DETERMINING THE ROLE OF THE CELLULAR IMMUNE RESPONSE AGAINST AQUAPORIN 4 IN THE CONTEXT OF NEUROMYELITIS OPTICA

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DEDICATION

To Bessie and Vicente Arellano, I want to thank you for all of the support you have given me leading up to this point. I only hope that I make you proud no matter what happens next in my life.

To my brothers Vince and Jeff, I thank you for the continual support throughout all of my studies. You have always helped to keep my feet on the ground and help me to relate to the world outside the protective bubble of academia.

To my mentor, Olaf, Stüve, your patience and wisdom has allowed me to grow more as an individual than I could have ever imagined in graduate school. You taught me to survive in the competitive world of research through being fearless and humble as I continue to grow into a senior scientist.

To all individuals who have acted within my thesis committee through out my graduate studies (Anne Satterthwaite, Todd Eager, Benjamin Greenberg, Nancy Street, and Nitin Karandikar), your guidance throughout my graduate career was critical for my success and I want to thank you for all of help you all provided in the troubleshooting of my project.

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by

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DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May 2014

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Publication No._____

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The University of Texas Southwestern Medical Center at Dallas, 2014

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Neuromyelitis optica (NMO) is a demyelinating inflammatory disorder of the central nervous system (CNS), which historically is clinically and pathologically defined as the co-occurrence of optic neuritis with myelitis. Thus far, all HLA haplotype analyses in cohorts of NMO patients suggest an association with *HLA-DRB1*03:01*. In the majority of patients, IgG against aquaporin-4 (AQP4) can be detected in the serum. AQP4 is expressed in numerous tissues: on the plasma membrane of principal cells and inner medullary collecting duct cells in the kidney, the endothelial cells lining of the gastrointestinal tract, and astrocytic foot processes of astrocytes within the CNS, just to name a few. The role of cellular immune responses against AQP4 in NMO has not been studied. Specifically, linear dominant determinants of AQP4 in the context of *HLA-DRB1*03:01* have not been identified. I hypothesize that $CD4^+$ T cells recognize linear determinants of AQP4 in the context of *HLA-DRB1*03:01*. I further hypothesize that NMO is co-initiated by AQP4-reactive CD4+ T cells.

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PRIOR PUBLICATIONS

Hussain R, Hayardeny L, Yarovinsky F, **Arellano B**, Deason K, Castro-Rojas C, St**ü**ve. O. Immune surveillance of the central nervous system in multiple sclerosis- Relevance for therapy and experimental models. Submitted.

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Altered peptide ligands (APL)

Antibody-dependent cellular cytotoxicity (ADCC)

Antigen-presenting cells (APCs)

Aquaporin 4 (AQP4)

B cell receptor (BCR)

Basic Local Alignment Search Tool (BLAST)

Basic Local Alignment Search Tool (BLAST)

Blood brain barrier (BBB)

CC chemokine receptor 7 (CCR7)

CC chemokine receptor 8 (CCR8)

Central nervous system (CNS)

Cerebral perivascular spaces (CPVS)

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Complement-dependent cytoxicity (CDC)

Complete Freund's adjuvant (CFA)

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Lymph nodes (LN)

Magnetic resonance imaging (MRI)

Major histocompatibility complex (MHC)

Monoclonal antibody (mAb)

Multiple Sclerosis (MS)

Myelin basic protein (MBP)

Myelin oligodendrocyte glycoprotein (MOG)

Myelin oligodendrocyte glycoprotein peptide 35-55 (MOG35–55)

National Center for Biotechnology Information (NCBI)

National Institute of Neurological Disorders and Stroke (NINDS),

Neuromyelitis Optica (NMO)

NMO spectrum disorder (NMOSD)

Nucleotide-binding oligomerization domain (NOD)

Optic neuritis (ON)

Pathogen-associated molecular patterns (PAMPs)

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INTRODUCTION AND LITERATURE REVIEW

Multiple Sclerosis – The Most Studied and Best Understood Autoimmune Disease of the Central Nervous System (CNS)

Multiple sclerosis (MS) is a debilitating neurological disorder characterized by the progressive destruction of the white matter found in the central nervous system (CNS). The disease typically manifests in individuals between their early to mid 20's to late 30's and is the leading neurological disorder leading to paralysis in young adults (1). According to the National Institute of Neurological Disorders and Stroke (NINDS), between 250,000 to 350,000 individuals have been diagnosed with MS in the United States alone, with an estimated 200 new cases diagnosed weekly (2). Studies show that the rate of disease incidence has slowly increased over the past few decades with the incidence rate in women comprising of 3.6 cases per 100,000 persons per year and 2.0 cases for men (3). Additionally, studies have found that while male disease incidences have stayed constant in recent years, the rate of disease incidence is increasing in women.

Individuals are diagnosed with MS after having experienced two disease attacks separated in time and space(4). Attacks can either be determined clinically or visualized utilizing magnetic resonance imaging (MRI) and gadolinium to observe the breakdown of the blood brain barrier (BBB) (5). Due to damage in the white

matter, MS clinically manifests with neurological deficits during phases of relapses, which may never fully resolve, and neurological disability that accumulates over time. Typically, patients experience loss of motor functions, speech, vision, and cognitive dysfunction, which are correlated to the lesions within the CNS (6, 7). In the late stages of the disease, atrophy occurs within the brain and spinal cord of patients as a result of the loss of axons and myelin (8, 9). The expanded disability status scale (EDSS) utilizes a numerical 0 to 10 rating that was generated for use by clinicians to quantify the impairment of their patients and is commonly used during clinical trials to examine the efficacy of therapies (10). It is currently unknown what initiates the demyelination, but research over the past 30 years has brought to light the important role the immune system plays in the pathogenesis of MS.

Clinical Subtypes

Multiple sclerosis comprises of multiple subtypes that vary based on clinical manifestations, but there appear to be two very clear pathological stages in the disease course: a relapsing-remitting stage followed by a progressive stage. (11) The majority of MS patients show a relapsing-remitting phenotype that is characterized by neurological deficits, co called exacerbations, followed by variable levels of recovery. Characteristically, relapsing-remitting patients will begin to transition into a progressive phenotype in which exacerbations cease but disability continues to worsen. It is estimated that 15% of patients are affected by a primary

progressive disease course in which neurological deterioration shows no form of relapse but rather is a continuous accumulation of disability over time (12). Typically, patients with relapsing-remitting MS (RRMS) respond to immunomodulatory therapies with many patients showing better prognosis and quality of life thanks to newly discovered treatments. Eventually, relapsing-remitting patients begin to show a progressive phenotype and typically stop responding to immunomodulatory therapies (13) (Figure 1.1). Unfortunately, there are no treatment options for the progressive stage of MS (14). This section will focus on the relapsing-remitting phenotype, as it is the primary studied subtype and has brought to light multiple key understanding to CNS autoimmunity.

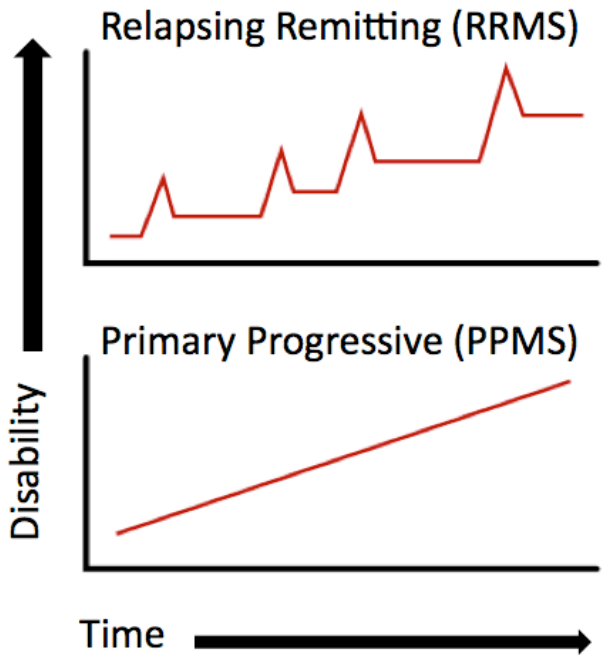

Figure 1.1 – The Two Predominant Disease Courses of Multiple Sclerosis

First described in *Lublin et al.*, MS patients accumulate disability over time. There are two stages in MS, a relapsing-remitting stage that will lead into a progressive stage, sometime patients will skip the relapsing remitting stage and go directly into a progressive stage. Though there are variations on the disease course, primary progressive MS (PPMS) and relapsing remitting MS (RRMS) are the predominant disease courses seen in patients.

Susceptibility to Multiple Sclerosis

Although there is no commonly agreed upon causative agent for the disease, familial studies strongly suggest a genetic component to the disease with an individual having a 30% increased likelihood of developing MS if both parents have the disease (15). It is widely accepted that specific major histocompatibility complex (MHC) II alleles are positively associated with the risk of developing MS (16). Disease associated haplotypes found in studies utilizing patients with the relapsing-remitting phenotype have been characterized in the MS animal model to further comprehend their role in CNS autoimmunity.

The MHC II allele *HLA-DRB1*15:01* is found to have a strong association in patients with MS. Patients appear to transcribe higher numbers of *HLA-DRB1*15:01* mRNA in peripheral blood mononuclear cells (PBMCs) and cerebrospinal fluid (CSF) cells as well as within thymic and CNS tissues (17). Expression of this allele is important mainly due to the ability of translated MHC II molecules to present antigens to $CD4^+$ T cell leading to activation and proliferation (18). The strong association between the MHC II allele and disease susceptibility is one of the strongest pieces of evidence that MS is a T cell-mediated autoimmune disease. The immunomodulatory therapies developed specifically for this disease aim toward inhibiting the inevitable reactivation of autoreactive immune cells against myelin antigens within the CNS. Comprehending and targeting specific pathways is critical for the creation of future therapies for CNS autoimmune diseases.

Environmental factors are also suggested to play a role in the disease. For example, populations that reside in a higher latitude in more westernized societies have higher disease incidences (19). When comparing European countries, a clear gradient appears with a statistically significant correlation between latitude and disease prevalence (20). It is believed that exposure to sunlight may be the greatest factor effected by latitude. More recently, high serum Vitamin D levels have been found to be a protective against the development of MS (21), linking the transcription of the *HLA-DRB1*15:01* gene to Vitamin D levels and bringing to light the role of this environmental factor in mediating the disease (22,23). This ultimately suggests that ultraviolet (UV) exposure may be the primary culprit in latitude associations to MS (24, 25).

Experimental Autoimmune Encephalomyelitis – The Animal model for CNS Autoimmunity

Within the relapsing-remitting subtype of MS are thought to be distinctive pathological features that drive the disease-associated lesions within the CNS (26). 1) Inflammation within the periphery leads to the localization of autoreactive immune cells to the CNS. 2) As a result, damage to the myelin sheath of oligodendrocyte cell body occurs (3) resulting in damage if not loss of axons in the area of inflammation. 4). Following the inflammatory event, gliosis occurs leading to axonal scaring paired with minimal remyelination of damaged axons, at which point it is believed relapses

tend to subside. Replicating these pathological features in rodent animal models has been critical for furthering the understanding of the critical pathways of CNS autoimmunity.

Experimental autoimmune encephalomyelitis (EAE) is the prototypical animal model of CNS autoimmunity. Similar to MS, the disease course varies based on animal strain and CNS antigen utilized between a monophasic, chronic, or relapsing clinical phenotypes . The underlying principle for initiating EAE in rodents, whether via active or passive induction, is the generation of myelin-protein-specific, autoreactive $CD4^+$ T cells though some EAE models use autoreactive B cells as the causative cell type (27). However, there are limitations to the EAE animal model. No EAE model replicates the progressive stage associated with the later stages of MS and therefore no therapies geared towards the more neurodegenerative disease course have been generated from this animal model. Having a reliable model that replicates the pathology of a disease course is critical for identifying key mechanisms. This animal model has allowed for the generation of therapies for patients with relapsing-remitting MS that have improved the health and quality of life patients.

Initiation of CNS Autoimmunity

Multiple immune cells have been identified in initiating and perpetuating MS disease activity. Special physiological considerations must be taken into account since the CNS constitutes a unique organ system because it is confined entirely within a bony space. A robust inflammatory response and the ensuing edema could easily result in severe CNS damage or even a fatal outcome, due to the restricted ability to expand due to space limitations.

Studies utilizing MS patient samples as well as various EAE animal models have allowed for a basic understanding of the initial CNS autoimmune events required for disease initiation and the necessary cell types that drive the relapsing-remitting phenotypes seen in MS, which is believed to be primarily driven by the immune system. There are three critical stages in CNS autoimmunity: 1) Generation of autoimmune cells specific for CNS proteins, 2) Localization of the autoimmune leukocytes into the CNS, 3) Reactivation of the autoimmune leukocytes within the CNS. Each of these stages has been shown to be critical for disease progression and have thusly been targeted pharmaceutically.

The Generation of Autoimmune Cells Specific for CNS proteins

Data suggests that MS is a $CD4^+$ T cell mediated disease (28). The generation and survival of autoreactive $CD4^+$ T cells relies heavily on interactions with MHC II expressing antigen-presenting cells (APCs). The different APCs play individual roles

that together lead to a progressive cycle that furthers the pathogenesis associated with the disease.

Role of the Innate Immune system

Within most tissues, the initial recognition of a pathogen is followed by activation of resident macrophages and tissue-specific dendritic cells (DCs). These innate cells express numerous surface receptors that identify pathogen-associated molecular patterns (PAMPs), as well as danger-associated molecular patterns (DAMPs). These intracellular and extracellular receptors include lectins, scavenger receptors, Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene (RIG)-I family receptors (29-31). Experimental depletion of resident myeloid cells results in reduced host protection against infection, reduced expression of soluble inflammatory mediators, and diminished chemoattraction (32-35).

Role of Innate Cells – Macrophages and Monocytes

The primary role of monocytes and macrophages is the activation of localized innate cells via cytokines and the phagocytosis of pathogens at the site of inflammation (36). These cells can be considered as the core cells responsible for innate protection throughout the body. The fate and survival of both cell types as well as their initial and ongoing role in the priming of adaptive immunity and tissue

homeostasis has been under intense investigation. Following the detection of a pathogen through TLRs, resident macrophages stimulate the extravasation of additional innate cells, such as neutrophils and monocytes, to the site of infection to combat pathogens (32, 33, 37). Subsequently, localized monocytes will differentiate into monocyte-derived macrophages to become the majority of myeloid cells within an inflammatory site. One group of investigators observed a prolonged retention of inflammatory monocyte–derived macrophages in the lesion caused by mild zymosan peritonitis (38-40). So, while resident macrophages may be the primary cell type activating the immune system at the site of infection, monocytes play an additive role through the generation of site-specific monocytes-derived macrophages at the site of inflammation. In combination, these cells localize and activate leukocytes that migrate to the site of inflammation.

