology and potential therapeutics for MS. Experimental autoimmune (or allergic) encephalomyelitis (EAE) is the demyelinating disease widely accepted and used to study MS in mice. EAE can be induced by active (Stromnes and Goverman 2006) or passive (Stromnes and Goverman 2006) induction. To perform active induction, a myelin peptide or protein is emulsified in complete Freud's adjuvant containing heat-killed tuberculosis. This is injected s.c. into the mouse on day 0 and an optional day 7 injection. Pertussis toxin (PTx) is also given i.p. at day 0 and 2. Passive induction is performed with the same protocol for active induction, followed by harvesting T cells from the lymph nodes of immunized mice, culturing T cells in vitro, and adoptively transferring these cells to naïve recipients. The mice that receive these adoptively transferred T cells will then exhibit disease symptoms without active disease induction. The model used determines which myelin peptide is chosen for studies, discussed later.

PTx use and its mechanism of action has been a topic of debate. The classical thought about its importance in EAE is that it helps to break down the BBB to allow infiltration of immune cells into the CNS. Addition of PTx has been shown in tissue culture to increase permeability in a cerebral endothelial barrier model (Bruckener 2003). However, this simple cause-effect relationship has been a challenge to prove in a mouse model. Most likely there are other factors at play in BBB breakdown, such as T cell activation and cytokine/chemokine production upon PTx injection. In mice transgenic (Tg) for CCL2 under a myelin promoter, mononuclear cell infiltrate was observed in the CNS and were shown to have crossed the endothelial basement membrane by histology (Fuentes, Durham et al. 1995). However, no clinical symptoms (Sifringer, Stefovska et al. 2007) or BBB

breakdown (Schellenberg, Buist et al. 2012) were observed unless PTx was given. These observations suggest that cytokines/chemokines are important for chemotaxis into the CNS, but that entry into the CNS alone does not cause disease. PTx was shown to increase mononuclear cell infiltrate and upregulate TNF-α and IL-1β mRNA to create a proinflammatory environment upon PTx exposure in that same model, demonstrating that the proinflammatory response may be responsible for disease induction and BBB breakdown (Sifringer, Stefovska et al. 2007). In the autoimmune disease model experimental autoimmune uveoretinitis (EAU), PTx has been shown to increase cytokine production and enhanced adaptive immune responses, providing further evidence that PTx acts through cytokine/chemokine responses and adaptive immune activation (Agarwal, Sun et al. 2002). In the same study, high doses (up to 10 ug in rats) of PTx was shown to inhibit disease induction, which has also been shown in the EAE model (Yin, Tang et al. 2014). Due to this, 200-250 ng PTx per mouse is used during disease induction.

As reviewed by Supachoke Mangmool and Hitoshi Kurose, PTx has both G-protein coupled receptor (GPCR) decoupling dependent and independent effects (Mangmool and Kurose 2011). Decoupling the GPCR was shown to inhibit T and B lymphocyte and neutrophil migration in vitro, which could explain why higher doses of PTx inhibit disease course through inefficient migration (Spangrude, Sacchi et al. 1985; Cyster and Goodnow 1995). However, PTx has also been shown to act in GPCR independent manners by activation of dendritic cells (DCs) to express CD86 and produce proinflammatory cytokine which are both involved in activation of the adaptive immune response (Wang, Yang et al. 2006). T cell activation (Gray, Huber et al. 1989) and TCR signal transduction (Schneider, Weiss et al. 2007) by PTx has also been shown which demonstrates the direct effects of PTx on activation of the adaptive immune response. PTx suppresses the number and function of

CD4+CD25+ T regulatory (Treg) cells in vivo, which would provide a more proinflammatory environment (Chen, Winkler-Pickett et al. 2006). Lastly, CD4+ T cell responses have been shown to be skewed toward a Th17 phenotype through IL-17 and IL-6 production in response to *Bordetella pertussis* infection and PTx in mice (Chen, Howard et al. 2007; Andreasen, Powell et al. 2009). Combining all factors described, PTx creates a proinflammatory environment via activation of the immune response to secrete proinflammatory cytokines and chemokines that boost the adaptive immune response. Leukocytes traffic into the CNS, attach to the endothelium via TNF-α induced VCAM-1 (Carlos, Schwartz et al. 1990) or other adhesion molecules induced by cytokines, and only cause disease with the appropriate proinflammary signals present through PTx, which will then cause BBB breakdown and more mononuclear cell infiltrate.

Several models have been utilized and are clearly defined to investigate different aspects of MS disease course. The myelin peptides myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), and myelin basic protein (MBP) have been used to induce disease in mice. For example, MOG35-55 induces primary progressive disease in C57Bl/6 whereas the same peptide induces relapsing-remitting disease in the NOD mouse strain (Slavin, Ewing et al. 1998; Kersh, Edwards et al. 2014). SJL/J mice follow a progressive-relapsing disease course where mice have an initial chronic phase followed by minor relapses when induced with PLP139-151 (McRae, Kennedy et al. 1992). An advantage of these models is the short time frame seen between disease induction and observation of clinical symptoms. Whereas MS in patients may take tens of years to develop, induction of disease in mice takes 10-20 days to see symptoms, depending on the model. This allows for relatively quick analysis of experiments compared to human clinical observation. Mice are also easily manipulated, bred, and stored. Full analysis of various organs can be performed at

multiple time points post-induction unlike humans in which case vital organs can only be analyzed post-mortem. Because of the relatively short lifetime of a mouse, experiments can also be observed over the lifetime of the mouse.

Demyelinating Disease Biology in Animals – A Model for Human Therapeutic Discovery

MS is studied in the demyelinating disease model due to its similarities to MS disease course. Disease onset is measured by the degree of paralysis of the animal with 0 = no disease, 1 = flaccid tail, 2 = moderate hind limb paralysis, 3 = total hind limb paralysis, 4 = moderate front limb paralysis, and 5 = moribund. This is similar to those MS patients who have gait difficulties and those with lower limb paralysis. Histologically, EAE and MS share similar features, such as inflammation with immune cell infiltrate and axon loss (Wujek, Bjartmar et al. 2002). In an MRI-based study, brains of mice exposed to EAE showed lesions typical of MS patients with accompanying inflammatory cell infiltrate surrounding these lesions (Rausch, Hiestand et al. 2003). The biology of demyelinating disease in humans has been either first discovered or supported via the use of the EAE mouse model. For example, the idea that T cells use adhesion molecules to cross the BBB was seen in mice which led to a novel therapeutic for MS. The expression of α4 integrin was measured to be higher in T cell clones to myelin peptide that were encephalitogenic compared to those clones that were not, and blocking α4 integrin using an antibody reduced disease severity in these mice (Baron, Madri et al. 1993). ICAM-1 and VCAM-1 were upregulated in the CNS of SJL/J mice afflicted with EAE and T cells expressing integrins to bind these adhesion molecules (Steffen, Butcher et al. 1994). The vast literature discussing the effects of blocking α4β1 integrin led to the production of Natalizumab (Tysabri) that is used as a front line therapy in MS patients today. Two other front line therapies, Copoxone (glatamir acetate)

and IFNβ therapy were also confirmed in animal models to reduce EAE symptoms (Abreu 1982; Lisak, Zweiman et al. 1983). Therapies for MS will be discussed in detail later. However, a preventive vaccine or better therapeutics for MS would be ideal, as patients respond differently to these medications. Animal models will be important in revealing novel insights into MS biology and uncovering new ways to treat MS patients since their biology and genetics are largely conserved compared to humans.

T Cell Biology

T cells play an important role in the adaptive immune response and have a wide variety of responses such as direct killing effects, activation of bystander cells, and regulation of the immune response. During the adaptive immune response, an antigen presenting cell (APC) processes antigen through two different pathways to present this antigen to T cells. Antigen from pathogens degraded in the cytosol is generally presented on major histocompatibility complex (MHC) class I molecules whereas extracellular antigens are processed in endosomes and loaded on MHC class II molecules. These different mechanisms evoke different types of T cells for different effects dependent on the type of evading pathogen for the optimal immune response. MHC class I will present peptide to CD8 T cells; however, MHC class II will present peptide to CD4 T cells. CD8 T cells are important for clearance of cytosolic pathogens, such as viruses. T cells that recognize peptide:MHC (pMHC) are then able to signal through their T cell receptor (TCR) for activation. This signaling is based on the magnitude of the response (affinity of TCR to pMHC) and contact time/duration of signal. The signal provides different outcomes of the cell including activation, anergy, exhaustion, and even deletion. This signaling in conjunction with the appropriate proinflammatory signals ensures that T cells target destruction of

foreign pathogens and not self. However, TCRs during autoimmune disease recognize self antigen as foreign antigen such that T cells will become activated when sensing these pMHCs. Signaling through the TCR during pMHC stimulation will terminally activate transcription factors to activate genes that control cell fate. Master transcription factors determine the differentiation of the cell in order to most appropriately respond to a sensed invader. CD8 and CD4 T cells interact with cells presenting antigen through both pMHC:TCR and adhesion molecules. Together, these interactions allow for formation of the cSMAC and pSMAC between cells, with TCR and costimulatory molecules in the cSMAC and adhesion molecules in the pSMAC. This allows effector cells to specifically target a neighboring cell by promoting directionality to released cytokines and effector molecules.

CD8 T cells actively kill cells presenting peptide on MHC class I and can secrete IFNγ to activate bystander immune cells. After SMAC formation, CD8 T cells can directly kill attached cells by releasing granules containing perforin and granzymes. These and other molecules such as Fas ligand and TNF are able to induce apoptosis in the attached cell. CD8 T cells have been associated with both pathogenic and regulatory roles in the CNS. Mice lacking CD8 T cells have reduced disease severity which suggests a pathogenic role for CD8s in MS (Bettini, Rosenthal et al. 2009). One potential role for CD8 T cells is the secretion of IL-17A to evoke a pathogenic Th17 response from CD4 T cells (Huber, Heink et al. 2013). However, it is important to note that other cell types when activated are most likely involved in the secretion in IL-17A which calls into question the magnitude of the role that IL-17A secreting CD8 T cells plays in the Th17 response. Opposing this is the thought that CD8 T cells play a regulatory role in MS. Studies show that CD8 T cells that are CNS antigen specific are able to dampen the antigen specific CD4 T cell response (York, Mendoza et al. 2010; Ortega, Kashi et al. 2013). Regulatory CD8 T cells are lost during exacerbations but

maintained during quiescent stages of MS disease course which suggests loss of regulatory CD8 T cell numbers and function during attacks (Baughman, Mendoza et al. 2011; Cunnusamy, Baughman et al. 2014).

CD4 T cells are helper cells that recognize peptide on MHC class II for activation and in turn then activate other immune cells via cytokine secretion. Different master transcription factors influenced by the proinflammatory environment control the differentiation of these cells. Bcl-6, Gata3, T-bet, Rorγt, and Foxp3 differentiate CD4 T cells into follicular helper T cells, Th2s, Th1s, Th17s, and Tregs, respectively. Different T cell lineages confer protection against foreign antigens in different ways. T follicular helper cells provide help to B cells during the germinal center reaction. Th2s provide protection against helminth infections by activating eosinophils, mast cells, and plasma cells through IL-4 and IL-5 secretion. Th1s secrete IFNγ to activate macrophages. Th17 cells activate fibroblasts and epithelial cells to secrete chemokines that guide neutrophils to the foreign antigen source. Finally, Tregs dampen the immune response via cytokine secretion. These cell types also have some degree of plasticity; for example, Th1s or Th17s can sometimes produce both IFNγ and IL-17A for effector functions instead of just one cytokine or the other. Understanding T cell biology is crucial for understanding the role that T cells play in MS as this disease is largely mediated by T cells.

The Role of CD4 T cells in Demyelinating Disease

T cells play a large role in the activation this disease, with Th17s playing a major role in disease progression. It was initially thought that Th1 cells were the main contributor to pathogenesis in MS. MBP-specific Th1 cells were shown to cause disease in B10.PL mice upon adoptive transfer to naïve recipients (Lafaille, Keere et al. 1997). Unexpectedly,

transferred Th2 cells were not shown to have a protective effect suggesting that other cell types other than the Th1/Th2 dichotomy could be involved in demyelinating disease course and that immune deviation was not sufficient to prevent disease. However, recent evidence points to Th17s as an initiator of disease course. Th17s were introduced as an inflammatory cell type independent of Th1s (Harrington, Hatton et al. 2005). In this same study, both type I (IFNα and IFNβ) and II interferons (IFNγ) were shown to inhibit Th17 development suggesting a potential pathway for amelioration of MS symptoms in those patients on interferon therapy. IL-23 was also shown to be crucial for disease induction whereas IL-12 was not (Cua, Sherlock et al. 2003). Therefore, Th17 cells are important for disease initiation during EAE. However, both cell types contribute to disease. Th1s vs. Th17s were shown to induce different types of EAE based on histology and chemokine profile (Kroenke, Carlson et al. 2008) which might explain why Th17s induce a more typical EAE phenotype whereas Th1s induce an atypical disease course (Domingues, Mues et al. 2010). Keeping in mind the heterogeneity of the helper T cell population and the varying disease courses that result from differences in this population may be important for downstream MS disease interventions.

The role of Th1s in disease progression has pathogenic effects as discussed, but also protective effects since 129/Sv mice resistant to EAE disease induction, when lacking the INFγ receptor, have been shown to have exacerbated disease outcomes (Willenborg, Fordham et al. 1996). Tregs also have been shown to play a protective role in MS. Supplementation of Tregs during EAE was shown to ameliorate disease symptoms (Kohm, Carpentier et al. 2002), possibility through IL-10 (Zhang, Koldzic et al. 2004). RRMS patients were then shown to have impaired/lack of suppressive function of Tregs (Viglietta, Baecher-Allan et al. 2004). These results showed that MS disease can be partially attributed to the lack of protection that normal Tregs would provide. Together, Th1s and Tregs

maintain a protective environment in order to limit disease severity in both the EAE model and in MS patients.

Genetics in MS

Multiple sclerosis is multifaceted in etiology. Genetics has been shown to play a role in MS pathology. Certain MHC class II genes such as HLA-DR2 and HLA-DR4 are associated with MS. Although DR2, or DRB1*1501-DQB1*0602, originally was assumed to have a dominant effect, a dose effect where two copies of this susceptibility haplotype increases risk for MS (Barcellos, Oksenberg et al. 2003). Using HLA-humanized mice, further investigation into this HLA haplotype shows that DQB1*0602 confers susceptibility to demyelinating disease rather than DRB1*1501 (Kaushansky, Altmann et al. 2012). GWAS studies, however, do not implicate DQB1*0602 as increasing susceptibility to MS compared to the DRB1*1501 haplotype alone (Patsopoulos, Barcellos et al. 2013). The same GWAS study does implicate other MHC alleles in MS as well as non-HLA alleles. DR4 has also been reported in MS (Marrosu, Muntoni et al. 1988). These genetic differences in MHC alleles predispose MS patients to disease. The differences in MHC alleles most likely allow for differences in thymic selection to myelin antigens, increasing or decreasing autoimmune risk. MHC molecules are important for presenting peptides to T cells. Depending on the fit of peptides into MHC molecules, the T cell response to antigen changes accordingly. Viral variant peptides, for example, have been suggested to bind sub optimally to MHC molecules resulting in altered T cell responses and activation (Shorter, Schnell et al. 2016). The MHC molecules that confer MS susceptibility risk could have similar altered peptide presentation leading to differences in thymic selection.

Other non-MHC alleles have also been associated with MS that are involved with T cell activation. Single-nucleotide polymorphisms (SNPs) within IL2RA and IL7RA genes were strongly associated with MS (Hafler, Compston et al. 2007). T cell activation is important for MS pathology in that CD4 T cells are the initiators of autoimmune disease. Dysregulation of activation may lead to unwanted T cell responses against self antigens. LCMV, for example, activates T cells to produce IL-2 and promote a Th1 response via the IL-2 receptor pathway along with high IFNγ production (Varga and Welsh 2000). Due to the association of the IL2RA to MS, this infection would be suspected to trigger autoimmunity. However, the opposing roles of IFNγ reported in literature have made the role of these proinflammatory cytokines in MS unclear.

Environmental factors in MS

Several factors are thought to play a role in MS disease pathology including genetic, immunologic, environmental factors, and luck. However, no one factor has been determined as a cause for MS. Multiple sclerosis only has a 25% concordance rate among monozygotic twins (Ramagopalan, Dyment et al. 2008). With a concordance rate strikingly low among genetically identical individuals, there must be a strong environmental component at play contributing to MS etiology. Insufficient vitamin D, gender, and infections are just a few of the environmental factors that have been highly linked to MS. The linkage between vitamin D and MS is still to be determined. Interestingly, vitamin D and the vitamin D receptor have been found to be required for disease initiation the animal model of MS (Wang, Marling et al. 2012). However, recently reviewed are the effects that vitamin D has on dampening Th17 responses and increase FoxP3+ Tregs (Hayes, Hubler et al. 2015). MS also has a female bias

over males. As reviewed, females are more likely to develop MS at a ratio of 2:1 or 3:1 depending on region due to a number of factors (Voskuhl and Gold 2012).

It has been shown that there is a higher risk for MS in the northern hemisphere than the southern hemisphere. In fact, those who are born in the southern hemisphere that move to the northern hemisphere assume the risk of the southern hemisphere, and vice versa. (Detels, Visscher et al. 1977; Elian, Nightingale et al. 1990; Compston and Coles 2008). This would suggest that there is a large environmental difference between the northern and southern hemispheres. Helminth infections, for example, are more common in the southern hemisphere compared to the northern hemisphere (Hotez, Brindley et al. 2008). These types of infections elicit a strong Th2 response. Several autoimmune diseases, such as MS, are Th1 and Th17 mediated. Helminth infections, therefore, will skew the response away from proinflammatory responses that result in autoimmunity. In addition, other infections could be skewed towards the northern or southern hemispheres. These differences in infections between the hemispheres could promote mechanisms that allow for differing control of CD4 T cell responses and influence susceptibility to MS.