It is unclear, however, whether the roles of macrophages and monocytes made in other compartments are equivalent in the specialized environment of the CNS. Many resident macrophages remain within the tissue throughout inflammation, suggesting that these cells impact inflammatory responses and the return to homeostasis. It was shown that the number of tissue-resident macrophages is maintained by local proliferative self-renewal, without the need for replenishment from the periphery, including in the brain (41). However, it is not known what happens to inflammatory monocyte-derived macrophages in the CNS after the inflammatory response is resolved. In other compartments, these cells have been

suggested to exit via the lymphatics or to recirculate (42-44). The CNS, however, does not have a lymphatic system. It is also a possibility that monocyte-derived macrophages in the CNS undergo *in situ* phenotypic conversion to become tissue-resident macrophages, which may occur during inflammation (45). Evidence of the kinetics of APC turnover in the cerebral perivascular spaces (CPVS) suggests that meningeal and perivascular monocytes are replaced over the course of several weeks by hematogenous cells under normal conditions, and that this turnover is accelerated during EAE (46). Additionally, localized monocytes may be released from the bone marrow due to CNS inflammatory cytokines, further generating CNS localized DCs and macrophages. (47) There also appears to be an ongoing migration of macrophages from peripheral blood into CPVS (48). This is critical primarily due to the correlation between CNS localized macrophages and the upregulation of the CC chemokine receptor 8 (CCR8). However, despite the uncertainty of the importance of CNS localized macrophages and monocytes, the role of microglia, the resident CNS macrophage, in CNS autoimmunity is well-defined.

Role of Innate Cells - Microglia

Microglia are the tissue-intrinsic monocyte-derived, myeloid cell within the brain and spinal cord, and are considered to be one of the key players in the reactivation of the cellular immune response against CNS proteins (49-51). It is currently thought that

microglia are a long-lived population of tissue macrophages, but it is unknown how cell populations of brain macrophages are maintained in homeostasis and during disease (50). The immune-restrained phenotype of microglia may be explained at least partly by the fact that, in contrast to other tissue macrophages, these cells are shielded from serum proteins that can selectively and potently activate macrophages (52).

The pathogenic role of microglia in CNS autoimmunity has been established in the EAE model of MS. By using the EAE model, several investigators have demonstrated the capability of microglia to initiate an adaptive autoimmune response against target CNS antigens. It is known that the induction of EAE after adoptive transfer of $CD4^+$ antigen-specific T cells requires restimulation with the cognate autoantigen within the CNS compartment (53). Experiments with radiation bone marrow chimeras, in which bone marrow–derived donor cells expressed a MHC II haplotype distinct from that present on recipient parenchymal microglia, have demonstrated that adoptive transfer of myelin-specific $CD4^+$ T cells restricted by the MHC II haplotype of the recipient parenchymal cells did not cause EAE. In contrast, myelin-specific $CD4^+$ T cells that were restricted by the MHC II haplotype of perivascular macrophages derived from the donor bone marrow inoculum led to clinical disease (54). Other investigators demonstrated that the elimination of all potential APCs except for $CD11c^+$ perivascular macrophages did not affect disease susceptibility in experimental animals (55). Another set of experiments, in which EAE

was actively induced with one myelin epitope, followed by adoptive transfer of unprimed T cell receptor (TCR) transgenic T cells specific for a different antigenic determinant, showed that the adoptively transferred T cells became activated and proliferated predominantly in the CNS (56). It is clear that once autoreactive T cells localize to the brain or spinal cord, microglia are fully capable of reactivating these cells to further CNS pathogenesis.

Role of Innate Cells - Dendritic Cells

Mature DCs are established as the most competent APCs in the initiation of immune responses. During the extravasation of immune cells to the site of inflammation, mature DCs are known to drain into the lymph nodes to activate naïve $CD4^+$ T cells in a CC chemokine receptor 7 (CCR7) dependent manner (57). In the EAE animal model, utilization of a kinase inhibitor on DCs led to a decreased production of IL-6 and IL-23 and subsequently a decrease in the polarization of myelin specific T cells towards the Th_1 and Th_{17} phenotype (58). Once these autoreactive T cells have been activated and enter the periphery, DCs may also play an important role in trafficking of these cells to the CNS and reactivating them in the brain (59).

There is overwhelming data that contradicts the old dogma that the healthy CNS parenchyma contains no DCs. Prodinger et al., using mice transcribing the green fluorescent protein (GFP) under the promoter of the DC marker CD11c, determined the distribution, phenotype, and source of $CD11c^+$ cells in non-diseased

brains (60). DCs were identified in periventricular areas, adjacent fiber tracts, and optical nerves. The majority of $CD11c^+$ cells were located within the juxtavascular parenchyma rather than the perivascular spaces. Most notably, imaging of DC transmigration across the blood brain barrier conferred a positive correlation between disease severity and the recruitment of DCs to the CNS in EAE (61).

In MS and EAE, DCs are readily detectable in the brain (60, 62-67). Fischer and Reichmann showed that CNS inflammation in EAE and toxoplasmic encephalitis was associated with the proliferation of $CD11b^+CD11c^+$ cells (68). These cells constituted up to 30% of the total $CD11b^+$ population of myeloid cells isolated from the brain. In both disease models, $CD11c^+$ cells were located in perivascular spaces and intraparenchymatic inflammatory sites. Morphologically, $CD11c^+$ cells from inflamed brain resembled bone marrow-derived DCs, and thus were identified as such. These cells secreted high levels of IL-12, and were more potent stimulators of naive or allogeneic T cell proliferation than microglia cells. Furthermore, DCs isolated from inflamed brain primed naive T cells from DO11.10 OVA specific TCR transgenic mice to produce IFNγ and IL-2. A functional maturation of brain DCs occurred following the onset of encephalitis (68). The role of DCs as relevant innate immune cells of the CNS was underscored by the work of Greter et al, who showed that $CD11c^+$ DCs alone are sufficient to present antigen *in vivo* in EAE to primed myelin-reactive T cells in order to mediate CNS inflammation and clinical disease (55).

It was also recently shown that steady-state DCs are capable of inducing peripheral T cell tolerance, and of regulating CNS inflammation. Yogev and colleagues used genetic approaches to deplete $CD11c^+$ DCs in mice (69) and found that experimental animals that lack DCs developed more severe EAE than control mice. DCs engineered to present a CNS-associated autoantigen in an inducible manner were capable of inducing peripheral T cell tolerance and EAE resistance. This tolerance coincides with an upregulation of the PD-1 receptor on antigen-specific T cells, which is necessary for DC-mediated induction of regulatory T cells (69). These findings were supported by the work of Zozulya et al, who demonstrated that intracerebral microinjection of DCs modified by tumor necrosis factor resulted in relative EAE disease resistance, and triggered the generation of IL-10-producing antigen-specific T cells in the periphery, and restricted IL-17 production within the CNS (66). Thus, while DCs are clearly critical cellular players in innate and adaptive immune responses within the CNS, DC responses can also be modulated to down regulate inflammation.

Role of Adaptive Cells

Unlike innate cells, the adaptive immune system plays a critical role in activating the immune system against a pathogen in an antigen specific manner. Both T and B cells express polyproteins on their cell surfaces that are diverse and specific for a single epitope. The T cell receptor (TCR) recognizes peptide/MHC II complexes,

requiring interaction with both the peptide and MHC II molecule to initiate TCR signaling (70). The B cell receptor is capable of binding to peptides in the linear or confirmation peptide sequence (71). Once the naïve B cell is activated, the binding domain of the BCR will generate the fragment antigen-binding (FAB) region of the secreted antibody (72). This attribute of the adaptive immune system allows for immune surveillance in an antigen specific manner that generates long-lived memory cells. The generation of these cells rely heavily upon interactions between T cells and professional antigen presenting cells: DCs, macrophages, microglia, and B cells in the context of CNS autoimmunity (73-76).

In order to prevent aberrant adaptive immune responses against self-antigen, several protective mechanisms are in place. The concept of central tolerance suggests that because of the antigen specificity of the TCR and BCR, T and B cell are screened against endogenous peptides/MHC II complexes or proteins (77). Because of the specificity of both receptors, newly developed T and B cells undergo negative selection within their respective primary lymphoid organs prior to being released into the periphery. Any cell found to be specific for endogenous epitopes are deleted to inhibit the generation of an adaptive memory immune response against host proteins (78). Once negative selection has occurred, naïve T and B cells localize to secondary lymphoid organs to await activation. It is thought that within autoimmunity, the break down of central tolerance may be what drives the memory immune response against self-antigens. By studying the roles of specific

cells of the adaptive immune system, and through understanding the critical mechanisms behind generating memory T and B cells specific for CNS antigens, therapies have been created to block the progression of CNS autoimmunity

Role of Adaptive Cells – T cells

There are many lines of evidence that suggest that MS is a T cell-mediated autoimmune disease. Among cells isolated from the inflammatory infiltrate in actively demyelinating MS lesions, approximately 10% are $CD4^+$ and $CD8^+$ T cells, whereas the remainder are monocyte-derived macrophages and CNS-intrinsic microglia (79). Additionally, the T-cell repertoire in individual MS patients appears to change during different stages of disease activity (80-82). When observing various studies examining EAE, it has been shown that CNS autoimmunity can be induced by the adoptive transfer of myelin-specific $CD4^+$ T cells into naïve recipient mice by injection (83). These observations in the EAE animal model do not reflect the entire complex immunopathology of MS. For instance, $CD4^+$ T cells are almost undetectable in chronic MS lesions (84). Thus, the prevailing dogma has been that MS is a disorder primarily mediated by antigen-specific $CD4^+$ T cells (85, 86).

More recent research has focused on the pathogenic role of different $CD4^+$ T cell phenotypes in MS and other autoimmune diseases. $CD4^+$ T helper (Th) cell subsets are currently distinguished by the cytokines they express (87). Th1 cells are defined as expressing IFNγ, TNF-β, IL-2, and NO (88) and activate myeloid cells to

stimulate and maintain cell-mediated immunity. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 and TGF-β. While Th1 cells are considered predominantly pro-inflammatory in autoimmunity, Th2 immune responses have been associated with immune regulation and disease recovery in patients with MS. Both IL-10 and IL-4 are capable of suppressing various aspects of the Th1 inflammatory response. TGF-β also plays a regulatory role in immune responses. The Th1-Th2 paradigm is insufficient, however, to associate one $CD4^+$ T cell phenotype with disease activity, and another with remission (89, 90). While Th1 and Th2 polarized cells can be observed in MS and other human autoimmune disorders, a clear distinction between these populations is not always possible. For instance, $CD4^+$ Th1 cells are capable of producing IL-10 and IFNγ during chronic infection and in experimental settings in mice and humans (91-96). Th17 cells are a distinct, more recently discovered lineage of $CD4^+$ T cells that may be very relevant to autoimmune diseases (97). Induction and maintenance of Th17 cells depend on either IL-23 provided by macrophages and dendritic cells or a TGF-β and IL-6 stimulus (98, 99). Th17 cells, in turn, express IL-17, which mediates proinflammatory responses, including allergic reactions (100). In the EAE animal model, adoptive transfer of Th17 cells is capable of driving disease pathogenesis dependent on the secretion of IL-17 and GM-CSF (101, 102).

$CD8^+$ T cells have also more recently been implicated in MS pathogenesis. Within the peripheral blood and CSF of MS patients, clonal and oligoclonal

expansion of $CD8^+$ T cells has been demonstrated (103-105). Compared with $CD8^+$ myelin-reactive T cells isolated from healthy individuals, $CD8^+$ T cells from patients with MS express a less naïve, more activated phenotype ($CD8^{hi}CD28^-CD57^+$) (106, 107). In some histopathological studies, $CD8^+$ T cells outnumber $CD4^+$ T cells, suggesting that cytotoxic T cells are driving the inflammatory process. $CD8^+$ T cells may also directly mediate axonal damage observed in MS lesions. Neurons were not thought to be vulnerable to T-cell-mediated injury. However, the observation that *in vitro* exposure to IFNγ induces neurons to express MHC class I molecules provides a plausible explanation for $CD8^+$ T-cell–mediated neuronal damage (108). Furthermore, activated $CD4^+$ and $CD8^+$ T cells were shown *in vitro* to align along axons and cause neuronal cell death by a cell contact-dependent mechanism independent of MHC (109). In fact, $CD8^+$ T cells directly establish contact with demyelinated axons, upon which cytotoxic mediators are released (110). *In vitro* experiments have further demonstrated that $CD8^+$ T lymphocytes are capable of transecting neurites (111).

Most TCRs consist of two linked polypeptides, α and β, which participate in the co-recognition of foreign antigen in the context of the antigen-MHC complex(112). This is a critical step in immune surveillance. However, a small subpopulation of circulating lymphocytes expresses γδ TCRs, which also mediate T cell activation. Clonal expansion of activated lymphocytes bearing the γδ TCR was demonstrated in the CSF of patients with recent-onset MS, but not from patients with chronic MS or

other neurological disorders (113). In the EAE model, mice deficient in γδ T cells have reduced CNS mononuclear cell infiltrates (114-116). γδ T cells may also down regulate CNS inflammation by promoting apoptosis in encephalitogenic αβ T cells. Interestingly, it was also recently shown that γδ T-cell–deficient mice are unable to recover from EAE (117, 118). Histopathologically, monocytes and lymphocytes persisted substantially longer in the CNS of deficient mice than in their wild-type counterpart. Therefore, although all of the T cells subsets discussed above appear to play a pathogenic role in MS and its animal model EAE, it is likely that the primary biological function of these cells is immune surveillance. Thus, targeting all T cells pharmacologically has potential risks and benefits to which therapies should target strictly pathogenic T cells as to allow for adequate immune surveillance.

Role of Adaptive Cells – B cells

The EAE model has recently led to numerous insights into B cell function in the pathogenesis of MS. Early investigations into the role of B cells in EAE utilized injections of anti-IgM antibodies to deplete B cells in rats prior to EAE induction. In these early studies, depletion of B cells prevented the induction of EAE (119). In follow-up experiments, EAE could be induced if experimental animal received myelin basic protein (MBP)-specific antiserum in addition to anti-IgM antibodies (120). Anti-IgM treated animals were also refractory to EAE induction by active immunization with MBP/complete Freund's adjuvant (CFA) (121). However, EAE could be induced

in a third of B cell depleted mice by adoptive transfer of MBP-specific encephalitogenic T cells, and this induction could be increased by the simultaneous administration of anti-MBP antibodies (121). A pathogenic role for Ig was further shown by the exacerbation of mouse and rat EAE following the administration of anti-MOG mAb (122).

Several investigators have shown susceptibility of B cell-deficient mice to EAE. B cell-deficient mice were first tested in the B10.PL (H-2u) mouse strain by disruption of the Ig μ heavy chain transmembrane exon (μMT). The mouse was subsequently backcrossed onto the C57BL/6 background (H-2b), in which active immunization with myelin oligodendrocyte glycoprotein peptide 35-55 ($MOG_{35–55}$) led to clinical EAE (123, 124). Interestingly, the dependence on B cells for the induction of EAE in the C57BL/6 mouse appears to be dependent on the source and nature of the MOG immunogen. In a study by Lyons et al., both C57BL/6 wild-type and B cell-deficient mice were susceptible to EAE following immunization with $MOG_{35–55}$ (125). In contrast, only wild-type mice were susceptible to EAE induced with whole protein recombinant MOG (rMOG) protein (125). Although the species source of the rMOG in this study was not indicated, subsequent studies demonstrated that the B cell dependence only occurred when human rMOG was used. Induction of EAE following immunization with rMOG from mouse or rat was shown to be B cell-independent (123, 126). Dittel et al. reported that the relapsing–remitting disease course in (B10.PLxSJL/J)F1 B cell-deficient mice was not altered as compared to wild-type

control mice (127). These data indicate a complex role for B cells in the pathogenesis and regulation of CNS autoimmunity and that the experimental outcome is highly dependent upon the EAE model used.

In addition to the secretion of auto-antibodies, recent studies indicate that B cells also function as APCs that facilitate the priming of myelin-specific T cells. Using a transgenic mouse expressing a MOG-specific TCR on the SJL/J (H-2s) background, a mouse strain that exhibits a relapsing–remitting EAE disease course, a high rate of spontaneous EAE was observed (128). Clinical disease activity in these mice was associated with a strong MOG-specific B cell response, and deposits of Ig and complement in CNS lesions (128). In addition, pathogenesis of the MOG-specific Ig was demonstrated by its enhancement of the severity of suboptimal EAE. Finally, depletion of B cells with an anti-CD20mAb reduced the incidence of spontaneous EAE (128). The efficacy and safety of anti-CD20 therapy has been found to be beneficial in patients with relapsing-remitting MS, though the drug does leave patients immune compromised (129, 130).

Using anti-mouse CD20 mAb, it has been shown that B cell depletion in C57BL/6 mice prior to induction of EAE by active immunization with $MOG_{35–55}$ exacerbates clinical disease (131). These data support prior observations of regulatory B cells in controlling CNS autoimmunity (123, 132, 133). Interestingly, clinical effects appear to depend on the timing of the B cell depletion in this EAE

model: When B cells were depleted just prior to disease onset, or after peak disease was established, no change in disease severity was noted (131). In contrast, EAE disease severity was reduced if B cells were depleted shortly after EAE onset (134). In a study utilizing human CD20 transgenic mice, B cell depletion was achieved using an anti-human CD20 mAb (135). B cell depletion prior to active EAE induction with MOG35–55 exacerbated EAE (135). In contrast, in the same experimental design, immunization with recombinant MOG (rMOG), which requires B cell recognition and processing, resulted in less severe EAE (135). Similar results were obtained if the B cells were depleted after the onset of EAE. Differences in the two outcomes were attributed to a reduction in MOG-specific Th1 and Th17 cells in the rMOG model, as compared to a relative lack of impediment of Th1 or Th17 differentiation in mice immunized with MOG35–55 (135). Additionally, Monson et al also showed that B cell depletion with rituximab in human CD20 transgenic mice was associated with diminished Delayed Type Hypersensitivity (DTH) and a reduction in T cell proliferation and IL-17 production during recall immune response experiments (136).

Neuromyelitis Optica (NMO) – A CNS Autoimmue Disorder with a Known Autoantigen

From what we have learned in studying MS, it is apparent that there are a vast number of potential mechanisms that drive pathogenesis in CNS autoimmunity. With this knowledge, multiple therapies have been generated for patients so that disease progression may be delayed for as long as possible. Treating patients in a disease specific fashion would limit disease progression early; however, one of the major hindrances to identifying biomarkers that would allow for earlier identification of the disorder is the lacking identity of the CNS autoantigen. It is unclear which of the many myelin protein initiates disease. Proteolipid protein (PLP), myelin basic protein (MBP), or myelin oligodendrocyte glycoprotein (MOG) may drive disease pathogenesis differently from patient to patient and all have been implicated in MS at some point (137, 138). In recent years neuromyelitis optica (NMO), previously thought to be a subtype of MS, has distinguished itself as a fully separate disorder via differences shown in pathology, genetic susceptibility, and most importantly, identification of the autoantigen. By studying the CNS autoimmune disorder NMO, researchers are now capable of studying a CNS autoimmune disorder in which the autoantigen is known and may now be able to study early interactions that lead to the generation of the autoimmune response.

Distinguishing NMO from MS

Only in the past decade have we identified the pathological differences that distinguish NMO as its own separate disease. Similarly to MS, there is a female preponderance. Additionally, the rate of relapses in NMO patients is 84.4% greater in NMO patients than in relapsing-remitting MS. In 2006, Wingerchuk et al. proposed a diagnostic protocol for distinguishing NMO from MS (139).

The anatomical areas afflicted by MS are distinct of those afflicted by NMO . As discussed earlier, MS patients show initial pathology predominantly within the brain parenchyma that is visualized traditionally by MRI with sites of acute inflammation identified on post contrast imagery(4). In contrast, NMO is characterized by lesions that are predominantly located in the optic nerve and spinal cord. NMO patients present with either optic neuritis and/or myelitis and with at least two of the three NMO specific criteria. 1) Contiguous spinal cord MRI lesions extending greater than three vertebral segments 2) A brain MRI that does not meet any diagnostic criteria for MS (140) 3) Presence of the NMO-IgG in the serum of patient.

Additionally, genetic susceptibility, such as haplotype association, appears to vary between the two autoimmune diseases. Specifically, while MS shows a positive association with the *HLA-DRB1*1501* allele (17, 22, 23), small cohort studies of NMO patients show both a positive association with the *HLA-DRB1*0301* allele and a negative association with MS-associated *HLA-DRB1*1501* allele (141, 142). With

both alleles resulting in the generation of MHC II polyproteins, the variation in haplotype associations may very well result in targeting of different autoantigens which lead to the variations in pathology that is seen between the disorders.

The most prominent difference however is the presence of the NMO-IgG in the serum of NMO patients. Originally identified in about 70% of optico-spinal MS patients, the NMO-IgG antibody was found to bind to mouse CNS tissue (143). This disease specific IgG has been the focus of multiple studies that have helped to distinguish NMO from MS and brings to light the how various immune cells may play different roles in CNS autoimmunity that was not previously characterized in MS. Seropositivity for NMO-IgG has been demonstrated to predict future relapse and/or later conversion to clinically confirmed NMO in patients with limited forms of NMO such as isolated optic neuritis (ON) or longitudinally extensive transverse myelitis (LETM) (144-147). Furthermore, NMO-IgG titers correlated with NMO disease activity or with the severity of opticospinal involvement in several independent studies (144, 148-150). Although absolute antibody titers varied widely and a disease-triggering threshold value was not apparent, serial assessment performed in those studies showed that clinical attacks were often preceded by a continuous increase in antibody titers, while NMO-IgG serum concentrations declined during recovery in individual patients and could be stably suppressed in response to various immunosuppressive treatments (148-150).

The presence of the NMO-IgG, and its clear association with disease progression and pathology, has allowed for the identification of the CNS autoantigen of NMO, while the autoantigen for MS is still unknown. In 2005, Lennon et al. determined that the NMO IgG antigen is present in CNS and non-CNS tissues and co-localizes with aquaporin 4 (AQP4) (151). AQP4 is a water channel found within tissues throughout the body with two primary isoforms, the M1 and the M23 (152, 153). Utilizing $AQP4^{-/-}$ mice, Verkman et al demonstrated that AQP4 maintains the concentration of water in a various tissues, as well as maintaining a proper concentration of ions in the extracellular matrix in the CNS (154). Despite the wide variation in binding characteristics, some antibodies were shown to bind with higher affinity to the shorter M23 isoform than to full-length M1-AQP4 (Figure 1.2) (155-157). Human NMO lesions display a marked loss of astrocytic AQP4 immunoreactivity combined with prominent deposits of IgG, IgM, and, as a sign of complement activation, the terminal membrane attack complex surrounding thickened blood vessels at sites of AQP4 loss (158-161). As a key feature, the astrocytic marker glial fibrillary acidic protein (GFAP) is lost in parallel with AQP4 lesions in many lesions, indicating astroglial loss, whereas myelin and neuronal axons are often preserved unlike that which is typically seen in MS lesions (159, 162). These findings are consistent with the assumption that the autoimmune response in NMO primarily attacks astrocytes and is initiated by autoantibody-mediated loss of AQP4.

Treatment of acute exacerbations involves high dose steroids and plasma exchange. However, it is currently unknown whether these interventions alter the natural course of the disease. Interestingly, approved immunomodulatory drugs for treatment of MS have been shown to have no benefit in patients with NMO, and may actually be harmful, including interferon beta (163, 164) and natalizumab (165). Instead, immunosuppressive regimens have been shown to be helpful. Currently accepted treatment regimens for NMO in the US include azathioprine plus prednisone, mycophenolate +/- prednisone, and the anti-CD20 monoclonal antibody, rituximab (166).

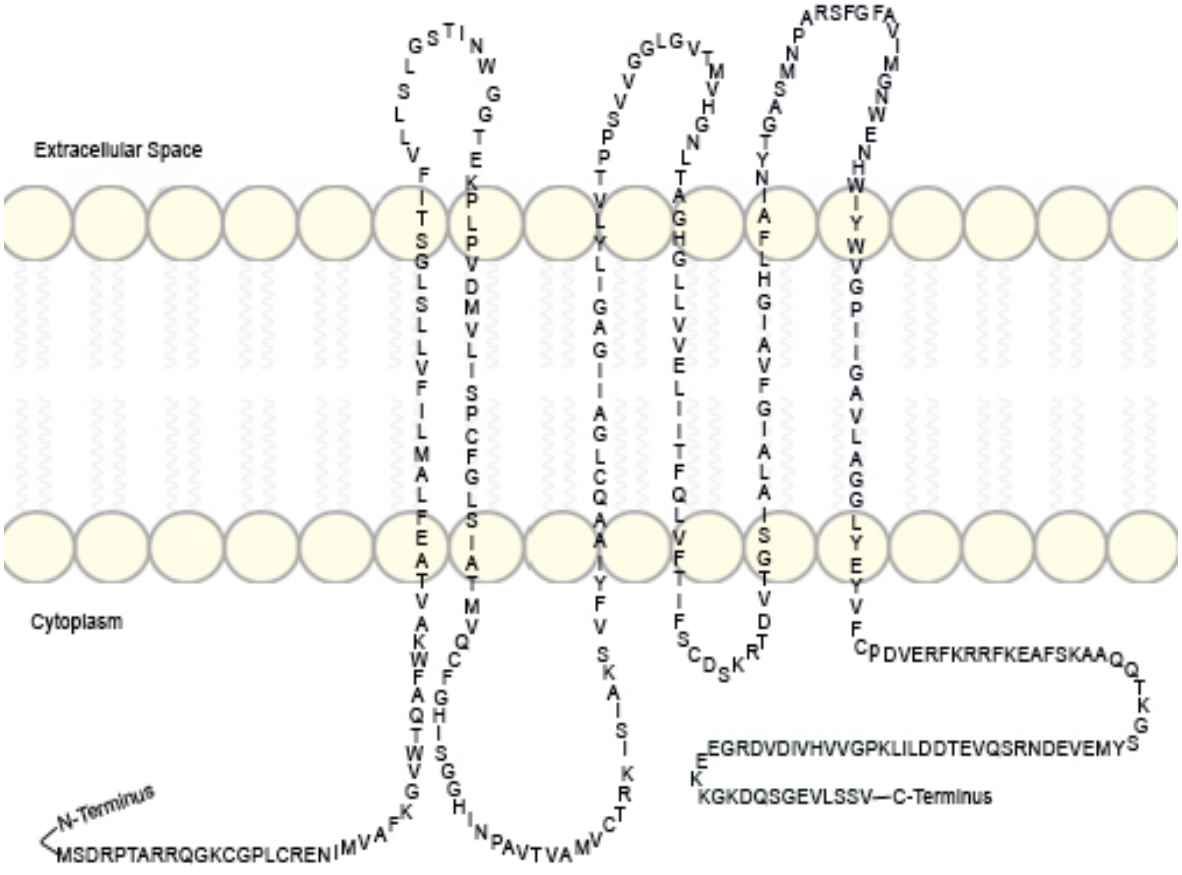

Figure 1.2 – The M1 Isoform of Human Aquaporin 4

AQP4 consists of six transmembrane α-helices. There are two isoforms of AQP4, M1 and M23, which differ in their n□terminus start site.

Pathology of NMO Driven by the NMO-IgG

If we further examine the role of the NMO-IgG in the progression of the disease, it becomes clear that the neuropathology associated with NMO is a result of the autoantibody. Understanding the role of astrocytes, their expression of AQP4, and how NMO-IgG drives pathology may lead to greater insights into therapeutic options.

Astrocytes are glial cells that make up a large portion of the CNS. There are two major subtypes of astrocytes, 1) protoplasmic astrocyte morphology exhibits multiple stem branches in a uniform distribution and is located in the grey matter of the CNS and 2) fibrous astrocyte morphology consists of long fiber-like processes found in the white matter. *In vitro* studies utilize Glial fibrillary acid protein (GFAP) as a detection marker for astrocytes. It is important to note that GFAP cannot be used as a constitutive marker for astrocytes due to the concentration of GFAP fluctuates in astrocytes with CNS health. Low expression levels of GFAP are associated with healthy CNS tissue while increased expression of GFAP in astrocytes is associated with astrogliosis, an abnormal increase in astrocyte cell counts due to neuronal trauma and is a precursor for scar formation (167).

Astrocytes are critical for the regulation of the cerebrovasculature to maintain the neuronal tissue. These glial cells support neuronal tissue through interacting with CNS blood vessels to transport energy substrates and neurotransmitter precursor proteins to neurons while also interacting with the synaptic space to maintain the extracellular matrix (168). At the synapse sites of neurons, astrocytes play a critical

role in the reuptake of neurotransmitters from the synaptic cleft (169). This prevents the accumulation of neurotransmitters such as glutamate that can cause excessive stimulation of the neuron, and ultimately in excitotoxicity (170, 171). The close proximity of the foot processes to the node of ranvier, the gaps between myelin sheaths, allows for the spatial buffering of excess potassium (K^+) surrounding the axons so that action potential homeostasis can occur (172) (173). The gap junctions between neighboring astrocytes also allow for the redistribution of these ions from areas of high [K^+] to low [K^+] (174).

All the while, astrocytes additionally supply the energy utilized by neuronal tissue. The astrocytic interactions between the blood vessels in the CNS lead to the release of prostaglandin E (PGE), arachidonic acids (AA), and nitric oxide (NO) (175). The secretion of these factors affects the vasodilatation of blood vessels and allows for the uptake of water and glucose into the CNS that is then transported to neurons in response to their metabolic demands. During times of high axonal action potential, glycogen is degraded into lactate and released by astrocytes to offer additional support to maintain the action potential occurring within the axons (176). Astrocytes utilize AQP4 to maintain their morphology and the concentration of water in the cerebral extracellular matrix(166, 177). It is clear that targeting AQP4 on the astrocytic foot processes in the CNS would prove detrimental to the survival of neuronal tissue due to their role in neurons acquiring metabolic substrates to generate energy and maintaining the extracellular space of the CNS.

There are two isotypes of AQP4 expressed on the astrocytic foot processes: the longer isoform M1 and the shorter M23 isoform (178). These proteins form tetramer structures, with the M23 isoform generating large orthogonal arrays of particles (OAP) with other M23-AQP4 tetramers (179). The M1 isoform tends to form individual tetramers and occasionally forms heteromers with the M23 isoform. It is the AQP4-M23 OAP that the NMO-IgG recognizes and binds to on transfected cells (180). Disruption of the AQP4 OAP assembly blocks binding of the NMO-IgG and delays NMO pathology (181).