Demyelinating Disease Modulation by Infections

Infections have been suggested as a potential explanation for the onset of MS. A few viruses in mice have been shown to either cause or exacerbate EAE symptoms. Theiler's virus has been used to study aspects of viral-induced demyelinating disease in susceptible mice (Tsunoda and Fujinami 1996). Use of this virus has shown that T cell reactivity to myelin antigens occurs sequentially, which supports the idea of epitope spread (Miller, Vanderlugt et al. 1997). This same study also provided evidence that epitope spread was not due to cross-reactivity of virus-specific T cells to a PLP epitope and occurred due to priming

of T cells to normally sequestered autoantigens, providing a potential mechanism into how viruses can induce or exacerbate autoimmune disease. However, a virus that actively causes MS in humans remains to be uncovered. Several pathogens and their peptides have been suspected to play a role in MS. During a *Candida albicans* fungal infection, EAE disease course was shown to be increased compared to controls (Fraga-Silva, Mimura et al. 2015). Fungal-specific T cells were thought to contribute to disease by producing harmful proinflammatory cytokines in the CNS. Bacteria have also been implicated in demyelinating disease. Mice infected during EAE disease course with *Streptococcus pneumoniae* exhibited a more severe disease course compared to controls (Herrmann, Kellert et al. 2006). Worsened disease course was accompanied by increased IL-6 and upregulation of MHC class II and costimulatory molecules. Finally, viruses have been associated with disease in MS patients. Viral peptides from Epstein-Barr virus (EBV), Influenza type A, Herpes simplex virus, and Adenovirus as well as a bacterial peptide from *Pseudomonas aeuroginosa* have all been reported to cross-react with myelin-specific T cell clones, suggesting a link between these infections and T cell mediated autoimmunity (Wucherpfennig and Strominger 1995). This cross-reactivity provides evidence for molecular mimicry. Molecular mimicry is the idea that an infection looks like self, and therefore produces T cells and antibodies that are cross-reactive between foreign and self-antigens. Therefore, it is thought that infections are able to trigger autoimmune disease and possibly be the initial cause of symptoms (Figure 1). However, the mechanism by which different infection types induce or worsen autoimmune disease may vary between infection types. In support of this, viral peptides with amino acid homology to myelin peptides have also been shown to induce EAE in mice without classical EAE induction with myelin peptides. For example, a herpesvirus *Saimiri* peptide with limited sequence homology to MBP not only had T cell crossreactivity to the myelin peptide, but

also was able to cause EAE disease symptoms on its own (Gautam, Liblau et al. 1998). Similarly, molecular mimics from *Candida albicans*, *Salmonella typhimurium*, and *E. coli* to PLP were expressed on viruses to determine if viral-expressing mimics could induce autoimmune disease (Ercolini, Croxford et al. 2007). Mild disease was seen in these mimic-expressing viruses, suggesting that viral vectors may be required to induce molecular mimicry mediated autoimmune disease. These studies together provide growing evidence for molecular mimicry in viral-induced autoimmune disease, epitope spread from infection-specific peptides to myelin specific peptides as well as providing the appropriate proinflammatory environment in which myelin specific T cells can be activated by bystander effect.

Although several infections may play a role in regulation of autoimmune disease, two viruses, Epstein-Barr virus (EBV) and human herpesvirus 6, have been associated to play a role in MS. Mice infected with an EBV equivalent, γHV-68, and then induced with EAE had a more severe disease course with increased CD8 T cells trafficking to the spinal cord and a greater Th1 proinflammatory response compared to uninfected mice induced with EAE (Casiraghi, Shanina et al. 2012). It is important to note that the infection itself did not cause disease in MOG-specific transgenic animals, suggesting that a viral insult itself was not sufficient to break tolerance to self-antigen. In MS patients, IgG titers of the EBV protein EBNA-1 were correlated with MRI lesions in CNS of MS patients as well as the lesion volume (Farrell, Antony et al. 2009). In addition, EBNA-1 specific T cells have been shown to cross-react with myelin antigens, hinting the role that molecular mimicry may play in EBV-induced demyelinating disease (Lunemann, Jelcic et al. 2008). Cross-reactive T cells were also shown to play a functional role in MS patients via secretion of INFγ, TNF, and IL-2, which would provide a proinflammatory environment. The lytic cycle of EBV has also been shown to correlate with MS disease course. In healthy donors and inactive MS patients,

more CD8 T cells for proteins involved with inactive EBV were seen, but more CD8 T cells for proteins involved with EBV activation were seen in active MS patients (Angelini, Serafini et al. 2013). However, EBV as a trigger for MS has been difficult to prove and remains a topic of debate.

The link between HHV-6 and multiple sclerosis is less clear. HHV-6 has been detected in the CSF of MS patients providing a link between infection and disease (Tejada-Simon, Zang et al. 2002; Yao, Honarmand et al. 2009). HHV-6 IgG titer has also been shown to be correlated with relapse risk in MS patients (Simpson, Taylor et al. 2012). HHV-6 reactivation has been shown to occur due to immune suppression via Natilizumab in MS patients, much like that of JC virus (Yao, Gagnon et al. 2008). This may cause more damage to the CNS in MS patients. Studies in MS patients with EBV and HHV-6 have provided insight into the potential of viral-induced autoimmunity in humans; however, more work is required to further associate viral infection with autoimmune disease and perhaps uncover an explanation into the induction of autoimmune disease by viral infection.

Protective Effects of Infections during Demyelinating Disease

Less attention has been attributed to the protective role that infections may play during autoimmune disease. Infection with Lactic Dehydrogenase Virus during EAE disease course was shown to produce positive disease outcomes (Inada and Mims 1986). Depending on when the infection was performed in relation to the time EAE was induced, EAE disease incidence was lower than non-infected mice (infection day 3 post-induction or 14 days pre-induction) or completely eradicated (infection given at same time of EAE induction). The day of symptom onset was delayed in these mice and severity of disease was slightly reduced. Previous infection with *Mycobacterium tuberculosis* (Mtb) and *Bordetella pertussis* has also been

shown to promote protection to EAE challenge (Lehmann and Ben-Nun 1992). However, caution in interpreting these results should be taken since Mtb and pertussis toxin are used to induce EAE. Therefore, previous immune responses to Mtb and pertussis toxin may affect induction efficacy. A recent paper involving *Plasmodium chabaudi* infection shows increased Treg generation may be responsible for lower disease course outcomes in mice cochallenged with EAE and *Plasmodium chabaudi* (Farias, Talaisys et al. 2011). However, several unexplored mechanisms could be responsible for how infection regulates disease course. Stimulation of inhibitory receptors on T cells, anti-inflammatory cytokines, altered antigen presentation, exhaustion, anergy, immune distraction, or immune deviation could all play a role in viral protection against autoimmune disease. Importantly, nonobese diabetic mice exhibiting diabetes symptoms similar to humans is prevented with lymphocytic choriomeningitis virus (LCMV) infection (Oldstone 1988). Although not a demyelinating disease, this finding shows that infections can offer protection against autoimmune diseases such as MS. More research should be performed to see how infections may protect against demyelinating autoimmune disease.

Protection via the hygiene hypothesis

The hygiene hypothesis would suggest that exposure to infections early in life can protect from rising diseases such as asthma and autoimmunity. Exposure to endotoxins early in life has been linked to protection from asthma and atopy (Braun-Fahrlander, Riedler et al. 2002). Animal models have reinforced the hygiene hypothesis concept. In nonobese diabetic (NOD) mice, a variety of infections have been shown to influence the incidence of type 1 diabetes. For example, raising NOD mice in specific pathogen free conditions instead of conventional housing increases diabetes incidence (Bach 2002). If Lewis rats are subjected to

pretreatment of complete Freund's adjuvant before inducing EAE with peptide, these rats become unresponsive to the induction (Hempel, Freitag et al. 1985). These results are similar to what was observed with our LCMV infections and *Listeria* infections performed on C57Bl/6 mice. However, *Listeria* invoked EAE under genetically susceptible conditions. These results highlight the interplay between genetics and environment such that individuals may need to have a genetic predisposition to autoimmune disease in order for environment (in this case infections) to trigger autoimmune disease. It would be of interest to determine, similar to the specific pathogen free condition experiments done in NOD mice, if LCMV or *Listeria* elicit greater EAE responses under these conditions. Perhaps the microbial environment that we develop early in life due to exposure to different microbes influences the ability to generate autoreactive T cell responses.

Current Therapies for MS

There are a wide variety of treatment options for MS patients. These medications are thought to alter the immune response to provide positive disease outcomes. Interferon β therapeutics are commonly used in MS patients due to their immunomodulatory effects. However, IFNβ therapy is not 100% protective. As previously stated, type I and II interferons may inhibit Th17 development, leading to a likely explanation of disease treatment. The reason behind differential efficacy in treatment seen in MS patients, however, remains unclear. Adverse events to IFNβ therapy have been documented as well (Jongen, Sindic et al. 2011).

Another immune-modifying treatment commonly used in MS patients is glatiramer acetate. Glatiramer acetate is a short polymer of amino acids that are found in myelin basic protein – a target of self-reactive T cells. Although the exact mechanism for this disease-

modifying treatment is not well understood, it is thought that the small protein distracts damaging myelin-specific T cells away from their original myelin target. As with IFNβ treatment, glatiramer acetate is not 100% effective. Research into the mechanism behind glatiramer acetate is required to improve upon this treatment strategy.

Natalizumab has also been used to treat MS patients. Its mechanism of action is more defined than the other listed treatments. This therapy utilizes a monoclonal antibody that targets α4 integrin, a molecule involved in cell adhesion. This integrin can interact with endothelial ligands VCAM-1, fibronectin, and MAdCAM-1. These interactions then allow immune cells to cross the endothelial layer and enter tissues such as the brain. Therefore, blocking this integrin prevents immune cells from trafficking into the CNS in MS patients. However, this therapy does not come without risks. For example, treatment with Natalizumab causes an immunosuppressed state in MS individuals. Due to this, polyomaviruses become unchecked in the CNS. This leads to the development of progressive multifocal leukoencephalopathy (PML), a fatal disease characterized by inflammation of the CNS. Nucleic acid of JC virus, a polyomaviruses, can be found in the CNS of MS patients on Natilizumab (Perez-Liz, Del Valle et al. 2008). T cell responses have been shown to be almost absent or dominated by anti-inflammatory IL-10 in MS patients that exhibited symptoms of PML (Perkins, Ryschkewitsch et al. 2012). This dysregulation of the immune response by Natalizumab is therefore not an ideal treatment for all MS patients.

Collectively, these therapeutics have been moderately successful in the treatment of MS patients; however, patients respond differently to these medications and there are risks associated with each treatment. There is no vaccine currently available for the treatment of MS. More research is required to develop more efficacious treatment options for the MS community.

Potential Therapeutics and Vaccination Strategies Toward Demyelinating Disease

Attenuated infectious vectors with peptide specific for a target antigen have been researched as potential vaccine strategies in order to boost the immune response against cancer (Mandl, Sigal et al. 1998; Starks, Bruhn et al. 2004). However, these studies sought to expand T cells that elicited a proinflammatory response to boost protective immunity against the cancer cells. Promoting a proinflammatory response in autoimmune diseases would not be ideal since this would encourage self-specific T cells to attack self instead of maintain tolerance. One avenue of potential protection is to include the self-peptide or an altered peptide ligand into a microbial vector to promote Tregs that suppress the immune system. Barnett et al. have shown that when the MBP encephalitogenic peptide was introduced into vaccinia virus, protection was observed when challenged with EAE (Barnett, Whitton et al. 1996). However, caution should be taken when choosing to vaccinate with self peptides as the myelin peptide PLP in vaccinia virus had the opposite effect and exacerbated autoimmunity (Barnett, Whitton et al. 1993). Peptides that are similar to the self-peptide, altered peptide ligands, have also been tested to determine their protective effects during demyelinating autoimmune disease as reviewed by Ahmed H. Badawi and Teruna J. Siahaan (Badawi and Siahaan 2012). With this information and the idea that Tregs are important in reducing demyelinating disease symptoms, it will be interesting to see if a vaccination strategy can be developed and utilized in order to boost a myelin-specific Treg response to reduce autoimmune disease symptoms.

2D Micropipette Adhesion Frequency Assay

To determine if affinity plays a role in infection-mediated autoimmunity, the micropipette system can be utilized to not only determine the frequency of antigen-specific CD4 T cells, but also to determine the affinity for these cells. Purified CD4 T cells and red blood cells (RBCs) coated in peptide:major histocompatibility complex (pMHC) using biotin and streptavidin to act as a surrogate antigen presenting cell (APC) are loaded into media under a microscope (Figure 2) (Edwards, Zarnitsyna et al. 2012). These cells are picked up separately via aspiration through two different micropipettes. One micropipette holds a T cell of interest while another micropipette holds a surrogate APC (Chesla, Selvaraj et al. 1998). The surrogate APC is brought into contact with the CD4 T cell and binding events are measured either as a 0 (no binding) or 1 (binding) for 50 touches to determine adhesion frequency. The adhesion frequency, the TCR density of the CD4 T cells (measured by flow cytometry), and the density of pMHC on the surrogate APC (also measured by flow cytometry) are parameters used to determine the affinity of a CD4 T cell through a mathematical model (Chesla, Selvaraj et al. 1998). The RBC membrane in this technique is highly flexible which allows us to detect a single T cell receptor (TCR):pMHC interaction sensitivity. With this sensitivity, the affinity of the TCR to its cognate antigen can be measured. The frequency of antigen-specific cells can also be determined by testing the number of cells that bind to the surrogate APC. The micropipette assay is advantageous to use to determine the frequency of low affinity autoimmune specific CD4 T cells which would not be measured accurately with other techniques as well as high affinity cells (Huang, Zarnitsyna et al. 2010). For example, it has been shown that flow cytometry underestimates the MOG-specific T cell response compared to the micropipette system (Sabatino, Huang et al. 2011).

Summary

MS is characterized as a demyelinating autoimmune disease. This disease is mediated by breakdown of the blood brain barrier, the appearance of lesions in both the white and grey matter, and the presence of myelin-specific T cells. CD4 T cells are the initiators of this disease, with the T cell response skewed towards a Th17 phenotype. These pathogenic Th17 cells create a damaging proinflammatory environment in the CNS. Tregs have an opposing role by maintaining tolerance and reducing disease pathology during demyelinating disease. Animals have been valuable in the research performed to study demyelinating autoimmune disease. Mouse disease models have not only revealed information regarding the biology of MS but also have furthered the advancement of treatments to ameliorate disease symptoms in patients. This research has led to the discovery of front line medications used today by MS patients. However, treatments are not ideal as they elicit different responses in MS patients and are not 100% effective. Interestingly, viruses have been shown to have some protective effects in mice exhibiting MS symptoms which provides a potential avenue of research in terms of developing novel therapies for MS patients. Similar results have been shown in the NOD model for the autoimmune disease type I diabetes, where LCMV infection prevented diabetes symptoms similar to humans. Vaccination strategies have been explored to ameliorate MS disease symptoms. Utilizing the self-peptide or altered peptide ligands are just two avenues by which protection may be achieved. To protect against autoimmune disease, Treg generation via vaccine may be critical. However, more research is required to better understand MS biology and the role that viruses play in autoimmune disease in order to develop new therapeutics and potential vaccine strategies for these patients.

Figure Legends

Figure 1. Schematic of molecular mimicry theory. An infection carries a peptide that looks like self, such as myelin oligodendrocyte glycoprotein (MOG). A person is then infected with this pathogen and generates pathogenic Th17s and Th1s to the peptide similar to MOG. Due to the cross reactivity of these cells to the foreign and self-peptide, this infection breaks tolerance and produces T cells that secrete proinflammatory cytokines to activate immune cells that damage the neuronal sheath and the neurons themselves, as well as oligodendrocytes that produce MOG. This leads to the paralysis and other symptoms observed in MS patients.

Figure 2. Representation of micropipette adhesion frequency assay. The T cell (left) is held by suction on a micropipette. This T cell has antigen-specific TCRs on its surface. This T cell is tested on the RBC (right). This RBC has been coated with biotin and streptavidin so that the biotinylated pMHC monomer may bind to the streptavidin. Cells are brought into contact and pulled apart in order to measure the affinity of the T cell.

Figures

Figure 1

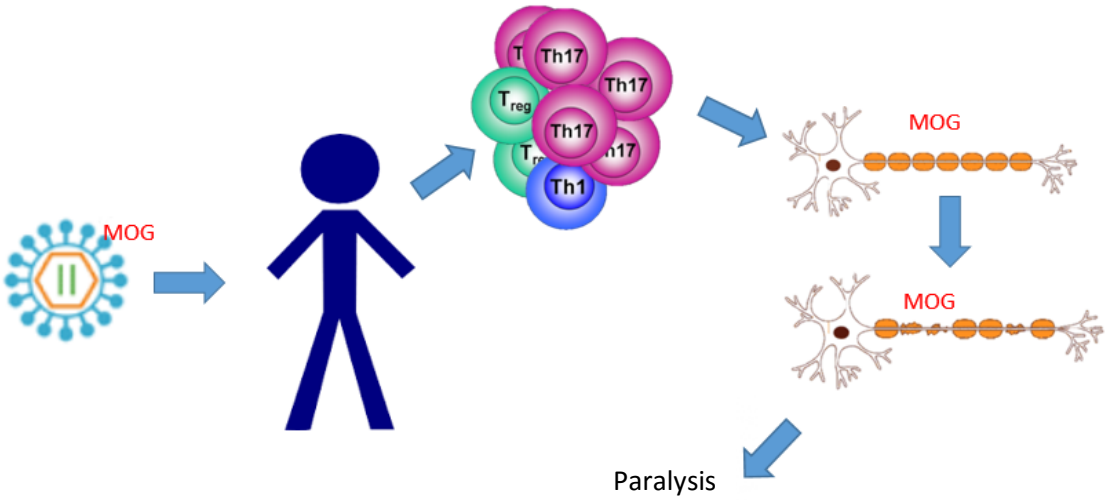

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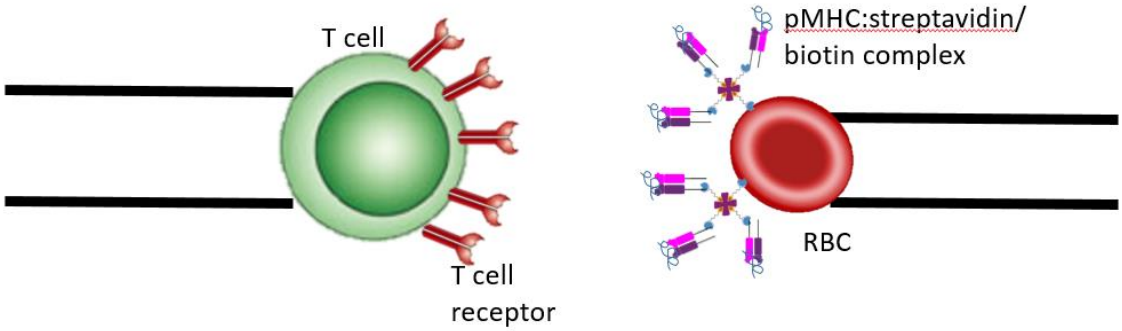
Figure 2


T cell
T cell
receptor
pMHC:streptavidin/
biotin complex
RBC

Chapter 2

Antigen specific Tregs are selectively expanded to prevent demyelinating disease

Jennifer Cosby, Ryan Martinez, Erin Theisen, John-Demian Sauer, and Brian Evavold

Abstract

Multiple mechanisms, including molecular mimicry, have been proposed to describe a relationship between infection and autoimmunity. Mimic epitopes are suspected to expand self-reactive T cells that can then target self-antigens, initiating demyelinating disease. We utilize a vaccine strain of *Listeria monocytogenes* containing myelin oligodendrocyte glycoprotein (Lm-MOG) to recapitulate the process of molecular mimicry. Upon infection, myelin-specific T cells are minimally expanded (~4 times) compared to naïve controls with ~85% as Tregs. This is substantially less expanded than the ~57 times which occurs upon immunization with MOG emulsified in CFA with pertussis toxin where ~15-20% are Tregs. We also investigated our infection model in a MOG deficient mouse that has altered thymic selection and an absence of MOG specific suppressive Tregs to determine whether MOG specific tolerance limits disease initiation by infection. Only in the MOG deficient mice does Lm-MOG initiate EAE with substantial numbers of MOG specific Teff (~282 times naïve controls) upon primary infection and low Treg frequencies (~5%). These data reveal two major findings: 1) MOG tolerance mechanisms are difficult to overcome and 2) deficient MOG tolerance mechanisms increase the risk for molecular mimicry induced demyelinating autoimmune disease.

Introduction

Multiple sclerosis is a demyelinating autoimmune disease characterized by a proinflammatory CD4 T cell response (Bruck 2005; Chen, Langrish et al. 2006). Conversely, Tregs have been implemented in controlling the effector CD4 T cell pool in order to prevent and/or inhibit disease. Tregs secrete IL-10, TGF-β, and IL-35 that can contribute to a suppressive environment, as has been investigated in the IBD mouse model (Asseman, Mauze et al. 1999; Read, Malmstrom et al. 2000; Collison, Workman et al. 2007). The mouse model of MS, experimental autoimmune encephalomyelitis (EAE), results in severe clinical symptoms compared to controls after Treg ablation (Koutrolos, Berer et al. 2014). MS patients have been reported to contain similar Treg frequencies compared to healthy controls (Putheti, Pettersson et al. 2004); however, functional Treg responses in MS patients is lacking (Haas, Hug et al. 2005; Venken, Hellings et al. 2008). Therefore, Tregs are an important mechanism to maintain peripheral tolerance.

Infections have been thought to break peripheral tolerance to self-antigens via multiple mechanisms, including molecular mimicry, epitope spread, release of sequestered antigens, and bystander activation (Vanderlugt and Miller 2002). During the process of molecular mimicry, a T cell can react to self antigen in the context of MHC and a similar foreign epitope (Lo, Woods et al. 2000). Therefore, the CD4 T cell can become activated by the foreign antigen and cross-react to the self antigen. CD4 T cells have been shown to cross-react between foreign and myelin antigens *ex vivo*, supporting the idea of molecular mimicry (Hausmann and Wucherpfennig 1997; Nelson, Beisang et al. 2015). In addition, molecular mimicry has been investigated in a mouse diabetes model. In these studies, the foreign gp66 LCMV antigen is introduced downstream of the rat insulin promoter (RIP), allowing for the gp66 antigen to become like a self antigen. When RIP-gp66 mice are

infected with LCMV, insulitis occurs from destruction of pancreatic β cells (von Herrath, Dockter et al. 1994). These results reveal that an infection could initiate autoimmune disease *in vivo*. However, these studies used the viral antigen as self which likely differs in tolerance mechanisms as opposed to an endogenous self-antigen.

Although molecular mimicry has been suspected to be a trigger for autoimmune disease, its role in suppressing autoimmune disease has been poorly described. Tregs are able to expand after an infectious insult (Farias, Talaisys et al. 2011; Boer, Joosten et al. 2015). In addition, dendritic cells can be stimulated by particular TLRs in order to become a regulatory DC that generates Tregs (Manicassamy, Ravindran et al. 2009). These Tregs can then promote an anti-inflammatory environment in order to suppress the T effector response to limit disease. The myelin antigens PLP and MBP have been previously introduced into vaccinia virus (Barnett, Whitton et al. 1996; Wang and Fujinami 1997). In these studies, the recombinant agent itself was not able to cause disease. EAE was exacerbated or reduced to PLP or MBP, respectively, when challenged to these antigens in CFA. The myelin antigen, MOG35-55, has not been studied in the context of infection.

In this study, the well-described MOG35-55 epitope was introduced into a vaccine strain of *Listeria* to serve as proof of concept that molecular mimicry may occur in nature. However, tolerance was harder to break than originally thought. Not only was classical disease prevented upon infection, but also myelin specific CD4 T cells minimally expanded (4 times over naïve controls) compared to the classical induction method of EAE (57 times over naïve controls). The effector myelin specific pool remained relatively unexpanded whereas Tregs were expanded. Tregs were not expanded to a control antigen. Together, these data suggest that myelin specific Tregs are selectively expanded to prevent EAE.

Materials and Methods

Mice

C57Bl/6 mice were purchased from Charles River Laboratories. C57Bl/6, Cα-/-, and MOG-/-, and FoxP3-GFP 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.

Lm-MOG infection and active EAE induction

Listeria monocytogenes LADD strain was engineered by Erin Theisen to express MOG35-55 (Lm-MOG) and was supplied by John-Demian Sauer's laboratory. Mice were infected i.v. with 1 X 10^7 CFU of Lm-MOG or Lm-gp66, similar to other studies (Lizotte, Baird et al. 2014). EAE was induced as previously described (Stromnes and Goverman 2006). Briefly, C57Bl/6 mice were induced with 200 μg MOG35-55 peptide, synthesized on a Prelude Synthesizer (Protein Technologies), emulsified in Complete Freud's adjuvant containing a final concentration of 2.5 mg/mL heat-inactivated *Mycobacterium tuberculosis* injected into the hind flank s.c. Mice received 250 ng pertussis toxin (List Biological Laboratories) i.p. on the day of induction and 2 days later. Disease severity was monitored via clinical scores and weights. Classical clinical scores were measured as follows: 0-no disease, 0.5-distal tail limp, 1-flaccid tail, 2-partial hind limb weakness, 2.5-severe hind limb weakness, 3-partial hind limb paralysis, 3.5-severe hind limb paralysis, 4-hind limb paralysis and cannot right self, 5-moribund/death. Atypical disease course was scored as follows: 1-mild head tilt, 2-severe head tilt, 3-body tilt, 4-axial rotation of body, 5-death.

Adoptive transfer and Treg Depletion

Five million CD4 T cell splenocytes were enriched via negative selection and transferred to T cell deficient mice. Infections were performed 1 day post-transfer.

Tetramer enrichment

Tetramer enrichment was performed as previously described (Moon, Chu et al. 2009). Cells were harvested from the spleen and pooled lymph nodes (inguinal, axial, brachial, cervical, mesenteric, and para-aortic LNs) of mice for analysis. Organs were processed into single cell suspensions using a 100 μm filter. Naïve cells were stained for two color and expanded populations were stained with one color tetramer (MOG-Ia^b PE and/or APC) at 4 μg/mL in Fc block for 1 hour at room temperature. Cells were washed with ice-cold FACS wash and then incubated with anti-PE magnetic beads for 30 minutes at room temperature. Cells were washed and enriched on a magnetic MACS column (Miltenyl Biotech). Cells were then surfaced stained for flow cytometry.

Extracellular surface staining

Cells surfaced stained with the following antibodies: anti-CD3ε (clone 142-2C11, BD Pharmigen), anti-CD4 (clone RM4-5, Biolegend), anti-CD8α (clone 53-6.7 Tonbo Biosciences), anti-220 (clone RA-6B2, eBioscience), anti-CD11c (clone HL3, BD Pharmigen), anti-F4/80 (clone BM8, eBioscience), and anti-CD44 (clone IM7, eBioscience). Surface staining took place on ice for 30 minutes. Flow cytometry was performed on a LSRII flow cytometer and data was analyzed using FloJo software. Cell counts were obtained by running samples with AccuCheck Counting Beads (ThermoFisher Scientific) on the flow cytometer.

Statistical analysis

Data were graphed and statistically analyzed using GraphPad Prism 6 software. Student's t tests were performed. GPower 3.1 was used to determine achieved statistical power of experiments.

Results

Disease is limited upon infection

To determine if infection alone could initiate demyelinating disease, C57Bl/6 mice were infected with Lm-MOG. An incidence of 0% of 5 infected mice displayed classical clinical EAE symptoms, whereas mice receiving peptide by the classical induction method (peptide emulsified in CFA) showed classical symptoms of EAE with an incidence of 12/13 mice (Figure 1A). Atypical disease with a head tilt or score of 2 on the atypical scale was observed in 1/5 infected mice (Abromson-Leeman, Bronson et al. 2004). No atypical disease was observed in classically induced EAE mice. The infected mouse also twirled when picked up by the tail, a phenotype that is not seen during typical disease course. These results suggest that infection alone is not sufficient to break tolerance to myelin antigen as classical disease incidence was 0%. Therefore, tolerance may be harder to break than once thought.

Genetically susceptible mice are at risk for infection-initiated disease

MOG-/- mice are genetically susceptible to infection since they have fewer Tregs to MOG35-55 and more effector CD4 T cells generated toward the antigen with higher affinity compared to an unaltered mouse (Martinez manuscript in preparation); therefore, these mice can model the defects seen in MS patients. To determine if infection initiates disease in these genetically susceptible mice, either C57Bl/6 or MOG-/- CD4 T cells were purified and transferred to a Cα-/- mouse prior to infection. Weight loss was observed in all mice, indicative of an active infection (Supplemental Figure 1). Post-primary infection, no control mice that received C57Bl/6 cells displayed neurological symptoms post-primary infection. These results confirm the difficulty to break tolerance in C57Bl/6 mice.

After MOG-/- transfer, both classical and atypical symptoms were observed in mice post-primary infection. Classical disease symptoms were observed in 10/18 (55.6%) mice and 2/18 (16.67%) displayed atypical symptoms upon primary infection. Average disease course for those mice that exhibited classical disease symptoms after primary infection is shown in Figure 1B. Average day of onset post-primary infection in mice with classical EAE symptoms was 10 days. Both chronic/unresolving symptoms and acute symptoms that resolved back to a score of 0 were observed. Mean clinical score of the 10 total classical EAE mice exhibiting disease was 2.4$\pm$ 0.2 after primary infection. Post-infection, weight will drop due to infection, rise upon bacterial clearance, and drop again due to clinical EAE onset/worsening of disease (Figure 1C). These results not only show that mice had an active infection, but also reconfirm the scoring system. Atypical mice did not resolve to a 0 post-primary infection. Together, these data suggest that genetically susceptible individuals are at risk for infection-initiated demyelinating disease.

Expansion of MOG-specific CD4 T cells is limited upon infection

Expansion of MOG-specific T cells in MOG-/- mice has previously been shown to be higher than in a wild-type C57Bl/6 mouse (Martinez manuscript in preparation). To determine if the amount of MOG-specific T cell expansion may play a role in initiation of autoimmune disease upon infection, C57Bl/6 mice and MOG-/- were infected and one week later tetramer enrichment was performed on these animals. The gating strategy used can be seen in Supplemental Figure 2 and Figure 2A. MOG-specific CD4 T cells significantly expanded compared to naïve controls (773 vs 195, Figure 2B). In MOG-/- mice, significant expansion was also observed (22,257 vs 79). Expansion was 282 times above naïve controls in the MOG-/- mice compared to only 4 times higher in the C57Bl/6

mice, suggesting that the capability of MOG-specific T cells to expand in genetically susceptible mice may play a role in initiating disease.

MOG-specific and Listeria-specific expansion are correlated in genetically susceptible mice

In order to initiate disease, T cell expansion to antigen may require reaching a particular threshold value in order to break tolerance to self-antigen. In the MOG-/- mice, this threshold seems to have been reached since T cell deficient mice reconstituted with MOG-/- CD4 T cells are able to exhibit disease symptoms, whereas unaltered C57Bl/6 mice rarely display disease. As a result, we were curious to know if the *Listeria*-specific (LLO) response correlated with the MOG-specific T cell response. Upon staining with LLO tetramer of pooled LNs and spleen, MOG-/- had less expansion to LLO than C57Bl/6 mice (60612 vs 141152, Figure 3A). This is most likely due to the competition and space limitations in order to expand to the two different antigens. In C57Bl/6 mice, MOG-specific T cells were not correlated with the LLO T cell response (Figure 3B). However, in MOG-/- mice, the two responses were strongly correlated with each other (Figure 3C). Therefore, predictions to determine autoimmune risk might be made based on responses to infectious insults.

Antigen-specific Tregs are generated upon infectious insult

Tregs are a major regulatory component during demyelinating disease course (Koutrolos, Berer et al. 2014). Due to this, Tregs were investigated upon infection. Tetramer enrichment was performed as in the expansion experiments. In C57Bl/6 mice, the majority of CD4 T cells were FoxP3+ (86%) while the majority of CD4 T cells in MOG-/- animals were FoxP3- (6.8%, Figure 4B). This suggests that there is a large effector population in the

MOG-/- animals contributing to disease. However, the C57Bl/6 animals are able to maintain tolerance because the few hundred CD4 T cells that do expand to MOG as shown in Figure 2B express FoxP3 post-infection. In addition, as shown in Figure 4C, these Tregs express classic Treg markers such as CD25, CD103, and GITR, confirming their Treg phenotype (Shevach, McHugh et al. 2001; Huehn, Siegmund et al. 2004; Shevach and Stephens 2006). Therefore, Tregs that expand in C57Bl/6 animals may be effectively limiting the self-reactive population to limit disease incidence. Expansion of Tregs is also specific to MOG expressed in *Listeria* upon infection compared to other antigens. In experiments where mice are infected with *Listeria* expressing the CD4 LCMV epitope gp66 (Lm-gp66), the Treg response is less than 3% (Figure 4B). Although the MOG-specific Treg total numbers average to be the same, the Teff/Treg ratio is much lower in the C57Bl/6 animals because of the similarity in Treg number (Figure 4B). This means that for every myelin-specifiic Teff, there in turn are more antigen-specific Tregs that are able to dampen the response to a MOG insult compared to a genetically susceptible MOG-/- animal.

Discussion

Although molecular mimicry has been proposed as a mechanism to initiate disease, we observe that breaking tolerance via infection is actually very difficult to overcome. Peptide emulsified in CFA produces ascending paralysis as a result of demyelinating disease at a high percentage (92% incidence). However, *Listeria* infection did not replicate classical EAE. In fact, no mice showed classical EAE symptoms. In addition, 1/5 mice exhibited atypical symptoms. Atypical EAE is rarely seen as a result of the classical induction method. Since classical EAE is not observed, the *Listeria* infection is unable to mount the correct immune responses in order to generate disease symptoms. Several factors may be at play that limits *Listeria*'s ability to break tolerance to MOG. For example, *Listeria* may not stimulate the correct innate immune pathways in order to mount an effective immune response. In fact, *Listeria* has been shown to activate signaling via TLR2. It has been previously published that TLR2-dependent ERK signaling in DCs programs these cells to generate Tregs (Manicassamy, Ravindran et al. 2009). MOG within *Listeria* can generate Tregs, whereas gp66 does not. This suggests that there may be multiple pathways downstream of TLR2 that may be activated in an antigen specific manner. Therefore, the antigen within the infectious agent may be responsible for inducing the suppressive capability of DCs. Genetically susceptible mice were able to generate Tregs to MOG expressed in *Listeria* as well. However, these Tregs were just not able to mount a functional response to limit the effector pool.

Thymic selection is also likely a contributing factor to the response during *Listeria* infection. Our lab has previously published that thymic selection is altered in genetically susceptible mice (MOG-/-) compared to wild-type controls (Martinez manuscript in preparation). Due to differences in thymic selection, nonfunctional Tregs and high affinity effector T cells are selected in genetically susceptible mice. This results in two contributing

factors in disease – a high CD4 effector T cell response and a lack of suppressive functional Tregs. As seen in genetically susceptible mice, antigen specific T cells mounted a high (282 times over naïve) antigen-specific T effector response compared to their wild-type (4 times over naïve) counterparts. Thymic selection of high affinity effector CD4 T cells likely contributed to the risk of demyelinating disease of genetically susceptible individuals due to infection. The minimal expansion observed in C57Bl/6 mice post-infection was not sufficient to induce classical EAE symptoms; however, there was 57 times expansion when EAE was induced by the classical method of MOG emulsified in CFA and Lm-MOG produced 282 times expansion of antigen-specific CD4 T cells. These results provide evidence that there is a particular theoretical threshold value that must be reached in order to initiate disease.

Functional Treg generation is also a result of thymic selection. In genetically susceptible mice, it has been shown that Tregs are less functional compared to their wild-type counterparts (Martinez manuscript in preparation). The generation of functional antigen-specific Tregs plays a major role in suppression of the antigen-specific effector pool as determined in these studies. By limiting the effector pool, antigen-specific T cells are not able to expand in order to reach the threshold value to cause disease in a wild-type mouse as described. This is an antigen specific phenomenon to the myelin antigen expressed in *Listeria* as gp66 did not show the same results. In fact, gp66 showed less than 3% Treg generation suggesting that the antigen expressed within *Listeria* provokes different responses. However, Tregs were ineffective at limiting the effector T cell pool in MOG-/- animals. Although antigen-specific Tregs are able to expand in genetically susceptible mice, the frequency of expansion was minimal (6.8%) compared to the high frequency of expansion seen in C57Bl/6 animals (86%). The limitation of Tregs to expand at a higher frequency most likely

allowed for the Teff response to overcome this peripheral tolerance mechanism post-infection. In MS patients, Treg functionality has been shown to be impaired (Haas, Hug et al. 2005; Venken, Hellings et al. 2008). Since tolerance mechanisms are deficient in MS patients and MOG-/- animals, it can be speculated that MS patients are susceptible to infection-mediated autoimmune disease.

Although this research focuses mostly on how the infection regulates a self-antigen response, the self-antigen response could also affect the immune response to infection. In fact, the LLO response was significantly lower in the MOG-/- animals compared to C57Bl/6 mice. The lower response could be as simple as a limitation in space to accommodate the larger MOG-specific CD4 T cell response seen in MOG-/- animals; however, there may be other reasons behind the response. For example, Treg generation has been shown to allow CD8 T cells to form a better memory response (Laidlaw, Cui et al. 2015). The same could hold true for CD4 T cells in the context of the Lm-MOG infection. The expansion of MOG and LLO specific CD4 T cells was correlated in genetically susceptible mice. Therefore, individuals that have a higher self-antigen response have a higher infection specific response. It would be interesting to be able to see if this held true for other infection antigens as well. If this were the case, predictions could be made as to who may be at risk for infection-based autoimmune disease based on the patient's response to foreign antigens.