There are two mechanisms by which NMO-IgG drives the destruction of astrocytes in an AQP4 dependent manner: antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytoxicity (CDC). Complement activation in response to NMO-IgG binding to astrocytic AQP4 and subsequent cell lysis likely constitutes the principal pathogenic effector mechanism, as demonstrated in both *ex vivo* and *in vivo* experimental settings. The NMO-IgG are comprised mostly of the IgG1 isotype (182-184) and has been shown to induce titer-dependent death of AQP4-transfected cell lines in vitro via CDC pathway (185-188). Also, levels of the anaphylatoxin C5a are elevated in the CSF of NMO-IgG-seropositive patients between attacks (189), which also supports our hypothesis of CDC driven destruction of the CNS . Despite this data, ADCC may potentially play an additive role in the antibodies pathogenesis seeing how lesion severity is enhanced by adding eosinophils, neutrophils, macrophages, or various soluble proinflammatory

cytokines to *in vitro* cell cultures examining the killing capabilities of the antibody against AQP4-expressing cells (190, 191). The most compelling piece of evidence, as demonstrated by an animal study, is that the intracerebral administration of IgG from human NMO sera together with human complement promotes a marked loss of AQP4 as the initial event, whereas astrocyte death, demyelination, and neuronal necrosis does not occur until later in NMO lesion evolution (192). In addition to macrophages/microglia and T cells populations typically seen in EAE lesions, the inflammatory infiltrate in this model comprises of numerous eosinophils and neutrophils, often in conjunction with signs of degranulation (158, 193). The intracerebral or intraperitoneal injection of neutrophil protease inhibitors effectively counteracted neuroinflammation and neural tissue damage in this mouse model of NMO (194), suggesting that neutrophils also act together with eosinophils as direct amplifiers of autoimmune tissue damage during NMO lesion development via ADCC.

While both pathways are capable of causing astrocytic destruction in a NMO-IgG dependent manner, it is apparent that complement localization to the CNS restricts NMO pathology. This is most apparent by animal models that perform intracranial injections of NMO-IgG to generate NMO pathology in rodent models (192, 195, 196). Without coinjections of human compliment, no disease phenotype occurs. In NMO pathology, astrocyte lysis occurs despite astrocytes expressing CD59, a glycophosphoinositol (GPI)- anchored membrane protein that inhibits terminal C5b-C9 complement to attach to the cell membrane (197). CD59 knockout

mice show an increased susceptibility to NMO-IgG induced transverse myelitis, generating extensive lesions similar to what is seen in NMO patients (198). It was once thought that complement canot enter the CNS due to the BBB, and that instead complement is synthesized within the CNS in response to injury(199, 200). Peripheral nerve damage leads to spinal cord microglia to upregulate the expression complement genes such as the C5 terminal complement gene (201). Once activated microglia begin secreting the initial complement proteins in the spinal cord, neighboring astrocytes may aid in driving NMO-IgG dependent CDC via the secretion the terminal complement components C6-C9 (202). And while C5b leads to CDC, the increased levels of C5a found in the CSF during exacerbation may recruit and activate infiltrating leukocytes, thus furthering pathology (203, 204). This accumulation of data suggests that NMO-IgG driven CDC begins a pathogenic cascade leading to further astrocyte destruction via ADCC.

Despite understanding how NMO-IgG drives astrocyte destruction, it is unclear why NMO pathology is localized to the spinal cord (SC) and optic nerve (ON) while leaving the brain intact. This is perplexing because astrocytes are present throughout grey and white matter within the CNS. In rare cases of NMO, NMO-IgG seropositive patients with abnormal brain scans typically show lesions in the regions of the brain with high AQP4 expression (205). AQP4 OAP are highly expressed in the spinal pial mater and ependymal surfaces that interact with the cerebrospinal fluid (206). Roemer et al. explains that while AQP4 is present

throughout the CNS, AQP4 immunoreactivity was limited in the white matter of the brain, but showed high reactivity throughout the white and grey matter in the spinal cord, with staining of the optic nerve exhibiting a similar pattern (160). Due to the similar staining for AQP4 between the SC and ON I speculate that the density of AQP4 may be driving the tissue specific pathology once NMO-IgG and complement is present within the CNS. Whether or not complement quantities and activation are differentially present in different anatomical areas of the CNS is currently a topic of speculation.

Generation of the NMO-IgG – A Critical Role for the Cellular Immune Response

Comprehending disease-specific mechanisms that drive the generation and secretion of the pathogenic autoantibody is clearly important. With the evidence that these autoreactive antibodies may not only serve as a diagnostic marker for NMO, but also act in a direct pathogenic manner, NMO is clearly a B cell-mediated disease. Given the current understandings of the requirements for antibody secretion by B cells, the AQP4-specific T-B cell interaction appears crucial to disease pathogenesis (Figure1.3). Understanding the necessary events that must occur to drive the activation and survival of these autoreactive cells is important in identifying targets for NMO specific therapies.

Like DCs and macrophages, B cells are professional APCs as defined by a constitutive expression of MHC class II. This is important seeing as the MHC II

coding *HLA-DRB1*03:01* allele has been positively associated with NMO while the MS associated *HLA-DRB1*15:01* allele shows a negative association in patients (141, 207, 208). In contrast to any other professional APC, B cells express an antigen-specific B cell receptor that enables them to recognize, bind, and internalize their antigen in a very efficient manner. After this initial step of specific antigen-recognition, B cells can then present the processed antigen in the context of MHC class II. As a consequence of these unique cellular features, antigen-specific B cells are very competent in the presentation of an antigen when their B cell receptor recognizes the same antigen as the responding T cell (209-211). Thus, besides constituting the source for the potentially pathogenic humoral response, B cells, and in particular AQP4-specific B cells, may serve as potent APCs for activation of AQP4 specific T cells. Recent findings suggest this B cell - T cell interplay may also occur in the target organ of the disease, namely the CNS. In the CSF of patients with NMO, B cells display signs of somatic B cell hypermutation, generally indicative of B cell antigen recognition within the target organ. Additionally, B cell recruiting and activating factors, such as BAFF or CXCL13 were found to be increased in the CSF of NMO patients (212-214). Also, CSF samples of NMO patients contain significantly higher amounts of the B cell survival cytokines, IL-6 and IL-17 (215, 216). Recent case reports using an antibody neutralizing the effect of IL-6 indicate that this cytokine could be indeed the key factor for progression of NMO. Administration of IL-6 receptor blocking tocilizumab was shown to be clinically beneficial in treatment-

resistant NMO patients (217, 218). This finding does suggest that IL-6 and the cells that produce it are involved in the pathogenesis of disease. Given that IL-6 together with TGF-beta is the central cytokine for promoting the development of Th17 cells are the predominantly known source of IL-17 during inflammation, these findings highlight that pro-inflammatory T cell (re-) activation continues in the CNS of NMO patients. Although the cellular source of Th17-polarizing IL-6 remains generally elusive, activated B cells produce substantial amounts of this pro-inflammatory cytokine and are therefore a promising candidate for its cellular source.

There is additional evidence from experimental and clinical investigations that supports the idea that a T cell response against AQP4 is required for the development of an AQP4 antibody. To some extent this is not entirely surprising, as IgG isotype switching, somatic hypermutation, and thus the maturation of a robust humoral response requires T helper function, which can be accomplished by Th17 cells (219). $CD3^+$ T cells are abundantly present in NMO lesions (158), predominantly of Th17 phenotype (216, 220-222). Th17 cells appear to facilitate the initiation and perpetuation of CNS autoimmune disease (97) and mediate proinflammatory and allergic responses via the secretion of IL-17, which is the critical cytokine needed to localize neutrophils to the sites of infection (223). Multiple studies have shown that activation of T cells from NMO patients leads to the secretion of Th17 associated cytokines such as IL-6, IL-21, and IL-17 (224, 225). In the setting of NMO, the increased levels of IL-6 found in the CSF of NMO patients

may also allow for the survival of AQP4 specific Th17 cells, while at the same time inhibiting $FOXP3^+$ T regulatory cells (226-228). IL-6 has also been shown to be important in the survival and secretion of NMO-IgG in plasmablasts isolated from NMO patients (229). The secretion of IL-17 may also play an additive role to the inflammatory environment of the lesion by localizing eosinophils and neutrophils in the CNS (223, 230).

With a clear role for AQP4 specific Th17 cells in disease pathogenesis, the identification of immunodominant linear determinants of human AQP4 would thus have important implications for understanding the etiology of NMO. Early mouse studies performed in C57BL/6 or SJL/J mice have shown that AQP4 is capable of stimulating a cellular immune response in mice (220, 231). Both mouse strains identified immunogenic peptides capable of leading to the proliferation of T cells *in vitro* that produced IL-2, IFNγ, GM-CSF, IL-10, and IL-17a (220, 231). Though this data in C57BL/6 and SJL/J mice identified AQP4 linear determinants that stimulate a cellular immune response in H-2, the C57BL/6 genetic background does not express H-2-IE, the equivalent gene of the human class II MHC molecule HLA-DR. Studies have shown that blocking of HLA-DR is capable of inhibiting the proliferation of T cells isolated from NMO patients that are AQP4 specific suggesting a crucial role for the MHC II molecule in the activation of AQP4 specific T cells (221). The identification of immunodominant linear determinants of human AQP4 may have important implications for understanding the etiology of NMO, and monitoring

disease activity in patients afflicted with this disorder. HLA-DRB1*03:01 restricted activation of $CD4^+$ T cells via dominant determinants of AQP4 may lead to cross-activation of B cells and NMO Ig isotype switching.

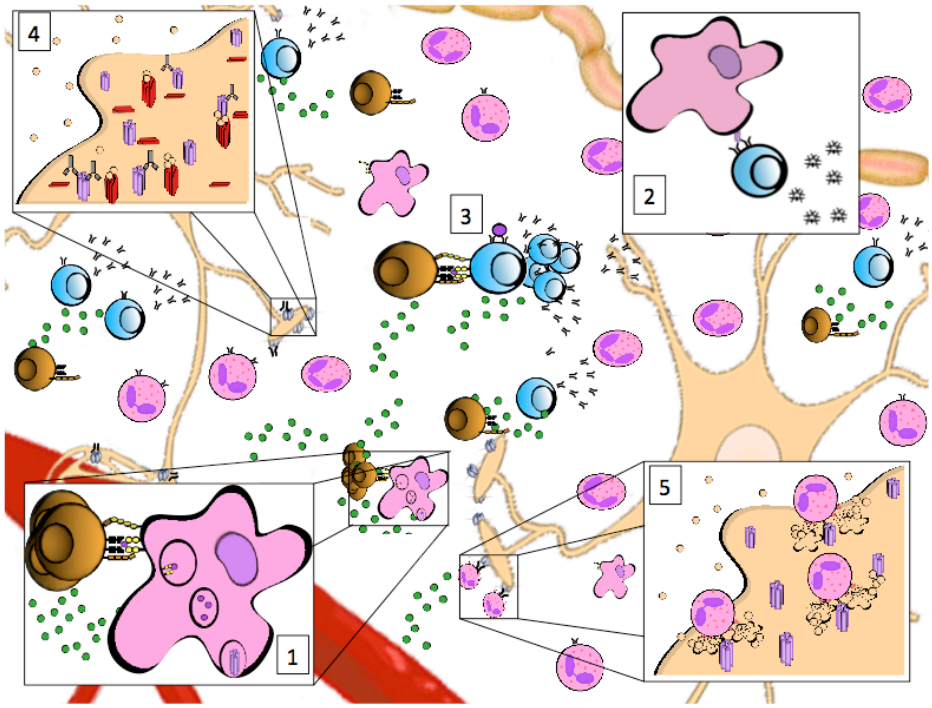

Figure 1.3 – A Pathogenic Model of NMO

With AQP4 established as being one autoantigen of NMO, it is likely that five events lead to the pathology seen in patients. 1) Activation or reactivation of AQP4 specific autoreactive $CD4^+$ T cells. 2) Activation of AQP4 specific B cells in the periphery. 3) Reactivation of AQP4 specific B cells by AQP4 specific $CD4^+$ T cells resulting in the secretion of anti-AQP4, IgG antibodies. As a result of these antibodies being present within the CNS, the destruction of astrocytes can occur via 4) complement-dependent cytotoxicity (CDC) or 5) antibody dependent cell mediated cytotoxicity (ADCC) targeting AQP4 on the astrocytic foot processes of these cells.

Project Summary

While the pathogenesis of NMO and MS have similarities, there are also significant differences. Most importantly, AQP4 has been identified as an autoantigen.

There are several lines of evidence that suggest that the cellular immune responses, in addition to humoral immune responses, may drive disease activity in a subset of patients: (1) Anti-AQP4-IgG is undetectable in a subset of patients with NMO, (2) in patients with anti-AQP4-IgG, antibody isotype switching from IgM to IgG could not occur without T cell involvement, (3) beneficial responses to B cell-specific therapies are not seen in all patients, and (4) treatments that target predominantly rapidly-dividing T cells benefit many patients. Also, all HLA haplotype analyses in small cohorts of NMO patients suggest a positive association with *HLA-DRB1*03:01*, a gene that codes for a MHC II molecule that presents linear antigens to $CD4^+$ T cells.

The role of cellular immune responses against human AQP4 (hAQP4) in NMO has not been determined. However, understanding the role of $CD4^+$ T cells in the inflammatory cascade of NMO is a pre-requisite to fully comprehend the pathological events that lead to the disease. I hypothesize that autoreactive T cells specific for linear hAQP4 determinants are present in NMO patients. I further hypothesize that the cellular immune responses against these linear determinants constitutes an important event in NMO immunopathogenesis: The generation of T cells specific for hAQP4. These cells likely play a critical role in the development of NMO through (1)

cytokine secretion; (2) formation of lymphoid follicles in the CNS; (3) attracting macrophages, neutrophils, and DCs to the CNS; (4) as well as cytotoxic T cell mediated cell apoptosis. Ultimately, studying cellular immune responses in NMO will likely lead to the development of novel, cell-specific, and potentially molecule-specific therapies. To test my hypotheses, I developed two specific aims.

Aim #1 – To identify the immunodominant linear hAQP4 determinants that generates a Th_1 and Th_{17} cellular immune response in *HLA-DRB1* 03:01* transgenic mice.

Utilizing humanized *HLA-DRB1*03:01* transgenic mice deficient in murine MHC II and on a B.10.Ab^{KO} background ($H2Ab^{-/-}$), and immunized with full length hAQP4, ELISpot assays will be performed to screen a set of overlapping, linear peptides, 20 amino acids in length, that span the entirety of hAQP4. These assays will quantify the Th1/Th17-like immune responses and identify the immunodominant peptide that stimulates the greatest cellular immune response against hAQP4.

Aim #2 – To identify encephalitogenic linear hAQP4 determinants that generate a Th_1 and Th_{17} cellular immune response in *HLA-DRB1* 03:01* transgenic mice.

By immunizing *HLA-DRB1*03:01* transgenic mice with immunogenic, linear determinants of hAQP4, an NMO animal model will be developed. The ideal phenotype will have neurological signs as well as immunological signs. The ideal animal model will have inflammation in the spinal cord and optic nerves, optic neuritis, paralysis, autoreactive T and B cells against AQP4, and the presence of an anti-AQP4 antibody found in the serum of the diseased animal.