Altogether, this research has provided new insight into the role of infection to sustain tolerance in healthy individuals with functional antigen-specific Tregs. Infections, although thought of as triggers for autoimmune disease, may actually help protect certain individuals from autoimmune disease by boosting peripheral tolerance. However, other individuals that lack a functional Treg response may be susceptible to infection-mediated

demyelinating disease. Determination of how to boost the antigen-specific Treg response and limit the antigen-specific effector T cell pool will be of interest in order to effectively treat MS patients.

Acknowledgements

The authors would like to thank Laurel Lawrence for breeding mice within Emory's animal facilities.

Figure legends

Figure 1. Infection initiates EAE in genetically susceptible mice. Classical EAE (EAE line) was induced with MOG emulsified in CFA with PTx given, or mice were infected with Lm-MOG (Lm-MOG line). A) Clincal scores were measured over time. T cell deficient mice were reconstituted with MOG-/- CD4 T cells and infected with Lm-MOG. B) Clincal scores and C) weights were measured over time. Mean±SEM are displayed for each.

Figure 2. Myelin specific CD4 T cell expansion is limited post-infection. C57Bl/6 or MOG-/- mice were either left naïve, classically induced for EAE, or directly infected with Lm-MOG. CD4 T cells were gated on lymphocytes, singlets, CD3ε+, F4/80-CDllc-B220-, CD4+, CD8-, CD44hi, MOG tetramer+. A) Sample staining for the CD44hi MOG tetramer+ gate in a MOG-/- mouse. B) Tetramer enrichment data from directly infected C57Bl/6 or MOG-/- mice. Student's t test used to determine significance at $p<0.05$.

Figure 3. LLO specific CD4 T cells are correlated in MOG-/-, but not C57Bl/6, mice. CD4 T cells were gated on lymphocytes, singlets, CD3ε+, F4/80-CDllc-B220-, CD4+, CD8-, CD44hi, MOG tetramer+ or LLO tetramer+. A) Tetramer staining performed on spleen and pooled lymphocytes day 7 post-infecition. Correlation analysis performed between MOG tetramer+ and LLO tetramer+ T cells in B) C57Bl/6 mice and C) MOG-/- mice. Student's t-test used to determine significance at $p<0.05$.

Figure 4. Tregs expanded in wild-type mice but not genetically susceptible mice. CD4 T cells were gated on lymphocytes, singlets, CD3ε+, F4/80-CDllc-B220-, CD4+, CD8-, CD44hi, MOG tetramer+, and FoxP3+ or FoxP3-. A) Example staining data of the Treg gate. B) Frequency and total numbers of antigen specifc Tregs of mice infected with Lm-MOG or Lm-gp66 in C57Bl/6 or MOG-/- mice. Teff/Treg ratio included. C) Frequency of antigen specific Tregs that express canonical Treg markers. Student's t test used to determine significance at $p<0.05$.

Supplemental Figure 1. Weight loss was monitored daily post-infection. Percentage of orignal weight loss is displayed for A) Mice that received C57Bl/6 CD4 T cells that did not exhibit disease symptoms, B) Mice that received MOG-/- CD4 T cells that did not exhibit disease symptoms and C) Mice that received MOG-/- CD4 T cells that did exhibit disease symptoms.

Supplemental Figure 2. Example staining data for flow cytometry. CD4 T cells were gated on lymphocytes, singlets, CD3ε+, F4/80-CDllc-B220-, CD4+, CD8-, CD44hi, MOG tetramer+ cells.

Figures

Figure 1.

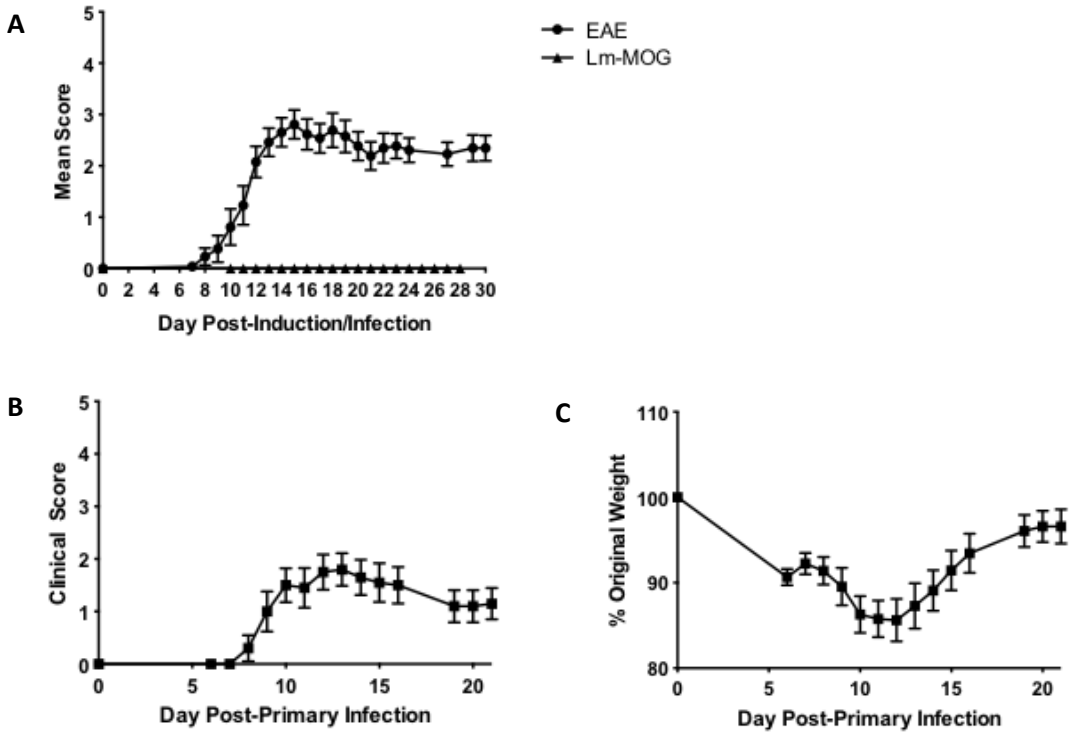
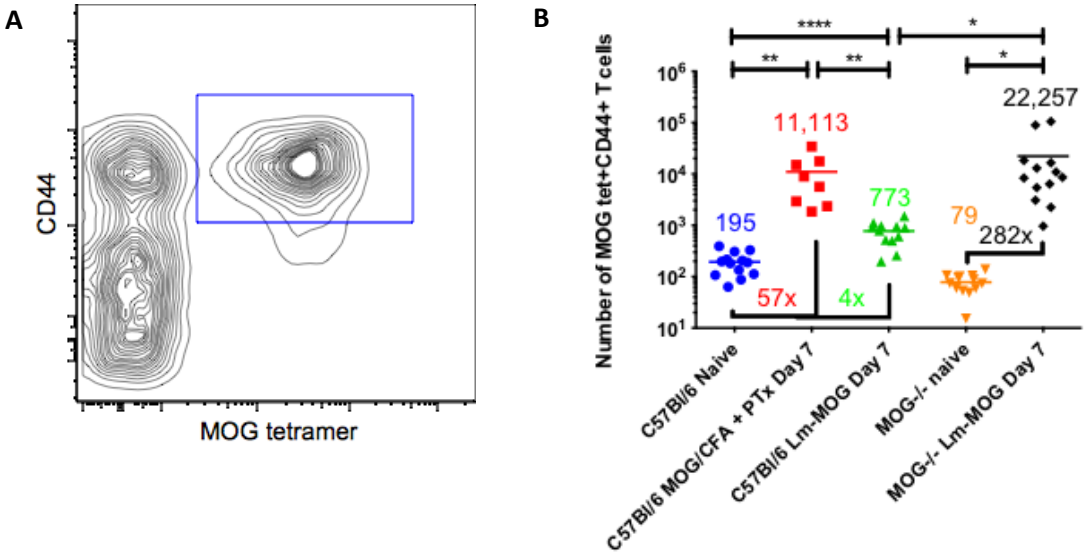


Figure 2.



Average of MOG-specific T cells

	CFA/MOG C57Bl/6	Lm-MOG C57Bl/6	Lm-MOG MOG -/-
Naïve	195	195	79
Day 7	11,113	773	22,257
Times Difference	57x	4x	282x

Figure 3

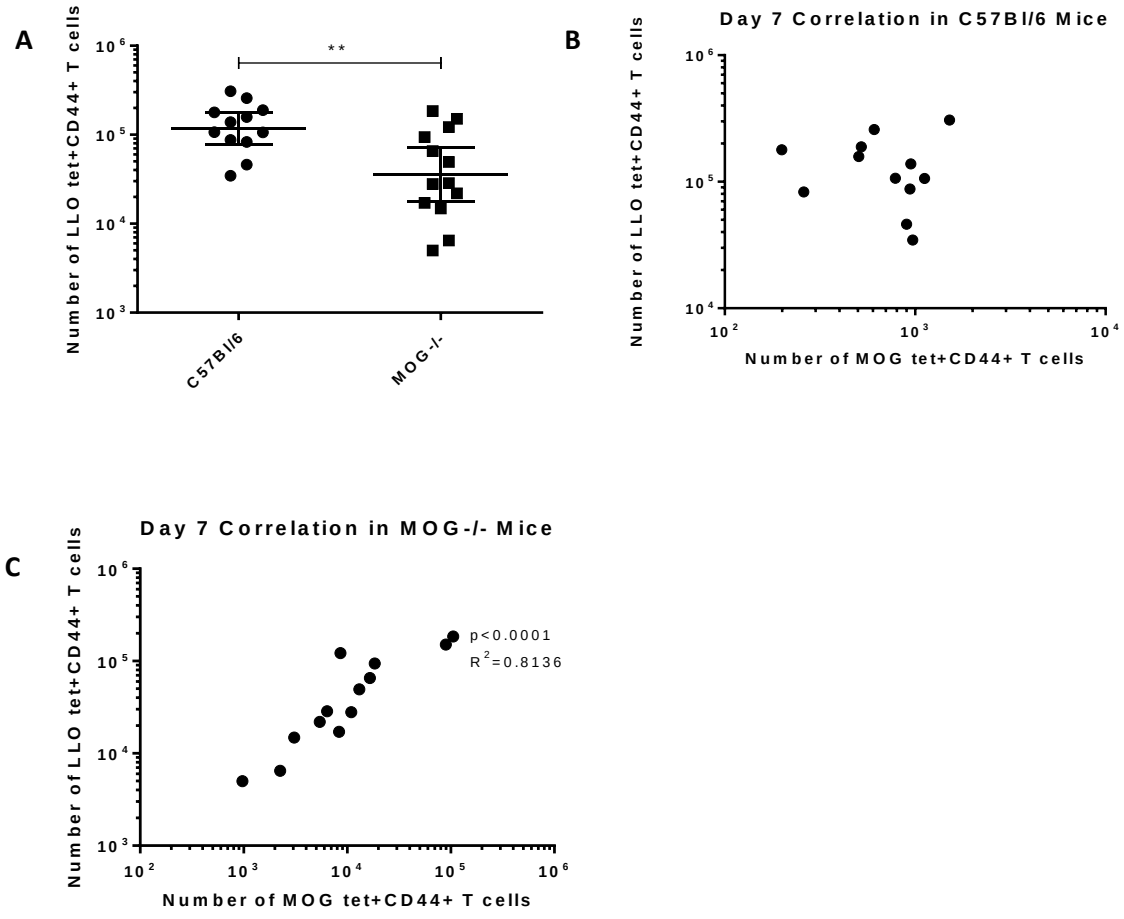
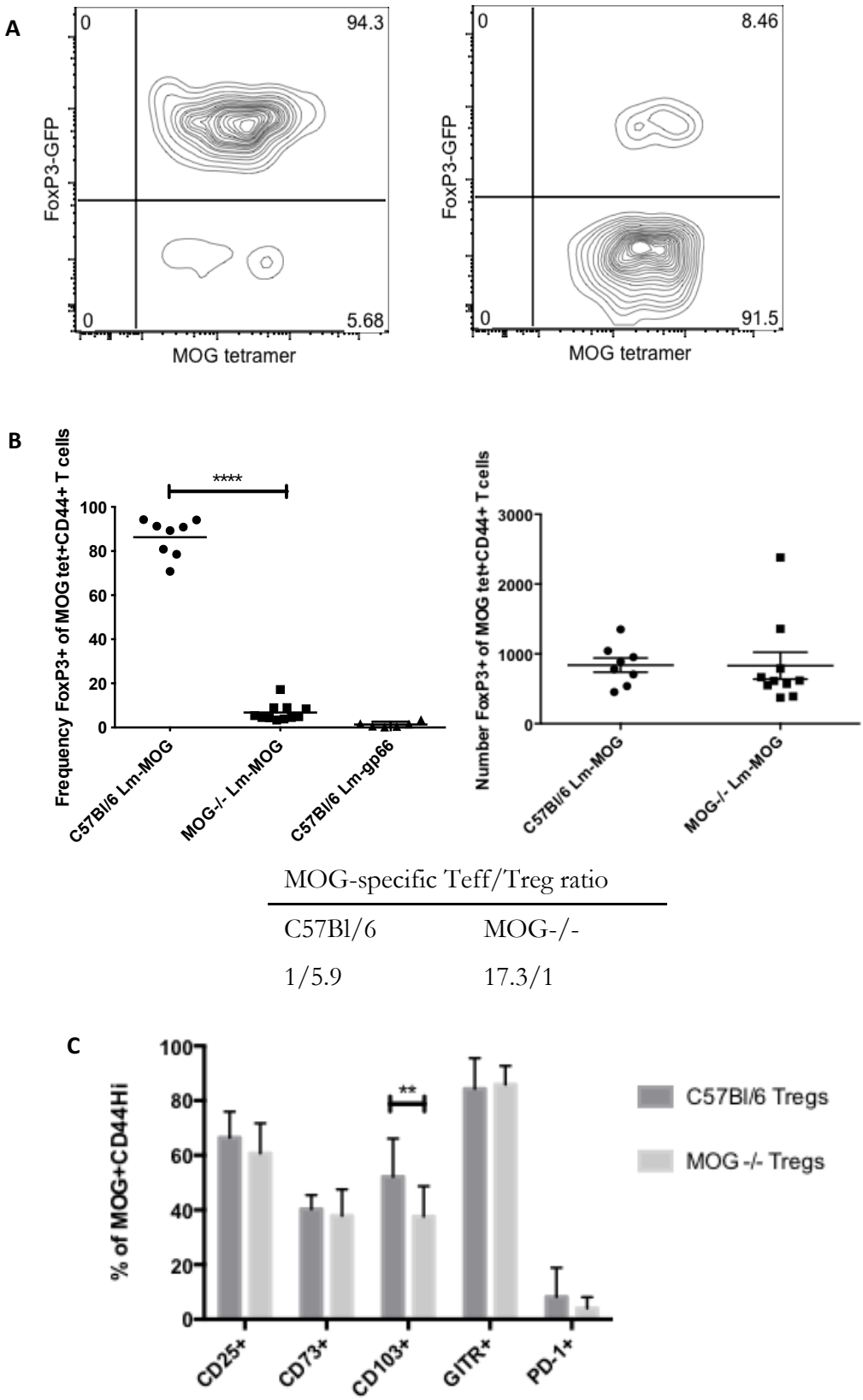
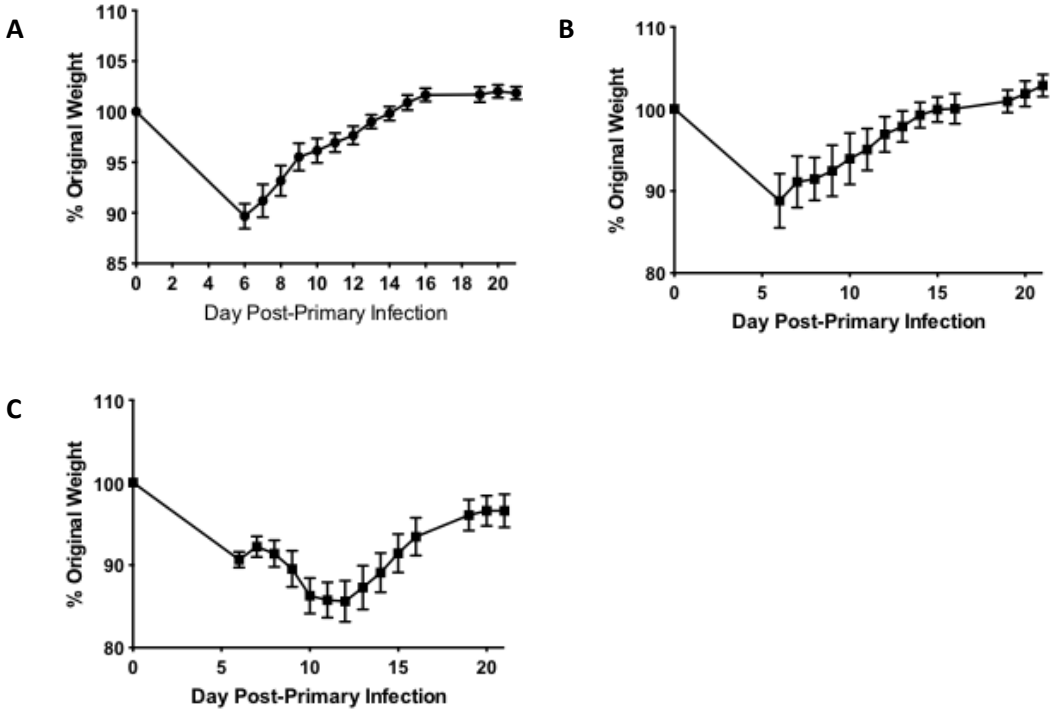

A
Number of LLO tet+CD44+ T cells
**
C57Bl/6
MOG-/-
B
Day 7 Correlation in C57Bl/6 Mice
Number of LLO tet+CD44+ T cells
Number of MOG tet+CD44+ T cells
C
Day 7 Correlation in MOG-/- Mice
Number of LLO tet+CD44+ T cells
p<0.0001
R2=0.8136
Number of MOG tet+CD44+ T cells

Figure 4

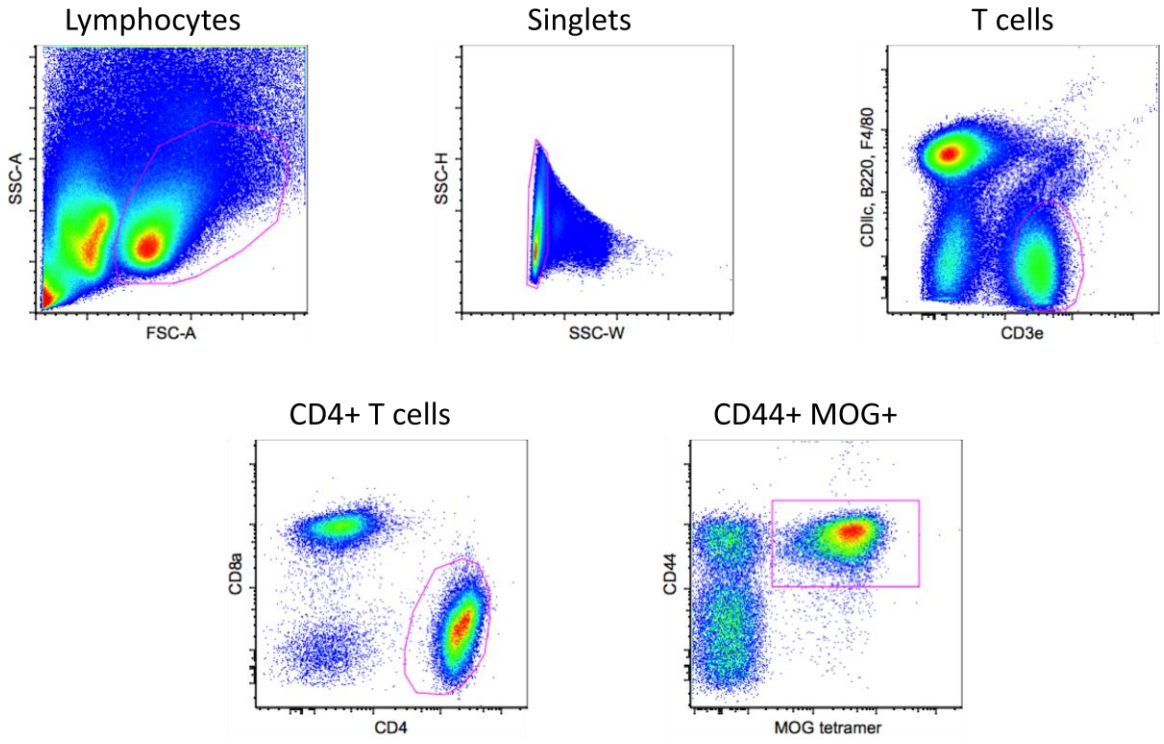


MOG-specific Teff/Treg ratio	
C57Bl/6	MOG-/-
1/5.9	17.3/1

Supplemental Figure 1



Supplemental Figure 2



Chapter 3

LCMV Delays EAE by limiting high affinity myelin CD4 T Cell Responses

Jennifer Cosby and Brian Evavold

Abstract

Infections are often proposed as the initial trigger of disease episodes or exacerbation of ongoing disease; however, their potential protective roles have been overlooked. In contrast to initiating of demyelinating disease, here we investigate how infections may offer protection. LCMV, an acute viral model, was used to determine viral effects on a well-established animal model of chronic-progressive autoimmune disease. Mice infected with LCMV had a delay in induction of paralysis as compared to uninfected controls with reduced numbers of myelin-specific CD4 T cells in the CNS that drive autoimmunity. The typical cytokine profile normally observed during demyelinating disease was altered. High affinity myelin specific CD4 T cells were shown to be absent in infected mice compared to uninfected controls. Thus, in contrast to the prevailing paradigm of infections driving autoimmune disease progression, we find that viral infection can lessen acute demyelinating disease.

Introduction

Multiple sclerosis is multifaceted in etiology. Genetically identical individuals, or monozygotic twins, have been shown to have a concordance rate of only 25% (Ramagopalan, Dyment et al. 2008). Due to this low concordance, there must be other factors at play. The argument for multiple sclerosis as an environmental disease has been discussed. For example, the northern hemisphere has a higher incidence of MS compared to the southern hemisphere (Compston and Coles 2008). It has been shown that if young individuals move from one hemisphere to the other, they adapt the risk of that hemisphere, suggesting that the environment of the hemisphere influences MS risk (Detels, Visscher et al. 1977; Elian, Nightingale et al. 1990). Infections have long been thought to be triggers of autoimmunity. For example, the mouse model for MS, experimental autoimmune encephalomyelitis (EAE), has a more severe disease course when previously infected with a mouse γ-herpesvirus (Casiraghi, Shanina et al. 2012). The virus, in this case, was thought to become reactivated to worsen clinical disease symptoms in mice. There are several proposed mechanisms in which infections are thought to activate the immune response against autoantigens. Bystander activation, epitope spread, molecular mimicry, and release of sequestered antigens have all been proposed as mechanisms which allow for infection to break tolerance to self-antigens (Vanderlugt and Miller 2002).

Although infections have been proposed as a potential trigger for autoimmune disease, much less studied is the role infection could play in protection of an individual from autoimmune disease. For example, LCMV has been shown to prevent diabetes in genetically susceptible NOD mice if given pre-symptoms (Oldstone, Ahmed et al. 1990). However, the mechanisms behind how infection regulates autoimmune disease remain unclear. It has been suggested that the types of viruses that individuals are exposed to over time (the individual's

virome) influences the host immune response (Virgin, Wherry et al. 2009; Virgin 2014). This in turn leads to changes in the susceptibility of that individual to infection. Therefore, the virome could be partially responsible for protection from autoimmune disease for that individual. Potential ways in which viruses that an individual is exposed to can influence protection may be by distracting the T cell effector immune response away from the site of disease, skewing the T cell response from a proinflammatory response to a non-inflammatory response, generating regulatory T cells, competing for space within the T cell pool, as well as many other factors.

Although viruses have been proposed to play a role as triggers in MS, little is known about how viruses may be protective in the context of MS. The parasite *Plasmodium chabaudi* has been shown to lessen disease symptoms during EAE disease course (Farias, Talaisys et al. 2011). This same study showed that Tregs were increased post-infection compared to uninfected controls as a potential mechanism for protection. However, there may be alternative mechanisms that could influence the potential for infections to offer protection. In this study, we investigate the mechanisms contributing to regulation of EAE disease onset resulting from acute viral infection. These experiments reveal that there are multiple mechanisms at play to regulate disease onset. The CD4 T cell response that drives demyelinating disease is diminished in the target organ - the CNS. The antigen specific T cell response appears to be altered due to infection. CD4 T cells producing key cytokines previously shown to play a role in EAE pathogenesis are lacking. In addition, the high affinity antigen specific T cell response is lacking.

Materials and Methods

Mice

C57Bl/6 mice were purchased from Charles River Laboratories. Female mice aged to 7-9 weeks old were used in experiments. 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.

EAE induction and infection

EAE was induced as previously described (Stromnes and Goverman 2006). Briefly, C57Bl/6 mice were induced with 200 μg MOG35-55 peptide, synthesized on a Prelude Synthesizer (Protein Technologies), emulsified in Complete Freud's adjuvant containing a final concentration of 2.5 mg/mL heat-inactivated *Mycobacterium tuberculosis* injected into the hind flank s.c. Mice received 250 ng pertussis toxin (List Biological Laboratories) i.p. on the day of induction and 2 days later. Disease severity was monitored via clinical scores and weights. Clinical scores were measured as follows: 0-no disease, 0.5-distal tail limp, 1-flaccid tail, 2-partial hind limb weakness, 2.5-severe hind limb weakness, 3-partial hind limb paralysis, 3.5-severe hind limb paralysis, 4-hind limb paralysis and cannot right self, 5-moribund/death. LCMV was given i.p. at 2×10^5 PFU per mouse one week or 10 days post-induction. Mice were sacrificed two weeks post-induction for phenotypic analysis unless otherwise stated. See Figure 1A for disease induction schematic.

Mononuclear cell isolation

Mice were subjected to perfusion by injecting PBS through the left ventricle of the heart. The brain and spinal cord were harvested from euthanized mice. CNS was then processed

through a 100 μm filter. The mononuclear cell layer was obtained using a Percoll (Sigma-Aldrich) gradient. Cells were washed with RPMI 1640 medium (Mediatech) containing 10% FBS (HyClone), 2 mM L-glutamine (Mediatech), 0.01 M HEPES buffer (Sigma-Aldrich), 100 μg/mL gentamicin (Mediatech), and 20 μM 2-ME (Sigma-Aldrich) and then used directly in experiments.

Extracellular surface staining

Cells surfaced stained with the following antibodies: anti-CD3ε (clone 142-2C11, BD Horizon), anti-CD4 (clone RM4-5, Biolegend), anti-CD8α (clone 53-6.7 Tonbo Biosciences), and anti-CD11b (clone M1/70, BD Pharmigen) for 15 minutes at room temperature. Flow cytometry was performed on a LSRII flow cytometer and data was analyzed using FloJo software. Cell counts were obtained by running samples with AccuCheck Counting Beads (ThermoFisher Scientific) on the flow cytometer.

Intracellular cytokine staining

Cells were stimulated in culture medium with 20 nM PMA (Fisher Biotech) and 1 μM ionomycin (Sigma-Aldrich) in the presence of 10 μg/mL Brefeldin A for 4.5 hours at 37°C. Cells were washed and surface stained as previously described. Cells were washed and then fixed with the BD Cytofix/cytoperm kit (BD Biosciences) for 30 minutes on ice in the dark. Cells were intracellularly stained with anti-IFNγ (clone XMG1.2, BD biosciences) and anti-IL-17A (clone ebio17B7, eBioscience). Flow cytometry was performed as described.

Micropipette adhesion frequency assay

Purified CD4 T cells and red blood cells (RBCs) coated in peptide:major histocompatibility complex (pMHC) monomers using biotin and streptavidin acted as a surrogate antigen presenting cell were loaded into a chamber created with two slides containing RPMI 1640 medium + 1% BSA (Huang, Zarnitsyna et al. 2010). Cells were allowed to settle and were then picked up via aspiration through two different micropipettes. The surrogate APC and CD4 T cell were brought into contact for two seconds and pulled away and binding events were recorded as either a 0 (no binding) or 1 (binding) for 50 touches to define an adhesion frequency. Cells included for analysis as antigen-specific were cells that had an adhesion frequency of >0.1. Cells that bound 100% to an RBC were resolved on a lower coated RBC. The adhesion frequency, the TCR density (measured by flow cytometry), and the density of pMHC on the RBC (also measured by flow cytometry) were used to determine the affinity of a CD4 T cell through a mathematical model (Chesla, Selvaraj et al. 1998). Frequency of MOG specific CD4 T cells was determined by the number of binders out of the total CD4 T cells ran.

Statistical analysis

Data were graphed and statistically analyzed using GraphPad Prism 6 software. Two-way ANOVA, Mann-Whitney, and student's t tests were performed.

Results

Acute LCMV infection delays disease course

C57Bl/6 control mice exhibited similar disease course and onset to that previously described in literature when induced with MOG peptide. However, mice that received infections had an overall less severe disease course over time (Figure 1B). Weight loss occurred at onset and peak of symptoms in control mice which supports the increase in scores seen in these mice. Those mice infected with LCMV did not lose weight over time compared to controls, confirming that LCMV mice had less severe disease at onset and peak time points (Figure 1C). Peak scores also trended lower in the infected group (Figure 1D). Peak scores in the control group averaged to 3.4$\pm$0.6 whereas the infected group had mean peak scores of 2.7$\pm$0.8. Determining the total area under the curve has been used as an indicator of duration of disease and overall severity (Kersh, Edwards et al. 2014). The total area under the curve in the infected group (20.4$\pm$10.8) was lower compared to controls (51.3$\pm$9.5), suggesting that infected mice had an overall less severe disease course (Figure 1E). In addition, disease course on average was significantly delayed in the infected group (Figure 1F). Mean onset for controls was 11$\pm$2 days while the infected group had a mean onset of 19$\pm$5 days post-induction. The protection seen was specific to LCMV as X31 influenza did not protect against EAE challenge (Supplemental Figure 1). Together, these data show that certain infections, but not all, protect against demyelinating disease.

LCMV reduces the CD4 T cell response

CD4 T cells have been identified as an important initiator of demyelinating disease. During LCMV infection, the frequency and total number of CD4 T cells was higher in the CNS (Figure 2A and 2B). CD4 frequency was 55% (21248 total CD4s) of the T cell population in

EAE controls, whereas infected mice had only 22% (5500 total CD4s) CD4 T cells in the CNS. Therefore, infection is preventing EAE at onset due to the lack of an initiating CD4 T cell response in the CNS. In the periphery, the CD4 T cell response by frequency and total numbers is lower compared to the EAE control group as well. CD4 frequency was 59% ($6.8x10^8$ total CD4s) of the T cell population in EAE controls, whereas infected mice had only 21% ($2.8x10^8$ total CD4s) CD4 T cells in the spleen (Figure 2C and 2D). This suggests that the CD4 T cell response is globally diminished and contributing to the lack of disease seen in the infected group during disease onset.

Cytokine profile is altered due to infection

With the lack of CD4 T cell response in the CNS, we were curious to investigate whether the cytokine response was diminished as well. IL-17A and IFNγ have both been implemented in EAE previously (Chen, Langrish et al. 2006; Pierson, Simmons et al. 2012). Out of the CD4 T cell population, the frequency of IL-17A producers is significantly decreased in the infected group, indicating that there is a diminished effector response for the initiation of EAE in this population (Figure 3A). This holds true for total numbers if IL-17A producers as well (Figure 3B). Although the frequency of IFNγ producers is increased in the group that received infections (Figure 3A), there is a lack of CD4 T cells in the CNS as discussed for Figure 2B. Therefore, the total numbers of IFNγ producers in the CNS is the same between the infected group and uninfected controls (Figure 3B). These data together suggest that the appropriate cytokine response required for recruitment and initiation of disease is absent in the infected group.

LCMV infection lowers the MOG-specific T cell frequency in the CNS

MOG-specific CD4 T cells are able to recognize peptide presented in the context of MHC. These cells are particularly important in driving an antigen-specific immune response in order to cause disease symptoms seen in EAE mice. In addition, tetramer staining for myelin antigens has been shown to be higher in MS patients compared to healthy controls (Raddassi, Kent et al. 2011). After tetramer staining for MOG-specific CD4 T cells in the CNS, mice infected with LCMV contained fewer MOG-specific T cells by frequency (Figure 4A). In fact, there was almost no tetramer staining in the infected group, whereas the EAE control group stained with tetramer. Total numbers of MOG-specific T cells also trended lower in the infected group compared to controls (Figure 4B). The affinity of the reaction between T cells and p:MHC for MOG is relatively low compared to foreign antigens; therefore, the tetramer reagent does not stain for MOG-specific T cells well. In order to increase sensitivity, the micropipette adhesion frequency assay was utilized to determine the MOG-specific T cell frequency. Using this assay, the MOG-specific T cell pool was determined to be lower in the infected group compared to controls, confirming the tetramer staining data (Figure 4C). Combined, these data suggest that the MOG-specific T cell response is lacking during disease onset.

High affinity MOG-specific T cells are absent during infection

Affinity has been previously suggested to play a role in contributing to demyelinating disease in MS patients. Patients tend to have higher affinity to myelin antigens compared to healthy donor controls (Bielekova, Sung et al. 2004). Therefore, we investigated the affinity for MOG during infection using the micropipette adhesion frequency assay. Affinity was significantly lower in the infected group, due to a lack of a high affinity MOG-specific CD4 T cell response (Figure 5). High affinity MOG specific CD4 T cells may be required in order

for disease initiation to occur. Without these cells in the infected group, disease onset is delayed.

Discussion

Genetic factors have been associated with MS. However, due to the low concordance of MS among monozygotic twins, MS is considered to be a highly environmental disease. Although infections have classically been thought of as triggers for MS, there have been a few infections shown to protect against autoimmune diseases. In our studies, a viral infection given pre-EAE symptoms has been shown to delay EAE onset. There are multiple mechanisms that may be at play in how infection is regulating demyelinating disease in this case. Firstly, the CD4 T cell response is reduced. CD4 T cell responses are shown here to be critical for initiating autoimmune disease. They are responsible for recruitment of innate immune effector cells that attack the myelin sheath leading to demyelination of the neuron. In addition, these innate cells then attack the oligodendrocytes that produce myelin, reducing the ability of neurons to remyelinate. It would be interesting to see in the case of LCMV infection if neutrophils and macrophages are reduced in the CNS well, as these cells are important to produce damaging cytokines or if the cytokine response in these cells is altered.

IL-17A and IFNγ have both been implicated in MS pathology. IL-17A specifically is important for initiation of disease pathology. We see a diminished IL-17A response post-infection, likely leading to an inability to mount an appropriate proinflammatory response to initiate disease. The role of IFNγ, however, has been widely debated. Studies have investigated how IFNγ may be proinflammatory, whereas others have documented its anti-inflammatory effects. A recent review clarifies these finding by describing how IFNγ suppresses inflammation in the brain, but not the spinal cord (Pierson, Simmons et al. 2012). Our results show that IFNγ was increased in the CNS and therefore playing a potentially anti-inflammatory role during disease course. Further studies are required to confirm the role

of IFNγ during CNS pathology. Use of the IFNγ receptor knockout mouse may be beneficial in order to determine the protective effect of IFNγ during acute viral infection.

Long debated is whether antigen specific T cells are important for initiating demyelinating disease or if a bulk CD4 T cell response is sufficient. Using the micropipette adhesion frequency assay, our lab has seen that the majority of CD4 T cells that enter the CNS after EAE challenge are antigen specific (Sabatino, Huang et al. 2011). High and low affinity T cells can be seen during this response, as is consistent with the data shown here. However, only low affinity MOG-specific T cells were observed in the CNS post-infection. With these data, it could be inferred that high affinity antigen-specific T cells are required for the initiation of disease symptoms. Therefore, the low affinity CD4 T cells seen during acute viral infection are not sufficient for disease initiation. However, the low affinity T cells may be important for maintaining disease, but their role during disease course remains unclear.