Materials and Methods

Whole Protein AQP4 and AQP4 Peptides

Whole protein AQP4 M1 was donated to us from Dr. William Harries of the Membrane Protein Expression Center & Center for Structures of Membrane Proteins Macromolecular Structure Group (UCSF, San Francisco, CA) (Figure 3.1). The twenty-amino acid-long synthetic peptides that overlap by ten amino acids across the entirety of human AQP4 (Table 3.1), $hAQP4_{298\text{-}300}$, fifteen-amino acid-long synthetic peptides overlapping by a single amino acid spanning the immunodominant $AQP4_{298\text{-}301}$ (Table 3.2), $mAQP4_{281\text{-}300}$, and the $hAQP4_{284\text{-}299}$ immunogenic region-alanine-scanning peptides (Table 4.2) and were generated by JPT Innovative Peptide Solutions, Berlin Germany.

Mice

Generation of transgenic mice expressing *HLA-DRB1*03:01* has been described previously (232). Briefly, DRB1*0301 (DR17) transgenic mice were generated by co-injection of an HLA DRα genomic fragment and a DRB1*03:01β gene fragment into (C57BL/6 • DBA/2) F_1X C57BL/6 embryos then backcross into the B10 mouse background. Once a B10.M-DRB1*03:01 was successfully backcrossed, the DR17 gene was then introduced into the class II-negative $H2Ab^0$ strain by mating the B10.M-DRBI*0301 line with the $B10.H2ab^{KO}$ line. All mice were bred and maintained

in a pathogen free mouse colony at the University of Texas Southwestern Medical Center according to the guidelines set forth by the National Institute of Health and institution. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UTSW.

Enzyme-linked Immunosorbent Spot Assay (ELIspot Assay)

The frequency of IFNγ, IL-17, and GM-CSF secreting $CD4^+$ T cells were determined by ELISpot assay. Groups of three male *HLA-DRB1*03:01* mice were inoculated in the inguinal and axillary regions with 100 μgs of whole-protein hAQP4 , $hAQP4_{281-300}$ or $mAQP4_{281-300}$ emulsified in complete Freund's adjuvant (CFA) containing 8mg/mL desiccated *M. Tuberculousis H37 RA* (DIFCO) in a 1:1 ratio. On day 10, lymph nodes and spleens were collected to generate single cell suspensions. Next, cells ($2.5\text{-}5.0x10^5$ cells/well) were incubated with a specified antigen (whole-length hAQP4 (50μgs/mL), hAQP4 overlapping peptides (50μgs/mL), $hAQP4_{281-300}$ (25 μgs/mL), $mAQP4_{281-300}$ (25 μgs/mL), $hAQP4_{281-300}$ overlapping peptides (25μgs/mL) $hAQP4_{66-79}$ (25μg/mL), a single hAQP4 alanine scanning peptide (25 μgs/mL), media only, or ConA (1μg/mL)) for 48 hours in 96 well ELISpot plates (Millipore MultiScreen 96-Well Plates). Capture and detection of cytokines were accomplished by using monoclonal antibodies (eBiosciences) specific for mouse IFNγ (Clone AN-18 (capture) and R4-6A2 (detection)), IL-17 (Clone eBio17CK15A5 (capture) and eBio17B7 (detection)), or GM-CSF (Clone MP1-22E9 (capture) and MP1-2231G6 (detection)). Spots were counted with an automated ELISpot plate reader

(Bioreader 5000, Biosys, Karben/Germany). All experiments were performed in triplicates and repeated at least once.

Generation of NMO Recombinant Antibody and Quantitative Immunofluorescence Microscopy

Recombinant monoclonal anti-hAQP4 antibody (NMO-rAb) and isotype control were generated from clonally-expanded plasmablasts recovered from the CSF of a seropositive NMO patient as described previously (233).

U87MG cells stably transfected with M23 hAQP4 were grown on coverslips and fixed with 4% paraformaldehyde for 15 minutes and then rinsed with 1X phosphate-buffered saline (PBS). Coverslips were subsequently blocked with 10% normal goat serum and then incubated with recombinant antibody (rAb) (10 ug/ml) with or without T cell peptide (5 ug/ml; 40-fold molar excess) in 5% normal goat serum overnight at 4ºC. Coverslips were washed 5 times with 1X PBS and then incubated with rabbit polyclonal anti-AQP4 (Santa Cruz #sc-20812) (4ug/ml) in 2% goat serum/0.1% triton X100 for 1 hour at room temperature. Coverslips were subsequently washed, and then incubated with goat anti-human-AlexaFluor 488 (Invitrogen #A-11013) and goat anti-rabbit-AlexaFluor 594 (Vector #DI-1594) in 2% goat serum for 1.5 hour at room temperature in the dark. Samples were then washed, fixed, and mounted with mounting media containing DAPI (Vector #H-1500).

Images were obtained using an Olympus Ix81 spinning disc confocal microscope, and the amount of red and green fluorescence was quantified using

Image J software. The ratio of green to red (G/R) fluorescence was measured in multiple independent fields, and the binding percentage was subsequently calculated by comparing the G/R ratio in the presence and absence of AQP4 peptide.

Experimental Autoimmune Encephalomyelitis (EAE) and AQP4 animal model

To induce active EAE, *HLA-DRB1*03:01* were immunized subcutaneously with CNS antigens emulsified in an equal volume of complete Freund Adjuvant (CFA) containing 8mg/mL H37Ra *M. Tuberculosis* (DIFICO) in each flank. Mice were eight to twelve-weeks of age. At the time of immunization and subsequently 48 hours later, mice received an intraperitoneal (IP) injection of pertussis toxin (Ptx) (200ng in 200μL PBS).

For the induction of disease by adoptive transfer, cells isolated from the lymph nodes (LN) of *HLA-DRB1*03:01* mice immunized with $hAQP4_{281-300}$, were removed and single-cell suspensions were prepared. The cells were cultured in RPMI 1640, supplemented with 5 x 10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum (HyClone, Logan, UT) and stimulated with 25 μgs/ml $hAQP4_{281-300}$ and 0.5ng/ml IL 12 in a 24 well plate for 72hrs. $5x10^6$ Cells per 200uls PBS were washed with PBS and injected IP into naïve *HLA-DRB1*03:01* mice; two independent experiments were conducted with a minimum of 3 mice per group.

For all experiments, individual animals were observed daily based on the EAE clinical scoring system as follows: 0 = no clinical disease, 1 = loss of tail tone, 2 = mild paraparesis, 3 = paraplegia, 4 = hindlimb and forelimb paralysis, 5 = moribund or death.

Histology

Following fixation in 10% buffered formalin, axially-sectioned spinal cord, and longitudinally-oriented optic nerves were processed and embedded in paraffin blocks. 4 µm sections were cut, mounted on Fisher Brand Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA), and stained with hematoxylin & eosin (Fisher Scientific, Pittsburgh, PA).

For the Luxol Fast Blue stained-sections, 6 µm thick sections of paraffin-embedded tissue were cut on a rotary microtome and mounted on Fisher Brand Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were deparaffinized and hydrated to 95% alcohol (StatLab Medical Products, McKinney, TX). Following heating of the sections in 0.1% Luxol Fast Blue (Sigma-Aldrich, St. Louis, MO) at 60° C for at least one hour, excess stain was rinsed off in 95% alcohol. The sections were rinsed in distilled water and placed in 0.5% lithium carbonate (Sigma-Aldrich, St. Louis, MO) for approximately 5 seconds. They were then differentiated in 70% alcohol for 25 seconds and rinsed in distilled water. Next, the sections were evaluated under microscopy and depending on the adequacy of differentiation, they were subjected to an additional round of 10-25 seconds of 0.5%

lithium carbonate, a 70% alcohol rinse for approximately 25 seconds, then rinse with distilled water. The sections were then dehydrated and cleared with xylene (StatLab Medical Products, McKinney, TX) and coverslipped with Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI).

Murine Pupillometry

The puppillary reflex of experimental mice are measured using the pupillometry system by Neuroptics Inc. (San Clemente, CA) previously described . Briefly, infrared cameras capture digital images of mouse pupils in darkness at a baseline level after sedation. After the baseline pupil size is determined, an intensity-calibrated light source emits a stimulus into one or both of the eyes and a custom program measures the pupil diameter designed to analyze pupil size, onset latency, constriction velocity, and response amplitude. The light stimulus consists of a flash of light at 2, 32, or 125 μWs.

T Cell Proliferation Assay

Ten days post immunization of $hAQP4_{281-300}$ or $mAQP4_{281-300}$ of *HLA-DRB1*03:01* transgenic mice not given Ptx, single cell suspensions were generated by isolating the LNs of the immunized mice. Utilizing the CellTrace Violet Proliferation kit (Life Technologies, Carlsbad, CA), $CD4^+$ T cell proliferation against antigens was determined. Briefly, isolated $20x10^6$ LN cells were incubated at 37°C for twenty minutes with 5μM CellTrace Violet in PBS. After incubation, cells were washed with

RPMI media twice, then incubated in a 96-well-round bottom plate at $1x10^6$ cells per well with specified antigen for 96 hours. Post incubation, cells were washed with staining FACS buffer (4% Fetal Calf Serum (FCS) in PBS) two times, then the Fc receptors were blocked with anti-CD16/32 (BD Biosciences, Franklin Lakes, NJ,) for 15 minutes at 4°C before staining with mAbs for 30 minutes at 4°C; for cells stained with intracellular anti-FOXP3, an additional incubation of cells in Biolegend's Fix/Perm prior to staining with anti-FOXP3 occurred preceding extracellular staining was necessary. Cells were stained utilizing the following monoclonal antibodies: CD3-PE-C7 (eBiosciences, San Diego CA, Cat.#25-0031-82), CD4-APC (BD Biosciences Franklin Lakes, NJ, Cat#553051), FOXp3-PE (BioLegend San Diego, CA, Cat#320019). Cells were run through a LSRII flow cytometer (BD Biosciences) with 50,000 cells being collected per sample and analyzed using FlowJo software (Tree Star, Ashland, OR).

MHC II Binding Assay

This assay was adapted from a protocol found in *Busch et al* (Busch, 1990 #4443). Briefly, spleens isolated from naïve *HLA-DRB1*03:01* mice were used to generate single cell suspensions. Once cells were isolated, $1x10^6$ splenocytes (Spl) were incubated with either biotinylated $hAQP4_{281-300}$, $mAQP4_{281-300}$, or $hAQP4_{284-299}$ alanine scanning peptides at a concentration of 10μgs/mL for 4 hours at 37°C in a 96-well plate. Post incubation, cells were washed two times with FACS buffer and

stained for flow cytometry utilizing avdiin-FITC (Biolegend) applying the previously described protocol. Once cells were stained with avidin-FITC, they were run through a BD Calibur flow cytometer, and Flowjo was utilized to quantify the percentage of FITC positive cells. Alanine scanning peptides (Table 4.2) utilized in the MHC II binding assay were biotinylated utilizing an EZ link NHS-Peg4-Biotinylation kit (Thermo Scientific, Waltham, MA) following the manufacturer's specifications.

Serum Antibody Enzyme-Linked Immunosorbent Assay (ELISA)

96-well Immulon 2HB flat-bottom microtiters plates were coated with 4μg/mL PBS plus $hAQP4_{281\text{-}300}$ or whole protein hAQP4 overnight at 4 degrees C. The following day, plates were washed using PBS-0.5%Tween and residual binding sites were blocked by incubating 100μLs/well of 1% BSA PBS for 1 hour. Post additional wash, 100μLs of 100 fold-diluted serum samples were incubated in designated well for 1 hour. Washed again, biotinylated secondary antibodies (IgM, IgG2a, or IgG2b) were resuspended at 2μgs/5mLs 1% BSA PBS and incubated for 1 hour. Post wash, Avidin-peroxidase was added to each well for 30 minutes. Plates were then washed for a final time with PBS-.5% tween then with DI water. 100μLs of TMB was added to each well to develop the assay and 100μLs of 2M HCL was added to stop the reaction. Plates were read on an Emax precision microplate reader (Molecular Devices) at 450nm. Concentrations were calculated based on standards curve generated from control antibodies.

Basic Local Alignment Search Tool (BLAST)

I performed a protein sequence homology search utilizing the NCBI BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence homology for the $hAQP4_{281-300}$ (EDNRSQVETDDLILKPGVVH) sequence and the $hAQP4_{288-294}$ (ETDDLIL) sequence were searched through the non-redundant protein sequences database utilizing the blastp (protein-protein BLAST) algorithm to search homologous peptide sequences.

MHC II Binding Prediction

The MHCII binding predictions were made on 3/7/2014 using the IEDB analysis resource Consensus tool. (235, 236)

Statistical Analysis

For parametric tests, data were checked for normality by using the Kolmogorov–Smirnov test. Normally distributed values were compared using the unpaired two-sided Student *t*-test. Correlations between continuous and categorical variables were assessed using the Mann-Whitney U-test. All experiments were repeated at least twice. All statistical tests were 2-sided and $p < 0.05$ indicated significance. All analyses were performed with Prism 5 for Windows (Graphpad, La Jolla, CA, USA).

RESULTS OF SPECIFIC AIM #1

Specific Aim #1 – To identify the immunodominant linear hAQP4 determinants that generates a Th_1 and Th_{17} cellular immune response in *HLA-DRB1* 03:01* transgenic mice.

Scientific Rationale

The generation of activated $CD4^+$ T cells requires the presentation of antigens on the MHC II molecule. In immunizing the *HLA-DRB1*03:01* transgenic mice with whole protein hAQP4, I am utilizing the lysosomal compartments within the APCs of the mice to break down the whole protein and present them on the MHC II molecule based on affinity binding characteristics of each peptide. This presentation will lead to the activation of the cellular immune response hopefully in a Th_1 or Th_{17} dependent manner. With these cells, I will perform in vitro studies that will identify the dominant linear determinant that is capable of causing antigen-specific cells to secrete IFNγ and IL-17 in a *HLA-DRB1*03:01* dependent manner. For this specific aim, I will be screening thirty-two, 20-amino acid-long peptides that overlap by 10 amino acids and span the entirety of M1 isotype of hAQP4 in *HLA-DRB1*03:01* transgenic mice (Table 3.1).

Aquaporin 4 Overlapping, 20-Amino-Acid Long Peptides.					
Peptide Number	**Sequence**	**AQP4**	**Peptide Number**	**Sequence**	**AQP4**
1	MSDRPTARRWGKCGPLCTRE	1-20	17	LVELIITFQLVFTIFASCDS	161-180
2	GKCGPLCTRENIMVAFKGVW	11-30	18	VFTIFASCDSKRTDVTGSIA	171-190
3	NIMVAFKGVWTQAFWKAVTA	21-40	19	KRTDVTGSIALAIGFSVAIG	181-200
4	TQAFWKAVTAEFLAMLIFVL	31-50	20	LAIGFSVAIGHLFAINYTGA	191-210
5	EFLAMLIFVLLSLGSTINWG	41-60	21	HLFAINYTGASMNPARSFGP	201-220
6	LSLGSTINWGGTEKPLPVDM	51-70	22	SMNPARSFGPAVIMGNWENH	211-230
7	GTEKPLPVDMVLISLCFGLS	61-80	23	AVIMGNWENHWIYWVGPIIG	221-240
8	VLISLCFGLSIATMVQCFGH	71-90	24	WIYWVGPIIGAVLAGGLYEY	231-250
9	IATMVQCFGHISGGHINPAV	81-100	25	AVLAGGLYEYVFCPDVEFKR	241-260
10	ISGGHINPAVTVAMVCTRKI	91-110	26	VFCPDVEFKRRFKEAFSKAA	251-270
11	TVAMVCTRKISIAKSVFYIA	101-120	27	RFKEAFSKAAQQTKGSYMEV	261-280
12	SIAKSVFYIAAQCLGAIIGA	111-130	28	QQTKGSYMEVEDNRSQVETD	271-290
13	AQCLGAIIGAGILYLVTPPS	121-140	29	EDNRSQVETDDLILKPGVVH	281-300
14	GILYLVTPPSVVGGLGVTMV	131-150	30	DLILKPGVVHVIDVDRGEEK	291-310
15	VVGGLGVTMVHGNLTAGHGL	141-160	31	VIDVDRGEEKKGKDQSGEVL	301-320
16	HGNLTAGHGLLVELIITFQL	151-170	32	VDRGEEKKGKDQSGEVLSSV	304-323

Table 3.1 – Human Aquaporin 4 Overlapping Peptides

Overlapping peptides of human AQP4 are twenty amino acids in length, overlapped every 10 amino acids, and spanned the entire sequence of the human AQP4 M1 isotype.