An interesting line of investigation would be to look at the trafficking of high affinity antigen specific CD4 T cells in the CNS and how it is altered via infection. After LCMV infection, MOG-specific T cell expansion seems to be similar in the periphery (data not shown). This suggests that lack of entry into the CNS is not due to lack of expansion. Therefore, CD4 T cells may not be receiving appropriate signals in order to traffic to the CNS. Alternatively, they may not upregulate integrins or other surface receptors responsible for crossing the blood-brain barrier. VLA-4, for example, has been implicated in MS pathology as blocking VLA-4 prevents EAE (Yednock, Cannon et al. 1992). CD4 T cells use VLA-4/VCAM-1 interactions in order to cross the blood-brain barrier and enter the CNS. In fact, Tysabri, which targets α4 integrin, is a drug commonly used to treat MS patients. However, patients using natalizumab are at risk for developing progressive multifocal leukoencephalopathy (Kleinschmidt-DeMasters and Tyler 2005). By deciphering the key

components involved in high affinity CD4 T cell entry into the CNS, we might determine safer and more appropriate targets to more effectively treat MS patients.

LCMV infection and most viruses elicit a strong CD8 T cell response. During EAE, CD8 T cells are shown to contribute to disease since adoptive transfer of CD8 T cells from EAE induced mice resulted in EAE symptoms (Ford and Evavold 2005). In our studies, CD8s were significantly higher in frequency in the CNS compared to uninfected controls (data not shown). However, they were not contributing to disease symptoms. The majority of these CD8s expressed IFNγ, much like the CD4s. In this case, IFNγ may be taking on a more neuroprotective role than a proinflammatory one. In addition, infection-specific T cells have been described to be seen in the CNS previously. Infection-specific T cells present in the CNS are thought to provide a mechanism for immunosurveillance to prevent infections from entering the CNS (Kwok, Miletic et al. 2002). This may be the case for observing CD8 T cells in the CNS of infected mice. Therefore, the CD8s seen in the CNS of the LCMV infected mice may be present as a security measure to ensure that the infection will not spread to such a vital organ as the CNS.

The hygiene hypothesis would suggest that exposure to infections early in life helps to prevent diseases that are increasing the in the northern hemisphere. For example, asthma and autoimmune diseases are on the rise in the northern hemisphere but not the southern. Our work would suggest that exposure to infections can be beneficial which supports the hygiene hypothesis theory. This also relates to how the virome can change immune responses over time. Different types of infections early in life trigger different responses. It could be argued that different infections would be present in the northern vs. the southern hemisphere. Therefore, the types of infections that are present by location could be

responsible for the increase in risk for asthma and autoimmune diseases seen in the northern hemisphere.

It would be interesting to determine what other infections are able to inhibit EAE and by what mechanisms. By uncovering the ways in which infections can prevent or inhibit autoimmune disease, we can then determine what pathways are the most effective targets for treating MS while limiting off-target effects.

Acknowledgements

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Figure Legends

Figure 1. EAE is less severe and delayed during LCMV infection. N=11 mice for EAE controls and N=12 for infected group. A) Mice were induced with 200 μg of MOG peptide emulsified in 5 mg/mL CFA on day 0 and 250 ng of pertussis toxin was injected. 2 days later, another pertussis toxin dose was administered. One week or 10 days after induction, mice were infected with 2×10^5 PFU of LCMV Armstrong. Mice were then either monitored for 30 days post-induction to determine overall disease course or sacrificed at day 14 post-induction for phenotypic analysis. B) Mice were observed for clinical signs of EAE and given a score as described in the methods section and C) weights were monitored. Mean±SEM are displayed with Two-way ANOVA performed to determine significance. D) Peak scores of mice are plotted per group. Mean±SD are shown. Mann-Whitney test was used to determine significance. E) The area under the curve per mouse was determined. Mean±SD are displayed. Student's t test was used to determine significance. F) The day of onset was determined per mouse. Scores 0.5 or above were used to determine day onset. Mean±SD are displayed. Student's t test was used to determine significance.

Figure 2. LCMV reduced the CD4 T cell response. N=5 mice for each. Mice were sacrificed at day 14 post-induction as seen in figure 1A. Cells were extracellularly stained and gated on lymphocytes, singlets, CD3ε+CDllb-, CD4+CD8α- cells. A) Frequency or B) total number of CD4+ T cells of the CD3ε gate are shown from the CNS. C) Frequency or B) total number of CD4+ T cells of the CD3ε gate are shown from the spleen. Student's t-test with $p<0.05$ used to determine significance.

Figure 3. Cytokine profile is altered post-infection. Mice were sacrificed at day 14 post-induction as seen in figure 1A. Cells were gated on lymphocytes, singlets, CD3ε+CDllb-, CD4+CD8- cells, followed by gating on IL-17A+ and/or IFNγ. A) Frequency or B) total numbers of cytokine producers are shown. Student's t-test with $p<0.05$ used to determine significance.

Figure 4. Myelin specific CD4 T cells are reduced in the CNS post-infection. Cells were gated on lymphocytes, singlets, CD3ε+CDllb-, CD4+CD8- cells, followed by gating on MOG tetramer+ cells. A) Frequency or B) total number of MOG tetramer+ CD4 T cells are shown from the CNS via flow cytometry. C) Micropipette adhesion frequency assay used to determine frequency of MOG-specific CD4 T cells. Student's t-test with $p<0.05$ used to determine significance.

Figure 5. High affinity MOG-specific CD4 T cells are absent post-infection. Micropipette adhesion frequency was performed as described. Affinity was determined per group. Geometric mean and confidence intervals are displayed. Student's t-test with $p<0.05$ used to determine significance between the two groups. Student's t-test was run on the log transformed data of affinities.

Supplemental Figure Legends.

Supplemental Figure 1. X31 influenza does not protect against demyelinating disease. EAE was induced in C57Bl/6 mice. Mice were then either infected with 10,000 PFU of X31 or were left uninfected as a control. Clinical scores were then assigned as EAE progressed over time.

Figures

Figure 1

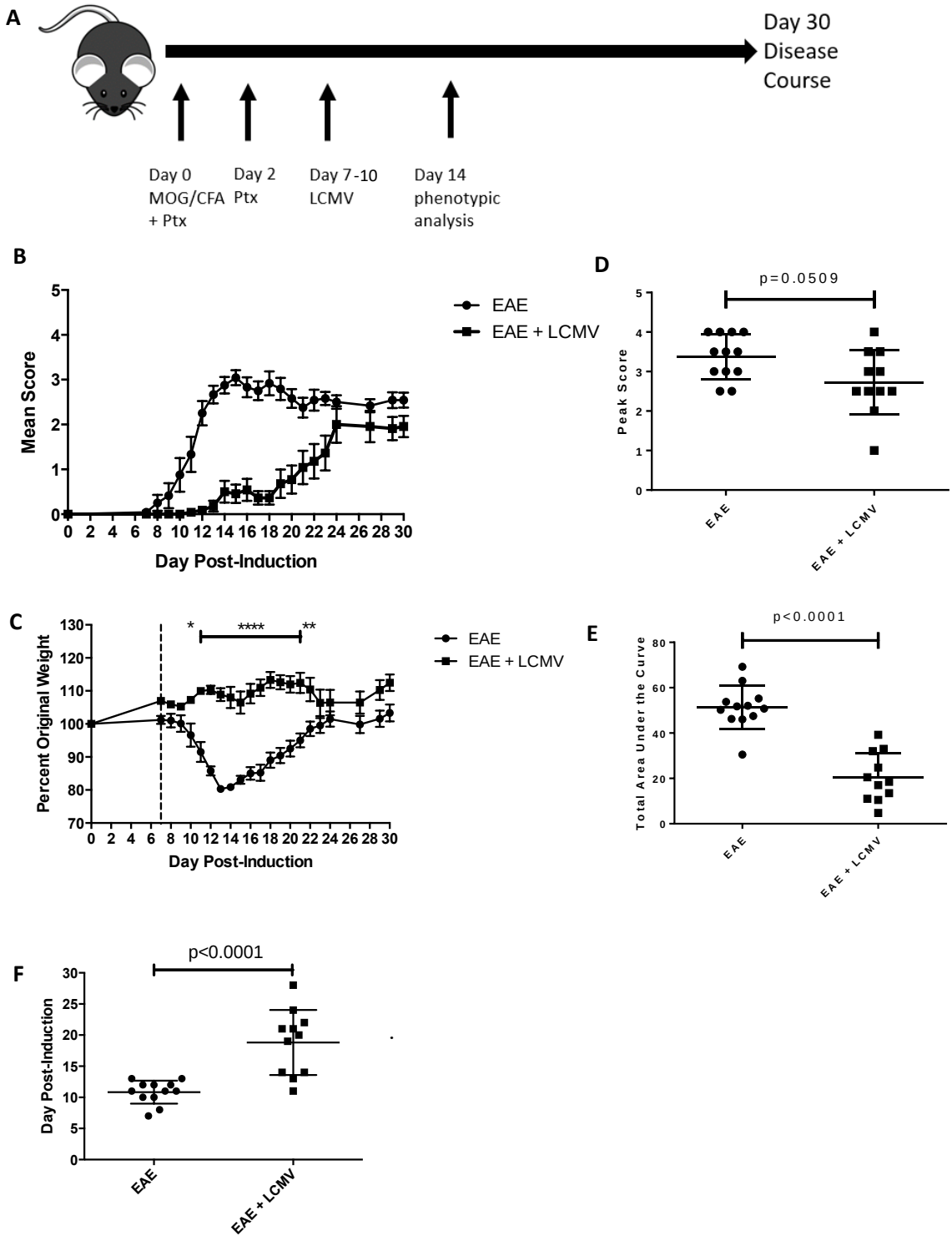


Figure 2

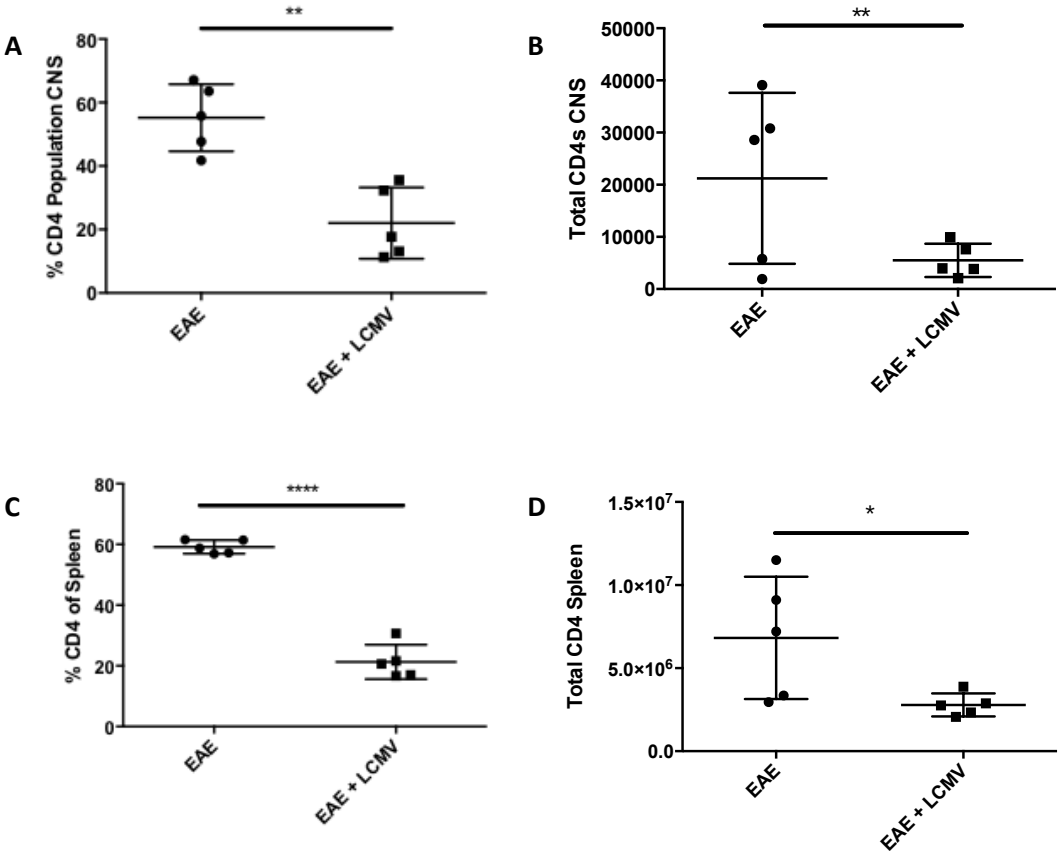

A
**
% CD4 Population CNS
80
60
40
20
0
EAE
EAE + LCMV
B
**
Total CD4s CNS
50000
40000
30000
20000
10000
0
EAE
EAE + LCMV
C
****
% CD4 of Spleen
80
60
40
20
0
EAE
EAE + LCMV
D
*
Total CD4 Spleen
1.5×10^7
1.0×10^7
5.0×10^6
0.0
EAE
EAE + LCMV

Figure 3

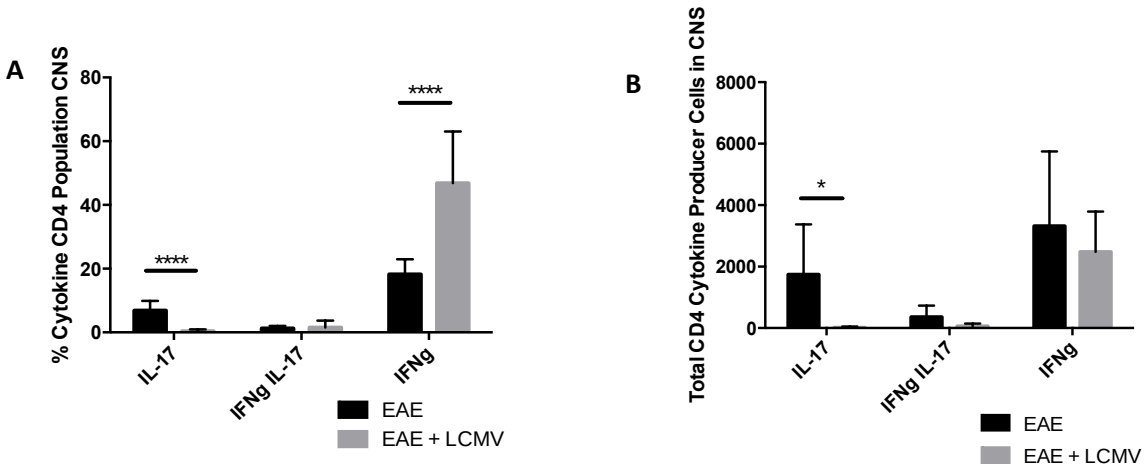

A
% Cytokine CD4 Population CNS
80
60
40
20
0
****
****
IL-17
IFNg IL-17
IFNg
EAE
EAE + LCMV
B
Total CD4 Cytokine Producer Cells in CNS
8000
6000
4000
2000
0
*
IL-17
IFNg IL-17
IFNg
EAE
EAE + LCMV

Figure 4

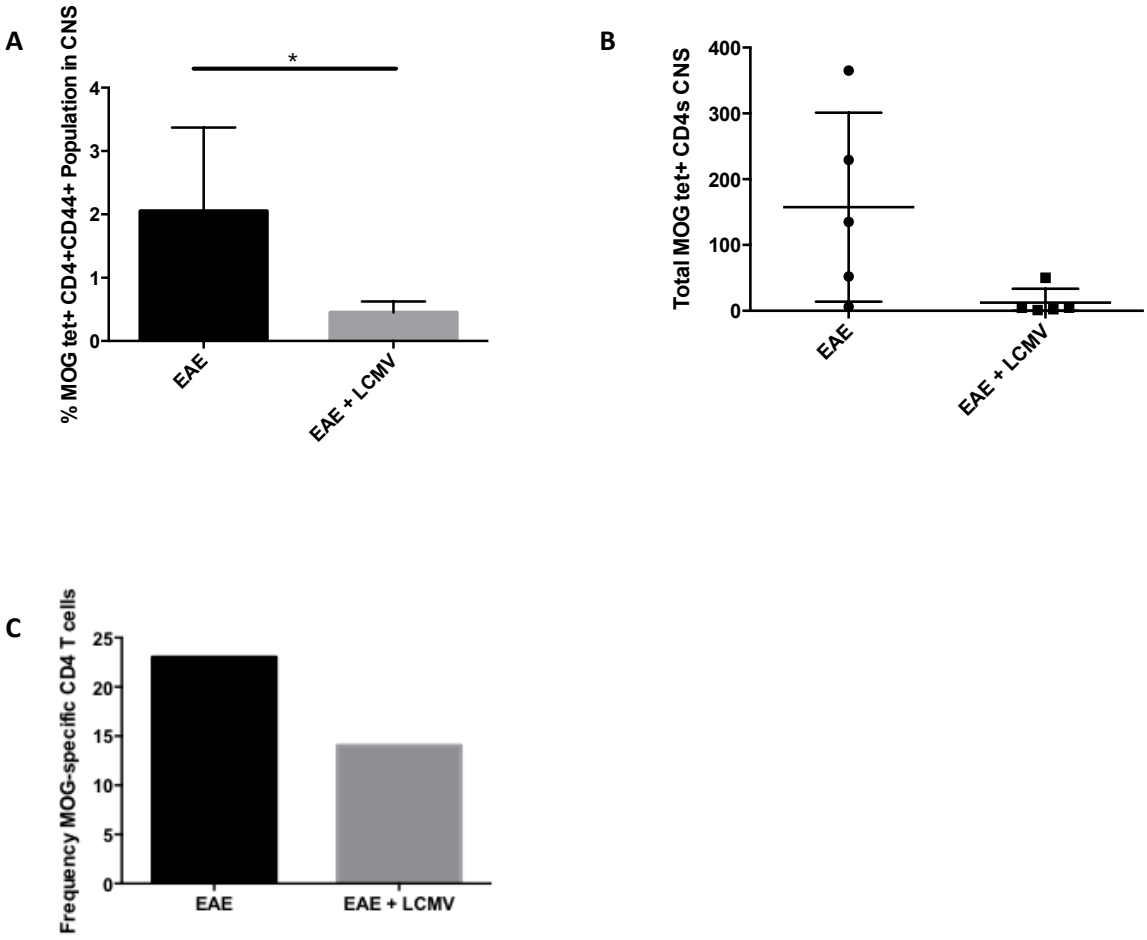
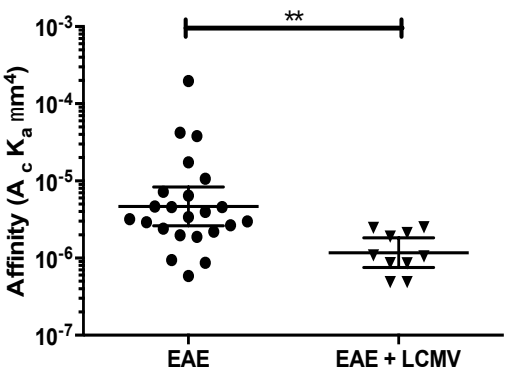

A
% MOG tet+ CD4+CD44+ Population in CNS
*
4
3
2
1
0
EAE
EAE + LCMV
B
Total MOG tet+ CD4s CNS
400
300
200
100
0
EAE
EAE + LCMV
C
Frequency MOG-specific CD4 T cells
25
20
15
10
5
0
EAE
EAE + LCMV

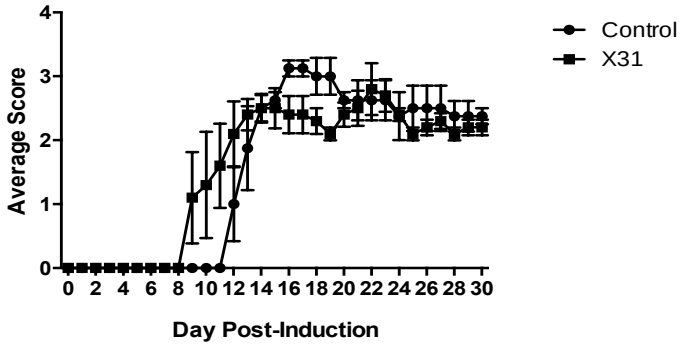
Figure 5



	EAE	EAE+LCMV
Affinity	4.68×10^{-6}	1.17×10^{-6}

Supplemental Figures

Supplemental Figure 1



Chapter 4

Discussion

Threshold for autoimmune disease.

The proposed mechanism for molecular mimicry is outlined in Figure 1. During this process, CD4 T cells are primed in the periphery to a myelin antigen mimic. These cells cross the blood-brain barrier and see the myelin antigen in the CNS, responding by secretion of cytokines and chemokines in order to recruit effector immune cells that demyelinate neurons. In our studies, we found that we could not induce classical autoimmune disease via infection in wild-type mice; however, demyelinating disease occurred in mice that received MOG-/- CD4 T cells. These data suggest that there is a particular threshold of MOG-specific CD4 T cell expansion that is required to be reached in order to cause disease. In wild-type mice, expansion is limited post-infection whereas MOG-/- CD4 T cells expand over a MOG/CFA + PTx control. We suggest that expansion is limited due to the generation of Tregs. In MOG-/- animals, thymic selection to MOG is altered compared to a wild-type mouse. Therefore, in mice where thymic selection is altered, the threshold for myelin-specific expansion is reached. These results illustrate the interplay between genetics and environment. Genetic susceptibilities in humans, such as expression of certain MHC molecules that dictate the precursor CD4 T cell pool, may predispose of individuals to this expansion. However, genetics alone does not seem to be adequate to induce disease. An

infectious insult, such as a molecular mimic, may then be required to induce demyelinating disease, as long as the theoretical expansion of T cells to myelin antigens is overcome.

Plasmodium chabaudi reduces EAE symptoms, possibly due to Treg generation (Farias, Talaisys et al. 2011). These studies do not analyze the antigen specificity of these Tregs. In our studies, individuals without genetic predisposition to autoimmune disease were able to expand Tregs to the molecular mimic, whereas genetically predisposed individuals were unable to generate functional Tregs. The inability to generate functional Tregs in MOG-/- mice allows for expansion of effector CD4 T cells over Tregs. The precursor pool of Treg cells between healthy controls and genetically susceptible individuals may be altered in such a way that functional Tregs are selected appropriately in healthy controls but not in individuals that develop MS. Therefore, functional Tregs are crucial to protect against molecular mimicry induced demyelinating disease which is driven by genetics and limits MOG-specific CD4 T cell expansion. Our studies provide new insight that antigen-specific Treg expansion to myelin antigens is important to maintain tolerance post-infection.

Protection during autoimmunity

Although most research focuses on how infections may trigger autoimmune disease, much less studied is the role that infections play in protection from autoimmune disease. In wild-type C57Bl/6 mice, Lm-MOG infection did not induce disease and generated a Treg response and LCMV infection delayed demyelinating disease onset. Interestingly, LCMV infection also prevents diabetes in genetically-prone NOD mice (Oldstone, Ahmed et al. 1990). To build on the Lm-MOG experiments, expansion in the periphery is one factor that allows for induction of autoimmune disease. Preliminary data would suggest that expansion between EAE control groups and mice infected with LCMV was similar in the periphery;

however, further experiments are required to confirm this expansion data. If consistent, this would suggest that there are other factors other than just a peripheral threshold value preventing autoimmune disease. In this case, there were fewer CD4 T cells in the CNS and high affinity myelin-specific CD4 T cells. This would suggest that high affinity myelin-specific CD4 T cells are unable to cross the blood-brain barrier and are prevented from entering the CNS. Therefore, in addition to a peripheral expansion threshold, there is most likely a threshold that must be reached in the target organ as well to induce disease. IL-17A is absent in the CNS as well. As Il-17A production is found normally during MS and EAE, the absence of this cytokine post-infection indicates that the typical proinflammatory environment found during EAE is unable to form in the CNS, potentially due to the reduced CD4 T cell response in the CNS. Altogether, these data suggest that the appropriate responses both in the periphery (Lm-MOG studies) and in the target organ (LCMV studies) are required in order to initiate demyelinating disease.

Future directions

The innate immune response plays a large role in autoimmune disease including MS pathology. Antigen presenting cells present antigen to T cells in the context of MHC. Innate cells are also recruited as effectors to attack the myelin sheath, the underlying neuron, and the oligodendrocytes that produce myelin. It would be interesting to explore in our infection systems whether the innate immune response is altered compared to controls. For example, are neutrophils and macrophages lacking in the CNS post-LCMV infection, leading to the delay in EAE onset? Does Lm-MOG allow for neutrophil and macrophage infiltration in

MOG-/- animals but not in C57Bl/6 mice? It would also be of interest to know if regulatory DCs are induced in animals lacking EAE.

Demyelination is a key component in MS pathology. Demyelination is generally determined by Luxol blue staining of the brain and spinal cord. It would be of interest to determine the degree and areas of demyelination in the CNS. In mice infected with LCMV, our studies would predict that demyelination may be delayed as seen by the delay in EAE clinical symptoms. In Lm-MOG infected C57Bl/6 mice, we would predict that demyelination rarely would occur due to the lack of EAE incidence seen. In those MOG-/- mice with EAE symptoms, we suspect that demyelination would occur. In the mice that atypical disease was displayed, it would be plausible that demyelination occurs in different areas compared to classically induced mice.

These studies mostly focus on the CD4 T cell response to myelin antigen. CD8 T cells have also been shown to play a role in EAE disease pathology (Ford and Evavold 2005). In our studies with LCMV, CD8s were increased in the CNS. However, we did not explore the antigen specificity of these cells. It would be interesting to note whether these CD8 T cells are involved in the protection observed post-infection. In addition, we could probe the antigen specific CD8 T cell response in the Lm-MOG infected mice to see if CD8s are suppressed in the C57Bl/6 mice but present in genetically susceptible mice.

Another line of investigation would be to determine if the autoimmune response inhibits or improves the response to the infection itself. During the *Listeria* response, LLO specific CD4 T cells were 2 times lower in the C57Bl/6 group compared to the MOG-/- group. This could imply that the LLO response was not as robust in the MOG-/- group compared to the C57Bl/6 group. However, these studies would need to be followed up with

secondary challenge experiments and bacterial CFU quantification to determine if the altered response leads to a differing biological effect.

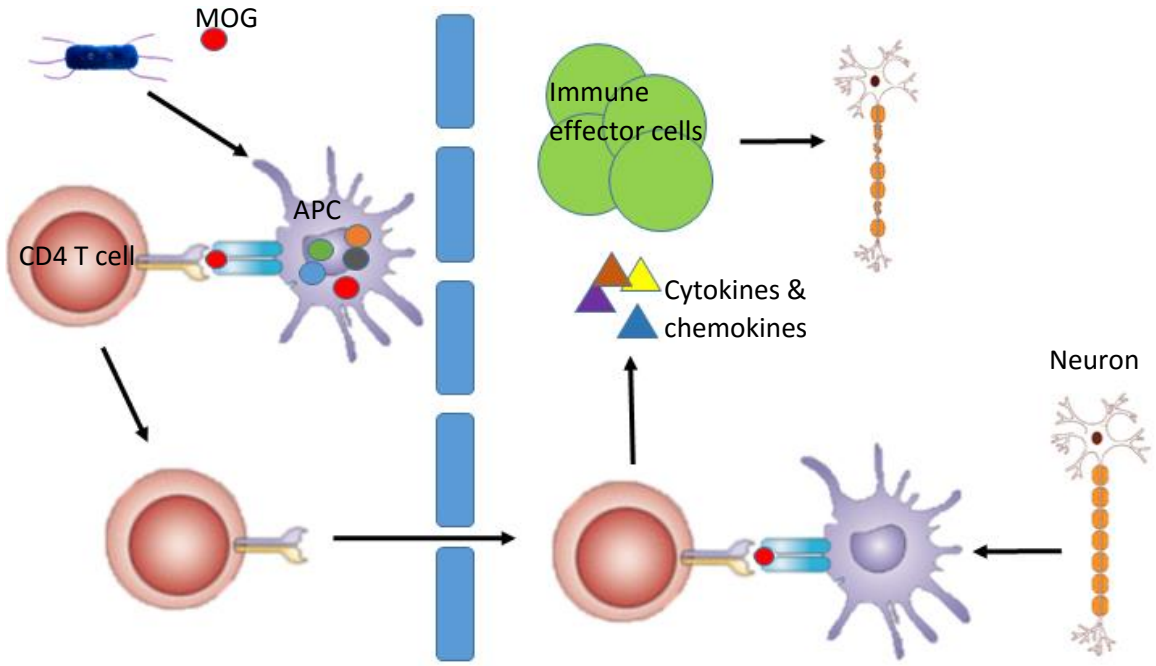
These studies have revealed multiple findings such as 1) infections are able to offer protection during EAE disease course, 2) tolerance to self is harder to break than once thought, and 3) genetically susceptible individuals are prone to infection-induced autoimmune disease. It will be interesting to see how future research uncovers the interplay between genetics and environment in the context of MS.

Figure Legends

Figure Legend 1. Model for molecular mimicry induced demyelinating disease. Infections carrying a peptide similar to MOG are taken up by APCs and presented to CD4 T cells in the context of an MHCII molecule. The CD4 T cell is then able to cross the blood-brain barrier and is able to recognize the MOG antigen via cross-reactivity of the TCR in the CNS. These CD4 T cells then secrete cytokines and chemokines in order to recruit immune effector cells. These cells cause damage to the myelin sheath, the underlying neuron, and the oligodendrocytes that produce myelin.

Figures

Figure 1



Literature Cited

Abreu, S. L. (1982). "Suppression of experimental allergic encephalomyelitis by interferon." Immunol Commun **11**(1): 1-7.

Abromson-Leeman, S., R. Bronson, et al. (2004). "T-cell properties determine disease site, clinical presentation, and cellular pathology of experimental autoimmune encephalomyelitis." Am J Pathol **165**(5): 1519-1533.

Agarwal, R. K., S. H. Sun, et al. (2002). "Pertussis toxin alters the innate and the adaptive immune responses in a pertussis-dependent model of autoimmunity." J Neuroimmunol **129**(1-2): 133-140.

Andreasen, C., D. A. Powell, et al. (2009). "Pertussis toxin stimulates IL-17 production in response to Bordetella pertussis infection in mice." PLoS One **4**(9): e7079.

Angelini, D. F., B. Serafini, et al. (2013). "Increased CD8+ T cell response to Epstein-Barr virus lytic antigens in the active phase of multiple sclerosis." PLoS Pathog **9**(4): e1003220.

Archibald, C. J., P. J. McGrath, et al. (1994). "Pain prevalence, severity and impact in a clinic sample of multiple sclerosis patients." Pain **58**(1): 89-93.

Asseman, C., S. Mauze, et al. (1999). "An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation." J Exp Med **190**(7): 995-1004.

Awad, S. A., J. B. Gajewski, et al. (1984). "Relationship between neurological and urological status in patients with multiple sclerosis." J Urol **132**(3): 499-502.

Bach, J. F. (2002). "The effect of infections on susceptibility to autoimmune and allergic diseases." N Engl J Med **347**(12): 911-920.

Badawi, A. H. and T. J. Siahaan (2012). "Immune modulating peptides for the treatment and suppression of multiple sclerosis." Clin Immunol **144**(2): 127-138.

Barcellos, L. F., J. R. Oksenberg, et al. (2003). "HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course." Am J Hum Genet **72**(3): 710-716.

Barnett, L. A., J. L. Whitton, et al. (1993). "Enhancement of autoimmune disease using recombinant vaccinia virus encoding myelin proteolipid protein." J Neuroimmunol **48**(1): 120.

Barnett, L. A., J. L. Whitton, et al. (1996). "Virus encoding an encephalitogenic peptide protects mice from experimental allergic encephalomyelitis." J Neuroimmunol **64**(2): 163-173.

Baron, J. L., J. A. Madri, et al. (1993). "Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma." J Exp Med **177**(1): 57-68.

Baughman, E. J., J. P. Mendoza, et al. (2011). "Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis." J Autoimmun **36**(2): 115-124.

Benedict, R. H., E. Wahlig, et al. (2005). "Predicting quality of life in multiple sclerosis: accounting for physical disability, fatigue, cognition, mood disorder, personality, and behavior change." J Neurol Sci **231**(1-2): 29-34.

Bettini, M., K. Rosenthal, et al. (2009). "Pathogenic MOG-reactive CD8+ T cells require MOG-reactive CD4+ T cells for sustained CNS inflammation during chronic EAE." J Neuroimmunol **213**(1-2): 60-68.

Betts, C. D., M. T. D'Mellow, et al. (1993). "Urinary symptoms and the neurological features of bladder dysfunction in multiple sclerosis." J Neurol Neurosurg Psychiatry **56**(3): 245-250.

Bielekova, B., M. H. Sung, et al. (2004). "Expansion and functional relevance of high-avidity myelin-specific CD4+ T cells in multiple sclerosis." J Immunol **172**(6): 3893-3904.

Bo, L., C. A. Vedeler, et al. (2003). "Subpial demyelination in the cerebral cortex of multiple sclerosis patients." J Neuropathol Exp Neurol **62**(7): 723-732.

Boer, M. C., S. A. Joosten, et al. (2015). "Regulatory T-Cells at the Interface between Human Host and Pathogens in Infectious Diseases and Vaccination." Front Immunol **6**: 217.

Braun-Fahrlander, C., J. Riedler, et al. (2002). "Environmental exposure to endotoxin and its relation to asthma in school-age children." N Engl J Med **347**(12): 869-877.

Brownell, B. and J. T. Hughes (1962). "The distribution of plaques in the cerebrum in multiple sclerosis." J Neurol Neurosurg Psychiatry **25**: 315-320.

Bruck, W. (2005). "The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage." J Neurol **252 Suppl 5**: v3-9.

Bruckener, K. E. (2003). "Permeabilization in a cerebral endothelial barrier model by pertussis toxin involves the PKC effector pathway and is abolished by elevated levels of cAMP." Journal of Cell Science **116**(9): 1837-1846.

Carlos, T. M., B. R. Schwartz, et al. (1990). "Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells." Blood **76**(5): 965-970.

Casiraghi, C., I. Shanina, et al. (2012). "Gammaherpesvirus latency accentuates EAE pathogenesis: relevance to Epstein-Barr virus and multiple sclerosis." PLoS Pathog **8**(5): e1002715.

Chang, A., M. C. Smith, et al. (2008). "Neurogenesis in the chronic lesions of multiple sclerosis." Brain **131**(Pt 9): 2366-2375.

Chen, X., O. M. Howard, et al. (2007). "Pertussis toxin by inducing IL-6 promotes the generation of IL-17-producing CD4 cells." J Immunol **178**(10): 6123-6129.

Chen, X., R. T. Winkler-Pickett, et al. (2006). "Pertussis toxin as an adjuvant suppresses the number and function of CD4+CD25+ T regulatory cells." Eur J Immunol **36**(3): 671-680.

Chen, Y., C. L. Langrish, et al. (2006). "Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis." J Clin Invest **116**(5): 1317-1326.

Chesla, S. E., P. Selvaraj, et al. (1998). "Measuring two-dimensional receptor-ligand binding kinetics by micropipette." Biophys J **75**(3): 1553-1572.

Collison, L. W., C. J. Workman, et al. (2007). "The inhibitory cytokine IL-35 contributes to regulatory T-cell function." Nature **450**(7169): 566-569.

Compston, A. and A. Coles (2008). "Multiple sclerosis." Lancet **372**(9648): 1502-1517.

Cua, D. J., J. Sherlock, et al. (2003). "Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain." Nature **421**(6924): 744-748.

Cunnusamy, K., E. J. Baughman, et al. (2014). "Disease exacerbation of multiple sclerosis is characterized by loss of terminally differentiated autoregulatory CD8+ T cells." Clin Immunol **152**(1-2): 115-126.

Cyster, J. G. and C. C. Goodnow (1995). "Pertussis toxin inhibits migration of B and T lymphocytes into splenic white pulp cords." J Exp Med **182**(2): 581-586.

De Stefano, N., P. M. Matthews, et al. (2003). "Evidence of early cortical atrophy in MS: relevance to white matter changes and disability." Neurology **60**(7): 1157-1162.

Detels, R., B. R. Visscher, et al. (1977). "Evidence for lower susceptibility to multiple sclerosis in Japanese-Americans." Am J Epidemiol **105**(4): 303-310.

Domingues, H. S., M. Mues, et al. (2010). "Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis." PLoS One **5**(11): e15531.

Edwards, L. J., V. I. Zarnitsyna, et al. (2012). "Insights into T cell recognition of antigen: significance of two-dimensional kinetic parameters." Front Immunol **3**: 86.

Elian, M., S. Nightingale, et al. (1990). "Multiple sclerosis among United Kingdom-born children of immigrants from the Indian subcontinent, Africa and the West Indies." J Neurol Neurosurg Psychiatry **53**(10): 906-911.

Ercolini, A. M., J. L. Croxford, et al. (2007). "Cross-reactivity between peptide mimics of the immunodominant myelin proteolipid protein epitope PLP139-151: Comparison of peptide priming in CFA vs. viral delivery." Journal of Neuroimmunology **186**(1-2): 5-18.

Farias, A. S., R. L. Talaisys, et al. (2011). "Regulatory T cell induction during Plasmodium chabaudi infection modifies the clinical course of experimental autoimmune encephalomyelitis." PLoS One **6**(3): e17849.

Farrell, R. A., D. Antony, et al. (2009). "Humoral immune response to EBV in multiple sclerosis is associated with disease activity on MRI." Neurology **73**(1): 32-38.