Results

*hAQP4$_{281-300}$ is the immunodominant linear determinant of hAQP4 in the context of HLA-DRB1*03:01*

ELISPot assays is an established and sensitive method for examining antigen-specific, cytokine secretion in low cell frequencies. Because the T cell repertoire is broad, and the frequency of AQP4 specific T cells are likely only in the range of 1 in $1x10^5$ to $1x10^6$ cells, I utilized ELISpot assay to quantify IFNγ and IL-17 secreting AQP4-reactive cells. Specifically, ELISpot assays were used to characterize the responses of lymph node (LN) cells and splenocytes (Spl) of *HLA-DRB1*03:01* mice immunized with whole protein hAQP4. Immunization with whole protein hAQP4 for the antigen were endocytosed and digested by antigen presenting cells (APCs), including myeloid cells and B cells, allowing for the cleaving and presentation of hAQP4 peptides fragments. These linear determinants of hAQP4 are then bound within the antigen-binding groove of MHC class II within the endosomes, and transported to the cell surface, where interactions with hAQP4-specific TCRs resulting in the activation and proliferation of $CD4^+$ T cells (237). My IFNγ and IL-17 ELISpot assays identified hAQP4$_{281-300}$ (peptide #29) as the immunodominant linear determinant in LN cells and Spl (Figure #3.1). Th_{17} immune responses by splenocytes against hAQP4$_{281-300}$ were not significantly different to those against whole-length hAQP4 (Figure 3.1B). None of the overlapping hAQP4

peptides, nor whole-length hAQP4 induced a IL-5-driven Th2 response (data not shown).

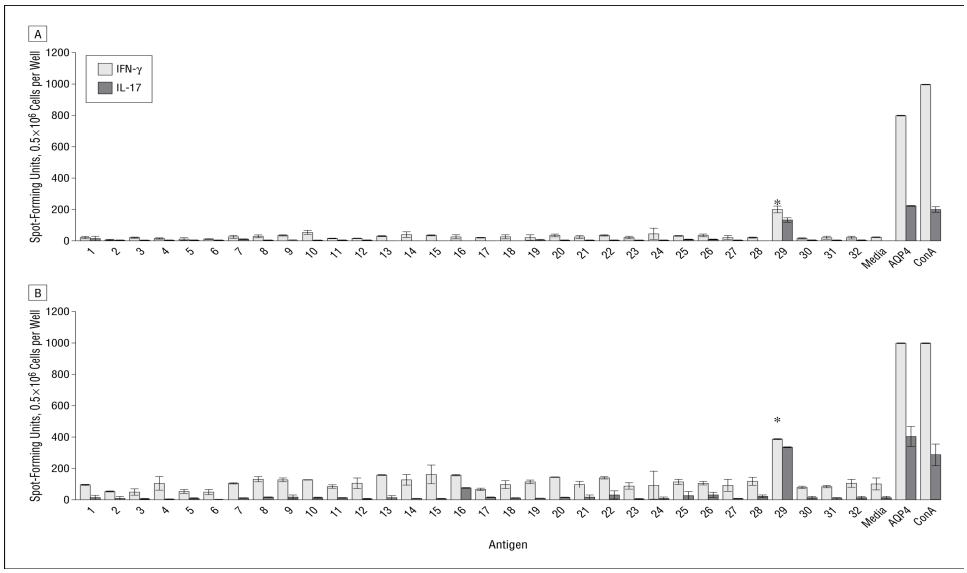

Figure 3.1 – AQP4$_{281-300}$ is the immunodominant, linear determinant of AQP4

HLA-DRB1*03:01 **transgenic mice were immunized with a full-length hAQP4-CFA emulsion resulting in each mouse recieving100μgs of antigen. On day 10, lymph nodes (A) and spleens (B) cells were collected from mice to generate single cell suspensions. Thereafter, cells (0.5x10^6 cells) were incubate for 48 hours in anti-**IFNγ **or anti-IL-17 coated 96-well ELISpot plates with single overlapping hAQP4 peptides (50μg/mL) (Table 1.), media only, full length hAQP4 (50μg/mL), or ConA (1μg/mL). Spot forming units (SFUs) represent the absolute number of cells that are secreting a specific cytokine in response to antigen in the well (*=P-Value<0.01).**

$hAQP4_{284-299}$ is the dominant immunogenic regions within $hAQP4_{281-300}$

Due to the biophysical properties of linear peptides, they are ideally 12-15 AA in length when bound in the antigen-binding groove of the MHC class II molecule and presented to $CD4^+$ T cells (238). In identifying the 12-15 amino acid immunogenic region of $hAQP4_{281-300,}$ I would be able to perform sequence homology studies that could identify potential pathogens that could drive the generation of these autoantigen-specific cells in NMO patients.

Thus, the immunodominant determinants within $hAQP4_{281-300}$ were identified by performing IFNγ and IL-17 ELISpot assays with 15mer peptides spanning the entire sequence of $hAQP4_{281-300}$ (Table 3.2). In LN cells and splenocytes from *HLA-DRB1*03:01* mice immunized with $AQP4_{281-300,}$ $AQP4_{284-298}$ induced a significantly higher Th17 response than other 15mers (Figure 3.2). $AQP4_{284-298}$ also induced the strongest GM-CSF-driven T helper response in splenocytes significant from other 15mers (data not shown). The secretion of GM-CSF by Th_1 and Th_{17} cells has been shown to be critical to drive EAE pathology through activating monocytes, macrophages, and DCs against myelin (59, 101, 102). Due to the insufficient number of lymph nodes cells, GM-CSF ELISpots could not be performed. $AQP4_{285-298}$ resulted in a significantly higher Th1 response than other 15mers in lymph nodes cells (Figure 3.2B). There was also a trend towards higher IFNγ secretion in splenocytes after recall with $AQP4_{285-298}$.

$AQP4_{281-300}$ Overlapping Peptides		
Number	**AA**	**Sequence**
1	280-294	VEDNRSQVETDDLIL
2	281-295	EDNRSQVETDDLILK
3	282-296	DNRSQVETDDLILKP
4	283-297	NRSQVETDDLILKPG
5	284-298	RSQVETDDLILKPGV
6	285-299	SQVETDDLILKPGVV
7	286-300	QVETDDLILKPGVVH
8	287-301	VETDDLILKPGVVHV

Table 3.2 – Aquaporin 4 $_{281-300}$ Overlapping Peptides

Overlapping peptides of human $AQP4_{281-300}$ that are fifteen amino acids in length and overlap one amino acid each.

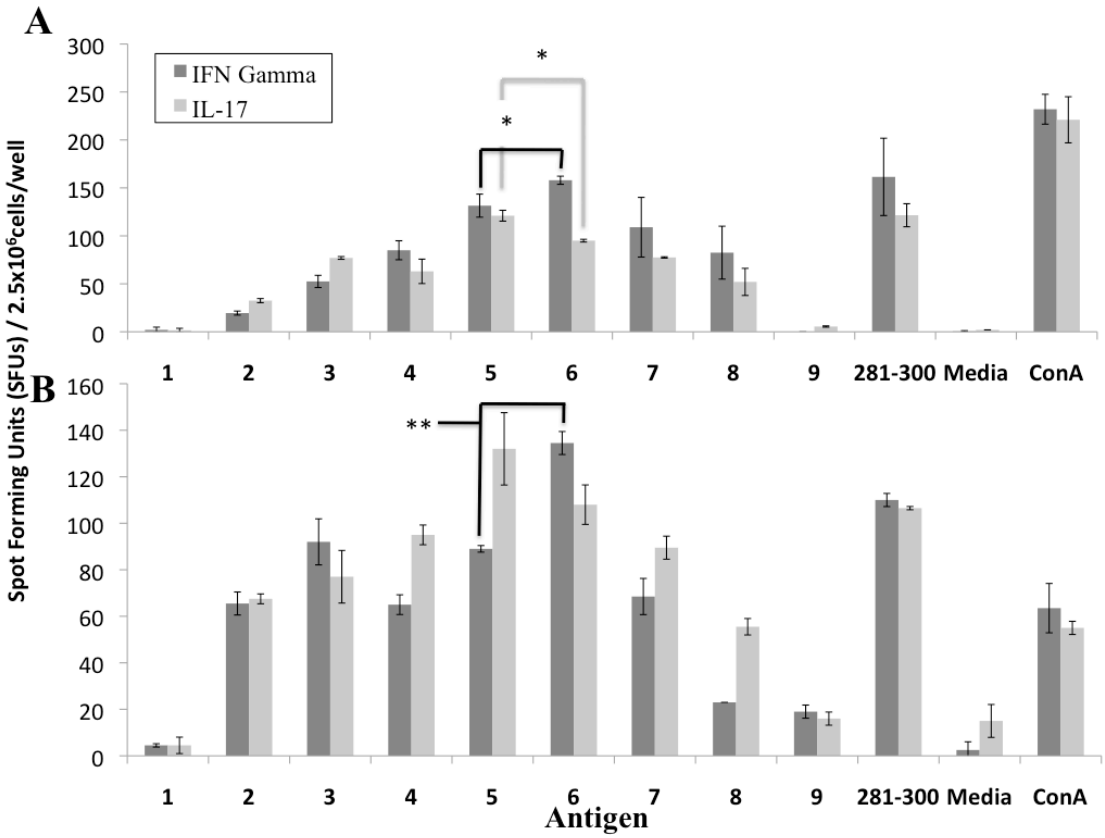

Figure 3.2 – $AQP4_{284-298}$ and $AQP4_{285-299}$ are the immunogenic regions of $AQP4_{281-300}$

HLA-DRB1*03:01 **transgenic mice were immunized with $AQP4_{281-300}$. On day 10, cells ($2.5x10^5$ cells) taken from the lymph nodes (A) and spleens (B) were incubate for 48 hours in anti-IFNγ or IL-17 coated ELISpot plates with a single overlapping peptide of AQP4 (50μg/mL) (Table 2.), $hAQP4_{281-300}$ (50μg/mL), media only, or ConA (1μg/mL). Spot forming units (SFUs) represent the absolute number of cells that are secreting a specific cytokine in response to antigen in the well (*=P-value <0.05 and **=P-value <0.01).**

hAQP4$_{281-300}$ does not inhibit the binding of NMO-recombinant antibodies to the surface of full-length hAQP4

With hAQP4$_{281-300}$ being the immunodominant peptide in *HLA-DRB1*03:01* transgenic mouse, it can be inferred that it may also be the target for certain AQP4-specific autoantibodies. In collaboration with Dr. Jeffrey Bennett lab at the University of Coloroda Denver, we subsequently examined whether the hAQP4$_{281-300}$ could inhibit the binding of NMO-recombinant antibodies (rAb) to surface AQP4 (Figure 3.3). Using a quantitative immunofluorescence-binding assay, we observed no significant inhibition of the binding of two NMO-rAbs in the presence of a 40-fold molar excess of hAQP4$_{281-300}$. This does not conclusively state that our peptide does not bind to NMO-antibodies, merely that the two rAbs generated from NMO patients are not specific for hAQP4$_{281-300}$. This immunogenic peptide still may play an important role in the generation of NMO-IgGs. B cells take up antigens and present antigens similar to most APCs, but are merely restricted by their BCR. Once whole protein AQP4 is taken up by a B cell, it is well within reason to think that hAQP4$_{281-300}$ could still be presented on the HLA-DRB1*03:01 MHC II molecule despite the specificity of that BCR for other regions present on whole protein AQP4.

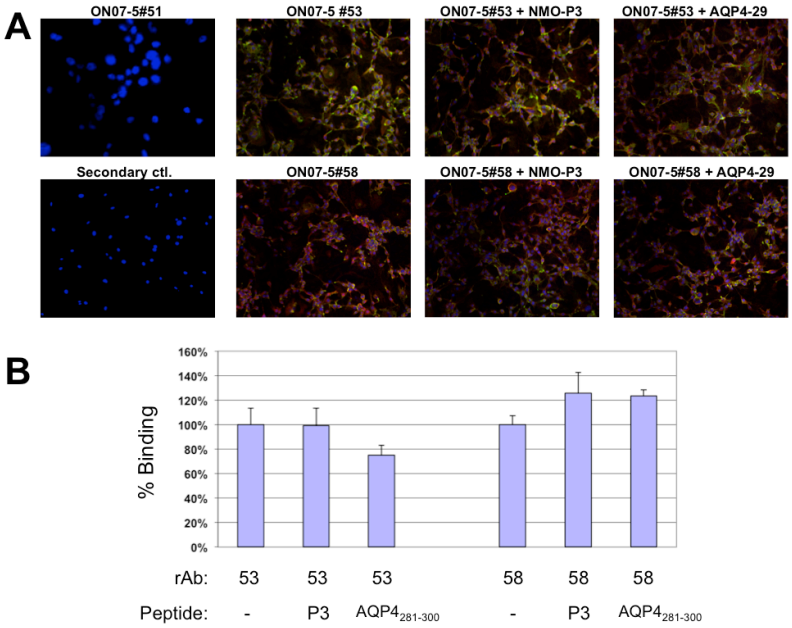

Figure 3.3 – Aquaporin $4_{281\text{-}300}$ ($AQP_{281\text{-}300}$) does not inhibit AQP4 antibody binding.

AQP4-specific and isotype control recombinant antibodies (rAbs) were incubated with a U87MG glial cell line stably transfected with the M23 isoform of human AQP4 in the presence or absence of AQP4 peptide. A, Fluorescence micrographs demonstrate robust binding of AQP4-specific rAbs 53 and 58 (green) to the transfected cell line. No staining was observed in the absence of rAb (secondary control) or when using an isotype control rAb (ON7-5 No. 51). A rabbit polyclonal anti-AQP4 antibody against the intracellular C-terminal portion of AQP4 was used as an internal control for AQP4 expression. B, Percentage binding of AQP4-specific rAbs 53 and 58 in the presence and absence of AQP4 peptides. NMO indicates neuromyelitis optica. Error bars indicate SE.

Summary of Results

The experiments I performed in this specific aim identified the immunodominant linear determinants of hAQP4 in the context of *HLA-DRB1*03:01*, as this HLA haplotype was recently associated with NMO in several patient cohorts (141, 207). Utilizing ELISpot assays, I determined the frequency of hAQP4-specific T cells and characterized their cytokine profiles to conclusively determine $hAQP4_{281\text{-}300}$ as the most immunogenic linear determinant within my *HLA-DRB1*03:01* transgenic mouse. The identification of $AQP4_{281\text{-}300}$ inferred that a cellular immune response against AQP4 is capable of occurring in the context of the disease associated haplotype and may play a critical role in NMO disease development and progression due to its ability to stimulate a pronounced Th_{17} immune response in the context of HLA-DRB1*03:01. Though my peptide was not capable of blocking the binding of recombinant NMO-IgG to AQP4 *in vitro,* it may still play a prominent role in the activation and survival of pathogenic B cells and lesion localizing innate cells such as eosinophils and neutrophils.