Fisk, J. D., A. Pontefract, et al. (1994). "The impact of fatigue on patients with multiple sclerosis." Can J Neurol Sci **21**(1): 9-14.

Ford, M. L. and B. D. Evavold (2005). "Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis." Eur J Immunol **35**(1): 76-85.

Fraga-Silva, T. F., L. A. Mimura, et al. (2015). "Experimental autoimmune encephalomyelitis development is aggravated by Candida albicans infection." J Immunol Res **2015**: 635052.

Fuentes, M. E., S. K. Durham, et al. (1995). "Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1." J Immunol **155**(12): 5769-5776.

Gautam, A. M., R. Liblau, et al. (1998). "A viral peptide with limited homology to a self peptide can induce clinical signs of experimental autoimmune encephalomyelitis." J Immunol **161**(1): 60-64.

Gray, L. S., K. S. Huber, et al. (1989). "Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein." J Immunol **142**(5): 1631-1638.

Haas, J., A. Hug, et al. (2005). "Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis." Eur J Immunol **35**(11): 3343-3352.

Hafler, D. A., A. Compston, et al. (2007). "Risk alleles for multiple sclerosis identified by a genomewide study." N Engl J Med **357**(9): 851-862.

Harrington, L. E., R. D. Hatton, et al. (2005). "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." Nat Immunol **6**(11): 1123-1132.

Hausmann, S. and K. W. Wucherpfennig (1997). "Activation of autoreactive T cells by peptides from human pathogens." Curr Opin Immunol **9**(6): 831-838.

Hayes, C. E., S. L. Hubler, et al. (2015). "Vitamin D Actions on CD4(+) T Cells in Autoimmune Disease." Front Immunol **6**: 100.

Hempel, K., A. Freitag, et al. (1985). "Unresponsiveness to experimental allergic encephalomyelitis in Lewis rats pretreated with complete Freund's adjuvant." Int Arch Allergy Appl Immunol **76**(3): 193-199.

Herrmann, I., M. Kellert, et al. (2006). "Streptococcus pneumoniae Infection aggravates experimental autoimmune encephalomyelitis via Toll-like receptor 2." Infect Immun **74**(8): 4841-4848.

Hotez, P. J., P. J. Brindley, et al. (2008). "Helminth infections: the great neglected tropical diseases." J Clin Invest **118**(4): 1311-1321.

Huang, J., V. I. Zarnitsyna, et al. (2010). "The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness." Nature **464**(7290): 932-936.

Huber, M., S. Heink, et al. (2013). "IL-17A secretion by CD8+ T cells supports Th17-mediated autoimmune encephalomyelitis." J Clin Invest **123**(1): 247-260.

Huehn, J., K. Siegmund, et al. (2004). "Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells." J Exp Med **199**(3): 303-313.

Inada, T. and C. A. Mims (1986). "Infection of mice with lactic dehydrogenase virus prevents development of experimental allergic encephalomyelitis." J Neuroimmunol **11**(1): 53-56.

Inglese, M., A. Ghezzi, et al. (2002). "Irreversible disability and tissue loss in multiple sclerosis: a conventional and magnetization transfer magnetic resonance imaging study of the optic nerves." Arch Neurol **59**(2): 250-255.

Jongen, P. J., C. Sindic, et al. (2011). "Adverse events of interferon beta-1a: a prospective multi-centre international ICH-GCP-based CRO-supported external validation study in daily practice." PLoS One **6**(10): e26568.

Kaushansky, N., D. M. Altmann, et al. (2012). "DQB1*0602 rather than DRB1*1501 confers susceptibility to multiple sclerosis-like disease induced by proteolipid protein (PLP)." J Neuroinflammation **9**: 29.

Kermode, A. G., A. J. Thompson, et al. (1990). "Breakdown of the blood-brain barrier precedes symptoms and other MRI signs of new lesions in multiple sclerosis. Pathogenetic and clinical implications." Brain **113 (Pt 5)**: 1477-1489.

Kersh, A. E., L. J. Edwards, et al. (2014). "Progression of relapsing-remitting demyelinating disease does not require increased TCR affinity or epitope spread." J Immunol **193**(9): 4429-4438.

Kirk, J., J. Plumb, et al. (2003). "Tight junctional abnormality in multiple sclerosis white matter affects all calibres of vessel and is associated with blood-brain barrier leakage and active demyelination." J Pathol **201**(2): 319-327.

Kleinschmidt-DeMasters, B. K. and K. L. Tyler (2005). "Progressive multifocal leukoencephalopathy complicating treatment with natalizumab and interferon beta-1a for multiple sclerosis." N Engl J Med **353**(4): 369-374.

Kohm, A. P., P. A. Carpentier, et al. (2002). "Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis." J Immunol **169**(9): 4712-4716.

Koutrolos, M., K. Berer, et al. (2014). "Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS." Acta Neuropathol Commun **2**: 163.

Kroenke, M. A., T. J. Carlson, et al. (2008). "IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition." J Exp Med **205**(7): 1535-1541.

Krupp, L. B., N. G. LaRocca, et al. (1989). "The fatigue severity scale. Application to patients with multiple sclerosis and systemic lupus erythematosus." Arch Neurol **46**(10): 1121-1123.

Kwok, L. Y., H. Miletic, et al. (2002). "Protective immunosurveillance of the central nervous system by Listeria-specific CD4 and CD8 T cells in systemic listeriosis in the absence of intracerebral Listeria." J Immunol **169**(4): 2010-2019.

Lafaille, J. J., F. V. Keere, et al. (1997). "Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease." J Exp Med **186**(2): 307-312.

Laidlaw, B. J., W. Cui, et al. (2015). "Production of IL-10 by CD4(+) regulatory T cells during the resolution of infection promotes the maturation of memory CD8(+) T cells." Nat Immunol **16**(8): 871-879.

Lehmann, D. and A. Ben-Nun (1992). "Bacterial agents protect against autoimmune disease. I. Mice pre-exposed to Bordetella pertussis or Mycobacterium tuberculosis are highly refractory to induction of experimental autoimmune encephalomyelitis." J Autoimmun **5**(6): 675-690.

Lisak, R. P., B. Zweiman, et al. (1983). "Effect of treatment with Copolymer 1 (Cop-1) on the in vivo and in vitro manifestations of experimental allergic encephalomyelitis (EAE)." J Neurol Sci **62**(1-3): 281-293.

Lizotte, P. H., J. R. Baird, et al. (2014). "Attenuated Listeria monocytogenes reprograms M2-polarized tumor-associated macrophages in ovarian cancer leading to iNOS-mediated tumor cell lysis." Oncoimmunology **3**: e28926.

Lo, W. F., A. S. Woods, et al. (2000). "Molecular mimicry mediated by MHC class Ib molecules after infection with gram-negative pathogens." Nat Med **6**(2): 215-218.

Lublin, F. D. and S. C. Reingold (1996). "Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis." Neurology **46**(4): 907-911.

Lublin, F. D., S. C. Reingold, et al. (2014). "Defining the clinical course of multiple sclerosis: the 2013 revisions." Neurology **83**(3): 278-286.

Lucchinetti, C., W. Bruck, et al. (2000). "Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination." Ann Neurol **47**(6): 707-717.

Lunemann, J. D., I. Jelcic, et al. (2008). "EBNA1-specific T cells from patients with multiple sclerosis cross react with myelin antigens and co-produce IFN-gamma and IL-2." J Exp Med **205**(8): 1763-1773.

Mandl, S., L. J. Sigal, et al. (1998). "Poliovirus vaccine vectors elicit antigen-specific cytotoxic T cells and protect mice against lethal challenge with malignant melanoma cells expressing a model antigen." Proc Natl Acad Sci U S A **95**(14): 8216-8221.

Mangmool, S. and H. Kurose (2011). "G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX)." Toxins (Basel) **3**(7): 884-899.

Manicassamy, S., R. Ravindran, et al. (2009). "Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity." Nat Med **15**(4): 401-409.

Marrosu, M. G., F. Muntoni, et al. (1988). "Sardinian multiple sclerosis is associated with HLA-DR4: a serologic and molecular analysis." Neurology **38**(11): 1749-1753.

Martin, C. L., B. A. Phillips, et al. (2006). "Gait and balance impairment in early multiple sclerosis in the absence of clinical disability." Mult Scler **12**(5): 620-628.

Martinez, R. (manuscript in preparation).

McDonald, W. I., A. Compston, et al. (2001). "Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis." Ann Neurol **50**(1): 121-127.

McRae, B. L., M. K. Kennedy, et al. (1992). "Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein." J Neuroimmunol **38**(3): 229-240.

Miller, S. D., C. L. Vanderlugt, et al. (1997). "Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading." Nat Med **3**(10): 1133-1136.

Moon, J. J., H. H. Chu, et al. (2009). "Tracking epitope-specific T cells." Nat Protoc **4**(4): 565-581.

Moulin, D. E., K. M. Foley, et al. (1988). "Pain syndromes in multiple sclerosis." Neurology **38**(12): 1830-1834.

Nelson, R. W., D. Beisang, et al. (2015). "T cell receptor cross-reactivity between similar foreign and self peptides influences naive cell population size and autoimmunity." Immunity **42**(1): 95-107.

Oldstone, M. B. (1988). "Prevention of type I diabetes in nonobese diabetic mice by virus infection." Science **239**(4839): 500-502.

Oldstone, M. B., R. Ahmed, et al. (1990). "Viruses as therapeutic agents. II. Viral reassortants map prevention of insulin-dependent diabetes mellitus to the small RNA of lymphocytic choriomeningitis virus." J Exp Med **171**(6): 2091-2100.

Ortega, S. B., V. P. Kashi, et al. (2013). "The disease-ameliorating function of autoregulatory CD8 T cells is mediated by targeting of encephalitogenic CD4 T cells in experimental autoimmune encephalomyelitis." J Immunol **191**(1): 117-126.

Patsopoulos, N. A., L. F. Barcellos, et al. (2013). "Fine-mapping the genetic association of the major histocompatibility complex in multiple sclerosis: HLA and non-HLA effects." PLoS Genet **9**(11): e1003926.

Patten, S. B., C. A. Beck, et al. (2003). "Major depression in multiple sclerosis: a population-based perspective." Neurology **61**(11): 1524-1527.

Perez-Liz, G., L. Del Valle, et al. (2008). "Detection of JC virus DNA fragments but not proteins in normal brain tissue." Ann Neurol **64**(4): 379-387.

Perkins, M. R., C. Ryschkewitsch, et al. (2012). "Changes in JC virus-specific T cell responses during natalizumab treatment and in natalizumab-associated progressive multifocal leukoencephalopathy." PLoS Pathog **8**(11): e1003014.

Pierson, E., S. B. Simmons, et al. (2012). "Mechanisms regulating regional localization of inflammation during CNS autoimmunity." Immunological Reviews **248**: 205-215.

Polman, C. H., S. C. Reingold, et al. (2011). "Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria." Ann Neurol **69**(2): 292-302.

Putheti, P., A. Pettersson, et al. (2004). "Circulating CD4+CD25+ T regulatory cells are not altered in multiple sclerosis and unaffected by disease-modulating drugs." J Clin Immunol **24**(2): 155-161.

Raddassi, K., S. C. Kent, et al. (2011). "Increased frequencies of myelin oligodendrocyte glycoprotein/MHC class II-binding CD4 cells in patients with multiple sclerosis." J Immunol **187**(2): 1039-1046.

Ramagopalan, S. V., D. A. Dyment, et al. (2008). "Genetic epidemiology: the use of old and new tools for multiple sclerosis." Trends Neurosci **31**(12): 645-652.

Rausch, M., P. Hiestand, et al. (2003). "MRI-based monitoring of inflammation and tissue damage in acute and chronic relapsing EAE." Magn Reson Med **50**(2): 309-314.

Read, S., V. Malmstrom, et al. (2000). "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation." J Exp Med **192**(2): 295-302.

Sabatino, J. J., Jr., J. Huang, et al. (2011). "High prevalence of low affinity peptide-MHC II tetramer-negative effectors during polyclonal CD4+ T cell responses." J Exp Med **208**(1): 81-90.

Schellenberg, A. E., R. Buist, et al. (2012). "Blood-brain barrier disruption in CCL2 transgenic mice during pertussis toxin-induced brain inflammation." Fluids Barriers CNS **9**(1): 10.

Schneider, O. D., A. A. Weiss, et al. (2007). "Pertussis toxin utilizes proximal components of the T-cell receptor complex to initiate signal transduction events in T cells." Infect Immun **75**(8): 4040-4049.

Sharief, M. K., M. A. Noori, et al. (1993). "Increased levels of circulating ICAM-1 in serum and cerebrospinal fluid of patients with active multiple sclerosis. Correlation with TNF-alpha and blood-brain barrier damage." J Neuroimmunol **43**(1-2): 15-21.

Shevach, E. M., R. S. McHugh, et al. (2001). "Control of T-cell activation by CD4+ CD25+ suppressor T cells." Immunological Reviews **182**: 58-67.

Shevach, E. M. and G. L. Stephens (2006). "The GITR-GITRL interaction: co-stimulation or contrasuppression of regulatory activity?" Nat Rev Immunol **6**(8): 613-618.

Shorter, S. K., F. J. Schnell, et al. (2016). "Viral Escape Mutant Epitope Maintains TCR Affinity for Antigen yet Curtails CD8 T Cell Responses." PLoS One **11**(2): e0149582.

Sifringer, M., V. Stefovska, et al. (2007). "The role of matrix metalloproteinases in infant traumatic brain injury." Neurobiol Dis **25**(3): 526-535.

Simpson, S., Jr., B. Taylor, et al. (2012). "Anti-HHV-6 IgG titer significantly predicts subsequent relapse risk in multiple sclerosis." Mult Scler **18**(6): 799-806.

Slavin, A., C. Ewing, et al. (1998). "Induction of a multiple sclerosis-like disease in mice with an immunodominant epitope of myelin oligodendrocyte glycoprotein." Autoimmunity **28**(2): 109-120.

Spangrude, G. J., F. Sacchi, et al. (1985). "Inhibition of lymphocyte and neutrophil chemotaxis by pertussis toxin." J Immunol **135**(6): 4135-4143.

Starks, H., K. W. Bruhn, et al. (2004). "Listeria monocytogenes as a vaccine vector: virulence attenuation or existing antivector immunity does not diminish therapeutic efficacy." J Immunol **173**(1): 420-427.

Steffen, B. J., E. C. Butcher, et al. (1994). "Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse." Am J Pathol **145**(1): 189-201.

Stromnes, I. M. and J. M. Goverman (2006). "Active induction of experimental allergic encephalomyelitis." Nat Protoc **1**(4): 1810-1819.

Stromnes, I. M. and J. M. Goverman (2006). "Passive induction of experimental allergic encephalomyelitis." Nat Protoc **1**(4): 1952-1960.

Tejada-Simon, M. V., Y. C. Zang, et al. (2002). "Detection of viral DNA and immune responses to the human herpesvirus 6 101-kilodalton virion protein in patients with multiple sclerosis and in controls." J Virol **76**(12): 6147-6154.

Tsunoda, I. and R. S. Fujinami (1996). "Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus." J Neuropathol Exp Neurol **55**(6): 673-686.

Vanderlugt, C. L. and S. D. Miller (2002). "Epitope spreading in immune-mediated diseases: implications for immunotherapy." Nat Rev Immunol **2**(2): 85-95.

Varga, S. M. and R. M. Welsh (2000). "High frequency of virus-specific interleukin-2-producing CD4(+) T cells and Th1 dominance during lymphocytic choriomeningitis virus infection." J Virol **74**(9): 4429-4432.

Venken, K., N. Hellings, et al. (2008). "Compromised CD4+ CD25(high) regulatory T-cell function in patients with relapsing-remitting multiple sclerosis is correlated with a reduced frequency of FOXP3-positive cells and reduced FOXP3 expression at the single-cell level." Immunology **123**(1): 79-89.

Viglietta, V., C. Baecher-Allan, et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med **199**(7): 971-979.

Virgin, H. W. (2014). "The virome in mammalian physiology and disease." Cell **157**(1): 142-150.

Virgin, H. W., E. J. Wherry, et al. (2009). "Redefining chronic viral infection." Cell **138**(1): 30-50.

von Herrath, M. G., J. Dockter, et al. (1994). "How virus induces a rapid or slow onset insulin-dependent diabetes mellitus in a transgenic model." Immunity **1**(3): 231-242.

Voskuhl, R. R. and S. M. Gold (2012). "Sex-related factors in multiple sclerosis susceptibility and progression." Nat Rev Neurol **8**(5): 255-263.

Wang, L. Y. and R. S. Fujinami (1997). "Enhancement of EAE and induction of autoantibodies to T-cell epitopes in mice infected with a recombinant vaccinia virus encoding myelin proteolipid protein." J Neuroimmunol **75**(1-2): 75-83.

Wang, Y., S. J. Marling, et al. (2012). "Development of experimental autoimmune encephalomyelitis (EAE) in mice requires vitamin D and the vitamin D receptor." Proc Natl Acad Sci U S A **109**(22): 8501-8504.

Wang, Z. Y., D. Yang, et al. (2006). "Induction of dendritic cell maturation by pertussis toxin and its B subunit differentially initiate Toll-like receptor 4-dependent signal transduction pathways." Exp Hematol **34**(8): 1115-1124.

Willenborg, D. O., S. Fordham, et al. (1996). "IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis." J Immunol **157**(8): 3223-3227.

Wucherpfennig, K. W. and J. L. Strominger (1995). "Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein." Cell **80**(5): 695-705.

Wujek, J. R., C. Bjartmar, et al. (2002). "Axon loss in the spinal cord determines permanent neurological disability in an animal model of multiple sclerosis." J Neuropathol Exp Neurol **61**(1): 23-32.

Yao, K., S. Gagnon, et al. (2008). "Reactivation of human herpesvirus-6 in natalizumab treated multiple sclerosis patients." PLoS One **3**(4): e2028.

Yao, K., S. Honarmand, et al. (2009). "Detection of human herpesvirus-6 in cerebrospinal fluid of patients with encephalitis." Ann Neurol **65**(3): 257-267.

Yednock, T. A., C. Cannon, et al. (1992). "Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin." Nature **356**(6364): 63-66.

Yin, J. X., Z. Tang, et al. (2014). "Pertussis toxin modulates microglia and T cell profile to protect experimental autoimmune encephalomyelitis." Neuropharmacology **81**: 1-5.

York, N. R., J. P. Mendoza, et al. (2010). "Immune regulatory CNS-reactive CD8+T cells in experimental autoimmune encephalomyelitis." J Autoimmun **35**(1): 33-44.

Zhang, X., D. N. Koldzic, et al. (2004). "IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells." Int Immunol **16**(2): 249-256.