Results for Specific Aim 2

Specific Aim #2– To identify encephalitogenic linear hAQP4 determinants that generate a Th_1 and Th_{17} cellular immune response in *HLA-DRb1* 03:01* transgenic mice.

Scientific Rationale

The generation of an NMO animal model requires an encephalitogenic cellular and humoral immune response against AQP4 in the spinal cord and optic nerve of the animal model. In this study, I intended to establish a T cell-mediated animal model of NMO in the context of *HLA-DRB1*03:01* utilizing the dominant linear peptide of hAQP4. The cellular immune response will hopefully activate the immune system against the AQP4 present in the CNS tissue, leading to similar NMO associated pathology. The ideal animal model would have inflammation in the spinal cord and optic nerves, lateral or bilateral optic neuritis, as well as progressive ascending paralysis due to a pathological drop out of astrocytes in spinal cord. With the generation of AQP4 specific T cells in the mice, I will perform a number of tests to examine whether an NMO disease phenotype occurs: 1) Histological studies will be performed to examine whether inflammatory cells infiltrate the CNS and cause demyelination. 2) Murine pupillometry will characterize the puppillary reflex of these mice to test for the occurrence of optic neuritis. 3) Mice will be examined daily for any signs of paralysis commonly seen in CNS autoimmune models. For this aim, I

will specifically be focusing on the $hAQP_{281-300}$ immunogenic peptide discovered in specific aim #1.

Results

*Active and passive immunization with $hAQP4_{281-300}$ does not induce clinical disease in HLA-DRB1*03:01 transgenic mice.*

I first examined whether active immunization of *HLA-DRB1*03:01* transgenic (Tg) mice with different AQP4 antigens results in clinical disease. A multitude of experimental procedures and conditions were tested to examine the encephalitogenic potential of hAQP4 peptides using the transgenic mice. In my specific aim #1, I determined that $hAQP4_{281-300}$ generated the strongest Th_1 and Th_{17} immune response. Knowing this, I utilized active immunization of the transgenic mice with CFA emulsions containing either recombinant whole protein hAQP4, $hAQP4_{281-300}$, or murine (m)$AQP4_{281-300}$ as my initial method of generating an animal model utilizing. In addition to the active immunization, mice received intraperitoneal injections of pertussis toxin (Ptx) on the day of immunization and two days later due to Ptx being necessary for driving CNS autoimmunity in the EAE animal model (239). This resulted in no visible signs of any CNS pathology. I further explored alternative methods to generate a T cells mediated NMO model. Some CNS autoimmune disease animal models require weekly booster immunization to generate disease phenotypes (240), so I proceeded with my active immunization

protocol followed by booster immunization at day fourteen with QuilA-AQP4 peptide emulsions made with incomplete Fraud's adjuvant (IFA); day 14 was chosen due to this day typically being the peak of disease in the control EAE animal model generated with Proteolipid protein $(PLP)_{91\text{-}110}$ in the transgenic *HLA-DRB1*03:01* mice (241), It is thought that booster immunizations might increase the encephalitogenic potential of the AQP4 peptides, overcoming the regulatory immune response, and drive CNS autoimmunity. No clinical disease course was seen in these experimental groups. Alternatively, since the inflammatory immune response must target $mAQP4_{281\text{-}300}$, I immunized mice with both $hAQP4_{281—300}$ and $mAQP4_{281\text{-}300}$ based off the thought that the strong cellular immune response against the $hAQP4_{281\text{-}300}$ might activate more encephalitogenic T cells specific for $mAQP4_{281\text{-}300}$ due to bystander activation. Similarly to other experimental protocols, no clinical disease course was seen utilizing this protocol (Figure #4.1 A).

In the adoptive transfer EAE model myelin-reactive activated $CD4^+$ T cells are transferred into a naïve recipient. This model has some propensities that are very different from actively-induced EAE: (1) The potential effects of control adjuvant and pertussis toxin on the innate immune system are eliminated, and (2) donor T cells are less dependent on reactivation with antigen in the CNS (53). This model was specifically developed to test the role of antigen-specific donor T cells in EAE pathogenesis (83). Since Th_1 and Th_{17} cells have been shown in the EAE model to be capable of causing disease when passively transferred, I next examined whether $hAQP4_{281\text{-}300}$-specific T cells could cause disease via adoptive transfer. My previous

experiments showed that $hAQP4_{281-300}$ generates a strong Th1 and Th17 immune responses in *HLA-DRB1*03:01* Tg mice. I utilized the passive transfer EAE disease model with the hope that the presence of immunogenic AQP4 specific Th_1 cells would localize to the CNS and drive a disease phenotype. In my experiments, LN cells were taken from $hAQP4_{281-300}$ or $mAQP4_{281-300}$ actively immunized transgenic mice and restimulated *in vitro* for seventy-two hours with immunized peptide at 10 μgs/mL and pro-inflammatory cytokines (IL-12 or IL-23). Post incubation, ten million cells were injected via intraperitoneal (IP) injection into each recipient mouse and observed for clinical disease phenotypes. In certain experimental groups, recipient mice were also irradiated to inhibit endogenous regulatory cells from blocking the activation of the adoptively transferred cells. (Representative data from the adoptive transfer of $hAQP4_{281-300}$–specific or $mAQP4_{281-300}$–specific $CD4^+$ Th_1 cells into *HLA-DRB1*03:01* recipient mice are shown in Figure #4.1B) Similarly to the actively immunize mice, no disease phenotype was visualized using either of these procedures. These results indicate that reactivation of $hAQP4_{281-300}$–specific or $mAQP4_{281-300}$–specific $CD4^+$ Th_1 cells does not occur in the CNS of *HLA-DRB1*03:01* recipient mice.

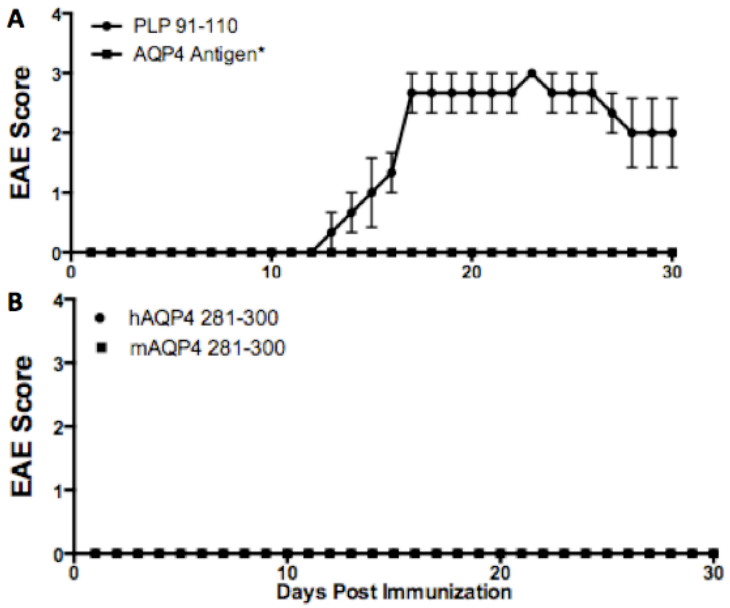

Figure 4.1 – HLA-DRB1*03:01 transgenic mice are disease resistant to active or passive EAE induction with human aquaporin 4.

(A) *HLA-DRB1*03:01* mice were actively immunized with proteolipid protein $(PLP)_{91\text{-}110}$ (100 μg/100μl/mouse; positive control), or varying AQP4 antigens*(whole protein hAQP4, $hAQP4_{281\text{-}300}$, $mAQP4_{281\text{-}300}$, $hAQP4_{281\text{-}300}$ with a Quil-A IFA booster on day 14 post-immunization, $mAQP4_{281\text{-}300}$ with a Quil-A IFA booster on day 14 post immunization, and $hAQP4_{281\text{-}300}$ plus $mAQP4_{281\text{-}300}$) emulsified in CFA. (B) Lymph node cells taken from *HLA-DRB1*03:01* mice immunized with $hAQP4_{281\text{-}300}$ or $mAQP4_{281\text{-}300}$ were restimulated for three days and passively transferred into *HLA-DRB1*03:01* mice. No disease phenotype was evident with any experimental paradigm.

To examine whether leukocytes infiltrated the CNS, histological studies were performed in mice that were actively immunized to examine whether infiltration of inflammatory cells or demyelination within the brain or spinal cord had occurred (Figure#4.2). Neither infiltration nor demyelination was seen by hematoxylin and eosin (H&E) and Luxol fast blue (LFB) staining, respectively. Examination of the optic nerve showed no inflammation or demyelination either (data not shown).

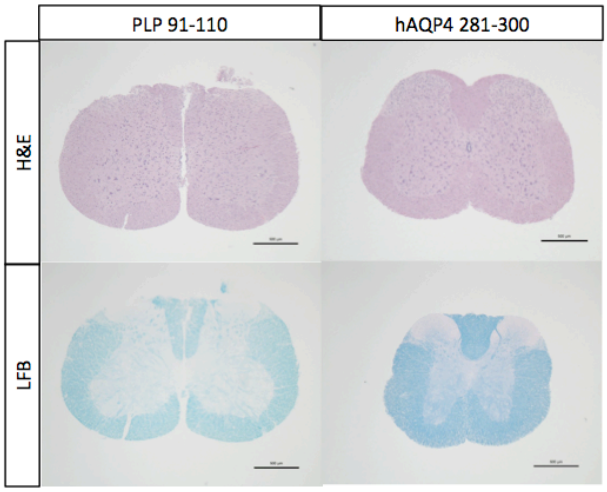

Figure 4.2 – Histology of spinal cord showed no infiltration or demylination in hAQP4 281-300 immunized mice

Paraffin sections were stained with haematoxlin eosin (H&E) and luxol fast blue (LFB). Representative sections of the spinal cords from PLP_{91-110} and $hAQP4_{281-300}$ immunized mice are shown. No inflammatory infiltrates or demyelination were observed with the spinal cord or the optic nerve in *HLA-DRB1*03:01* mice in which EAE was actively or passively induced.

The absence of any functional deficits was further corroborated through measurement of the pupillary reflex by murine pupillometry on day 15 post immunization (Figure #4.3). Mice actively immunized with hAQP4$_{281-300}$ and the control antigen PLP$_{91-110}$ did not show altered pupillary responses, further substantiating our previous findings that no encephalitogenic inflammation had occurred within the optic nerve.

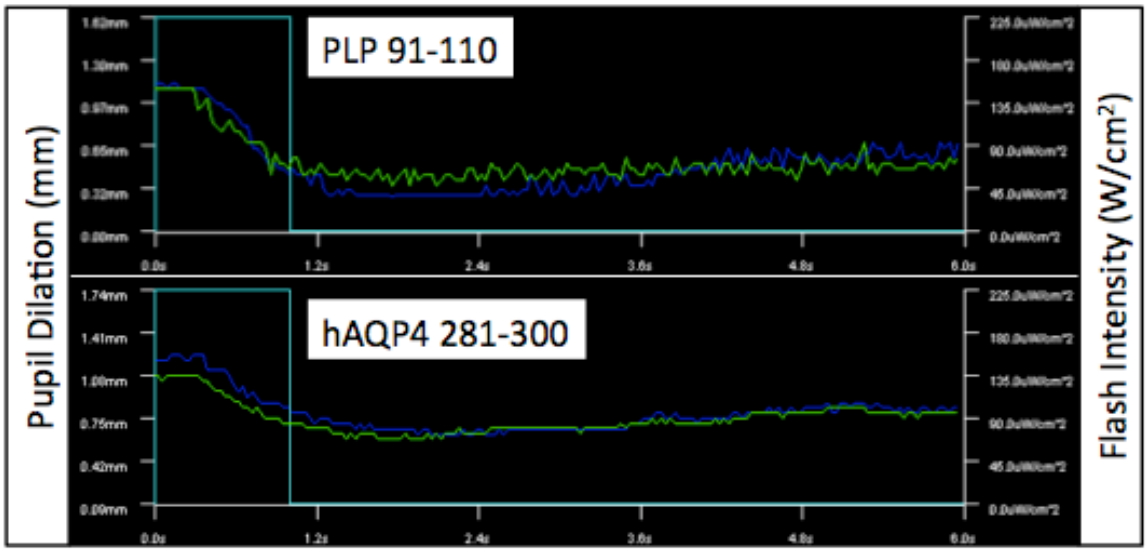

Figure 4.3 – Optic neuritis was not present in hAQP4 or PLP$_{91-110}$ immunized transgenic mice

Fifteen days post immunization of *HLA-DRB1*03:01* transgenic mice with PLP$_{91-110}$ or hAQP4$_{281-300}$, pupillary reflex was measured via a mouse pupillometry. Mice actively immunized with hAQP4$_{281-300}$ and the control antigen PLP$_{91-110}$ did not show altered pupillary responses.

$hAQP4_{281-300}$ specific T cells do not proliferate against $mAQP4_{281-300}$.

The complete disease resistance of HLA-DRB1*03:01 to active and passive disease induction was disappointing. Generating a $CD4^+$ T cell-mediated EAE animal model requires identification of the dominant and encephalitogenic peptide in the context of MHC II by the T cell receptor TCR (239). Moreover, immunogenic peptide analogs from different species have been shown to have varying effects on the expression of clinical disease (242). It is important to note that with regard to amino acid sequence, hAQP4 is not identical to mAQP4 protein. According to the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) website, there is a 93% protein-protein sequence homology between hAQP4 and mAQP4. Within the immunogenic $hAQP4_{281-300}$, there is a single amino acid mutation between the human peptide and the mouse analog: An aspartic acid (D) in the human peptide to glutamic acid (E) in the mouse (Figure #4.4). Both are negatively charged acidic amino acids that contain a carboxylic acid at the end of their side-chains. The only difference between the two amino acids is an additional methyl group in the side chain of glutamic acid. Thus, their biophysical properties are very similar. However, it still seemed conceivable that this amino acid substitution leads either to an inability of $mAQP4_{281-300}$ to bind to HLA-DRB1*03:01 or to be recognized by the $B.10.Ab^{KO}$ TCR.

Figure 4.4 – Sequence Homology Between Human and Murine $AQP4_{281\text{-}300}$.

There is a single aspartic acid (D) to glutamic acid (E) mutation at the $AQP4_{290}$ position that may play a role in the interactions between the peptide binding to the *HLA-DRB1*03:01* MHC II molecule or the B.10.Ab^{KO} TCR of AQP4 specific T cells.

To determine differences in immunogenicity between $hAQP4_{281-300}$ and $mAQP4_{281-300}$, I first examined whether hAQP4 specific T cells were capable of proliferating against $mAQP4_{281-300}$. Within proliferating $CD4^+$ T cells are two populations of cells: (1) $CD4^+$ high, and (2) $CD4^+$ intermediate cells. $CD4^+$ intermediate T cells have a greater expression of the regulatory transcription factor FOXp3 suggesting that they possess T regulatory (Treg) properties (243-245). In contrast, $CD4^+$ high are considered T effector cells. In lymph node cells of *HLA-DRB1*03:01* mice immunized with $hAQP4_{281-300}$ I determined a significant proliferation of both T effector cells and Tregs when $hAQP4_{281-300}$ was used as the recall antigen. In contrast, there was no proliferative response to $mAQP4_{281-300}$ (Figure #3.5). ELISpot assay revealed an increased frequency of IFNγ and IL-17 producing cells (Figure #3.6) when $hAQP4_{281-300}$ was the recall antigen, but not with $mAQP4_{281-300}$. These observations suggest that the aspartic acid residue plays a critical role in either the presentation of $hAQP4_{281-300}$ in the context of *HLA-DRB1*03:01*., or in the recognition of $hAQP4_{281-300}$ by the $B.10.Ab^{KO}$ TCR.

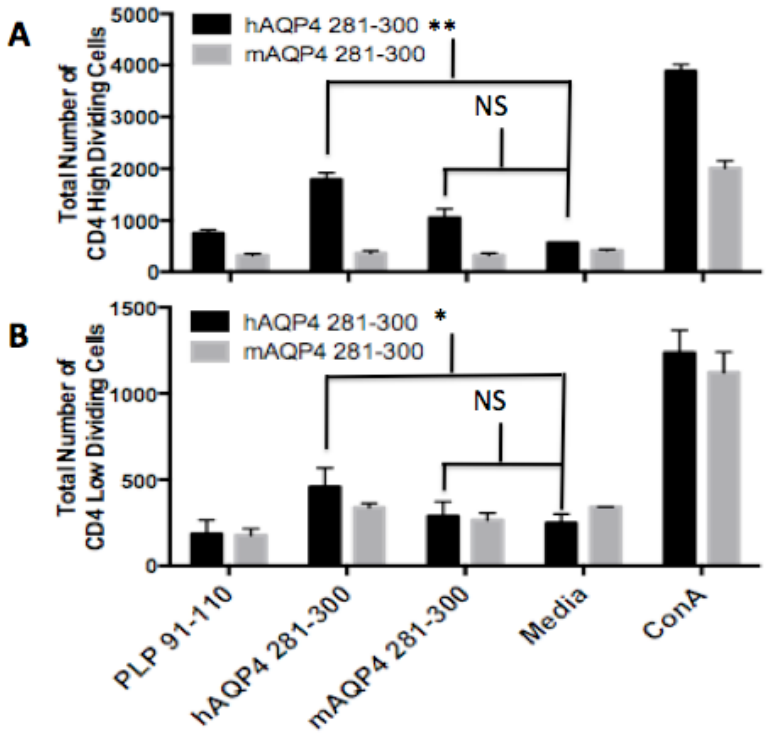

Figure 4.5 – hAQP4 specific T cells to do proliferate in response to $mAQP4_{281-300}$.

Ten days post immunization of *HLA-DRB1*03:01* transgenic mice with either $hAQP4_{281-300}$ (black) or $mAQP4_{281-300}$ (grey), lymph node cells taken from these mice, stained with CellTrace Violet then restimulated for 96 hours with PLP_{91-110} (25μg/mL), $hAQP4_{281-300}$ (25μg/mL), $mAQP4_{281-300}$ (25μg/mL), media, or ConA (1μg/mL). Data represents the total number of A) $CD3^+$ $CD4^+$ high T cells (T effectors) and B) $CD3^+$ $CD4^+$ intermediate T cells (T regulatory). (*=P-value <0.05 and **=P-value <0.01).

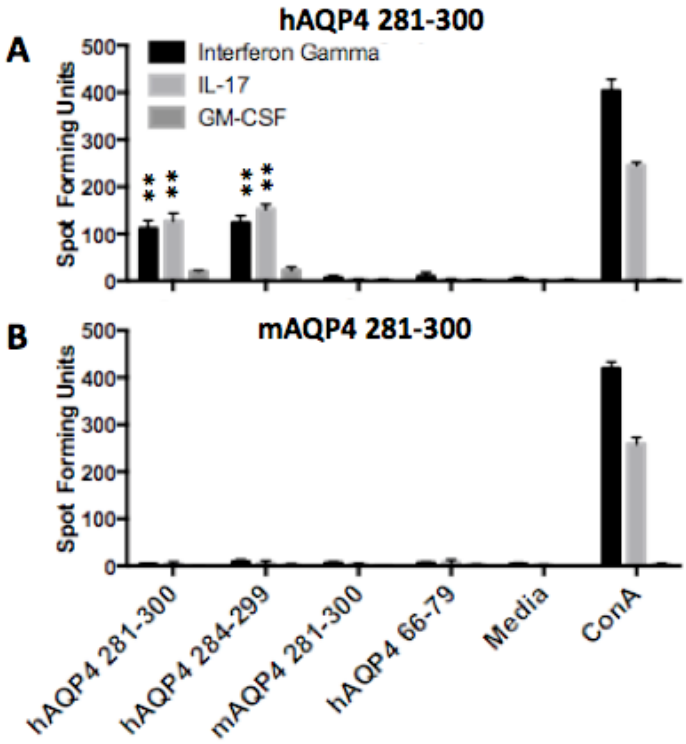

Figure 4.6 – $hAQP4_{281-300}$ specific T cells do not generate a Th_1 or Th_{17} immune response against $mAQP4_{281-300}$

Ten days post immunization of *HLA-DRB1*03:01* transgenic mice with either $hAQP4_{281-300}$ (A) or $mAQP4_{281-300}$ (B), lymph node cells taken and restimulated with $hAQP4_{281-300}$ (25μg/mL), $hAQP4_{284-299}$ (25μg/mL), $mAQP4_{281-300}$ (25μg/mL), $hAQP4_{66-79}$, media, or ConA (1μg/mL) for 48 hours in IFNγ, IL-17, or GM-CSF ELISpot plates. (*=P-value <0.05 and **=P-value <0.01).

*$hAQP4_{281-300}$ and $mAQP4_{281-300}$ binds to the HLA-DRB1*03:01 MHC II molecule*

With no cellular immune response against $mAQP4_{281-300}$, I next examined whether the single amino acid mutation affected the anchoring of the mouse peptide to the *HLA-DRB1*03:01* MHC II molecule. To identify critical residues of the AQP4 peptides, I generated alanine-scanning peptides that replaced each amino acid with an alanine to aid in distinguishing anchor residues from contact residues (Table #4.1). Since I previously identified $hAQP4_{284-299}$ to be the immunogenic region within $hAQP4_{281-299}$, the alanine scanning peptides assessed only these residues.

Utilizing a flow-cytometry-based MHC II binding assay, I was able to delineate between anchor residues and TCR contact residues. To perform the flow cytometry based MHC-peptide binding assay, peptides were biotinylated so that when presented on the MHC II molecule, a FITC-Avidin would distinguish peptides that were capable of being presented from those that could not. In comparing the percent of FITC-Avidin positive cells, I identified amino acids 288E and 294L as the main anchor residues that interact with the *HLA-DRB1*03:01* MHC II molecule (Figure #4.7). The remaining residues, including the 290D residue that distinguishes $mAQP4_{281-300}$ and $hAQP4_{281-300}$, were not required. As a negative control, Alanine scanning peptides were tested in C57BL/6 mice to examine the critical residues for binding to the *H-2b* MHC II molecule. Despite $hAQP4_{281-300}$ being able to be presented on the *H-2b* MHC II molecule, the critical residues were not similar to the critical residues necessary for binding to the *HLA-DRB1*03:01* MHC II molecule.

This observation may explain why we were unable to elicit cellular immune response against hAQP4$_{281\text{-}300}$ in C67BL/6 mice immunized with this peptide. (data not shown)

Human AQP4 Alanine Scanning Peptides

Peptide	Amino Acid Sequence
Human 284-299	RSQVETDDLILKPGVV
Mouse 284-299	RSQVETEDLILKPGVV
R284A	ASQVETDDLILKPGVV
S285A	RAQVETDDLILKPGVV
Q286A	RSAVETDDLILKPGVV
V287A	RSQAETDDLILKPGVV
E288A	RSQVATDDLILKPGVV
T289A	RSQVEADDLILKPGVV
D290A	RSQVETADLILKPGVV
D291A	RSQVETDALILKPGVV
L292A	RSQVETDDAILKPGVV
I293A	RSQVETDDLALKPGVV
L294A	RSQVETDDLIAKPGVV
K295A	RSQVETDDLILAPGVV
P296A	RSQVETDDLILKAGVV
G297A	RSQVETDDLILKPAVV
V298A	RSQVETDDLILKPGAV
V299A	RSQVETDDLILKPGVA

Table 4.1 – hAQP4$_{284\text{-}299}$ Alanine Scanning Peptides.

The immunogenic region of hAQP4$_{281\text{-}300}$, hAQP4$_{284\text{-}299}$, was utilized to generate alanine scanning peptides at which each peptide sequence has a single alanine residue mutation.

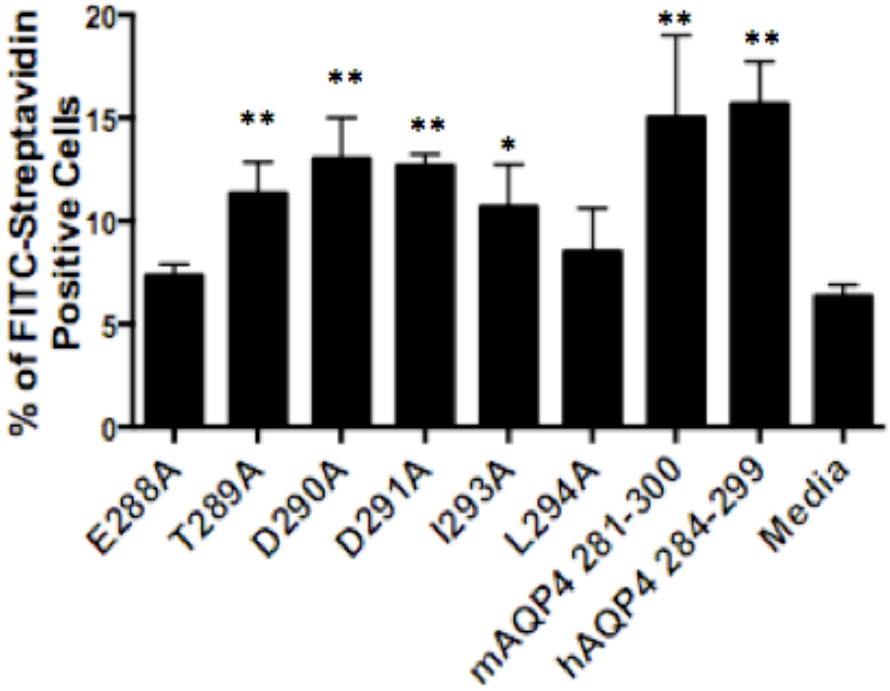

Figure 4.7 – MHC II binding assay identified critical residues for binding of hAQP4$_{281\text{-}300}$ to the *HLA-DRB1*03:01* MHC II molecule.

Splenocytes taken from a *HLA-DRB1*03:01* transgenic mouse were incubated for 12 hours in the presence of biotinylated hAQP4 alanine scanning peptides. Post incubation, cells were stained utilizing FITC-Avidin and antigen positive cells were quantified. (*=P-value <0.05 and **=P-value <0.01).

mAQP4$_{281-300}$ is not recognized by the B.10.Ab^{KO} *TCR*

With mAQP4$_{281-300}$ being capable of being presented on the *HLA-DRB1*03:01* MHC II molecule, I next examined whether the single amino acid mutation inhibited the contact with the hAQP4$_{281-300}$ specific B.10.Ab^{KO} TCR. Utilizing the Alanine-scanning peptides, I was capable of identifying all of the critical residues that are required for generating the cellular immune response against the peptide. In performing IFNγ and IL-17 ELISpot assays, it was determined that peptides 288E, 289T, 290D, 291D, 293I, and 294L are critical for that activation of the T cell. Seeing as 288E and 294L were determined to be MHC II anchor residues, the remaining residues play a critical role in the contact between the peptide and B.10.Ab^{KO} TCR (Figure #4.8). This method was used first as to distinguish all residues necessary for reactivating T cells against the peptide.

Consequently, these data suggest that the mAQP4$_{281-300}$ binds to the *HLA-DRB1*03:01* molecule via the same anchor residues and is thus able to be presented on the MHC II molecule in our transgenic mice. This suggests that the D to E mutation between hAQP4 and mAQP4 peptides prevent the activation of hAQP4-specific T cells against mAQP4$_{281-300}$. I hypothesize that mAQP4$_{281-300}$-specific B.10.Ab^{KO} TCRs may be negatively selected against in the thymus to protect against the generation of a cellular immune response against endogenous self-peptides.

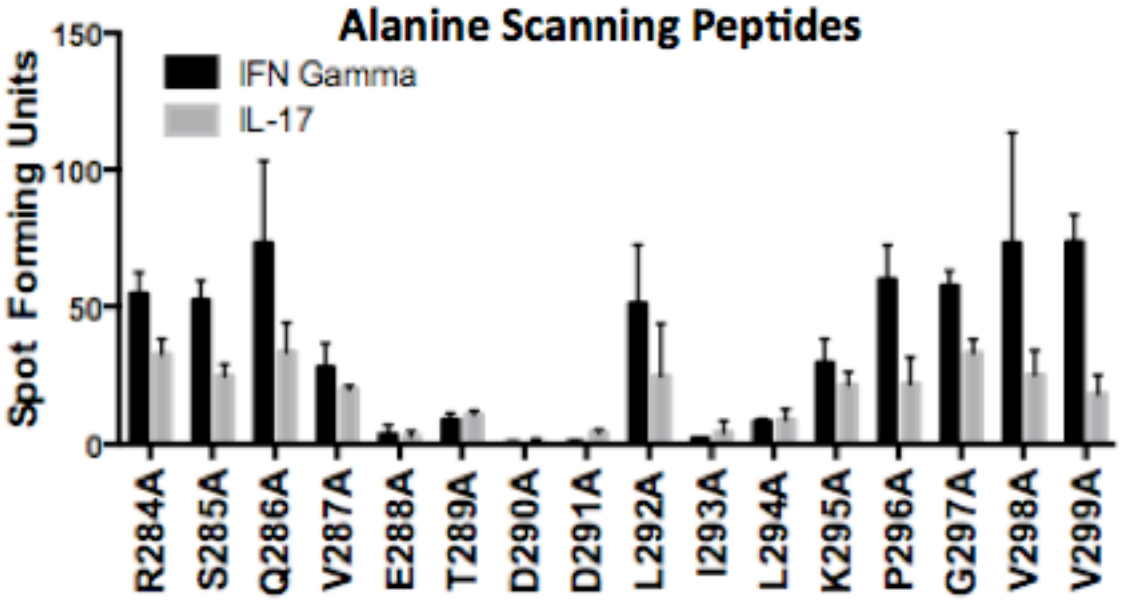

Figure 4.8 – Alanine mutations in critical residues affect the Th_1 and Th_{17} phenotype.

Ten days post immunization of *HLA-DRB1*03:01* transgenic mice with $hAQP4_{281-300,}$ lymph node cells taken and restimulated with hAQP4 alanine scanning peptides (25μg/mL) for 48 hours in IFNγ and IL-17 ELISpot plates (*=P-value <0.05 and **=P-value <0.01).

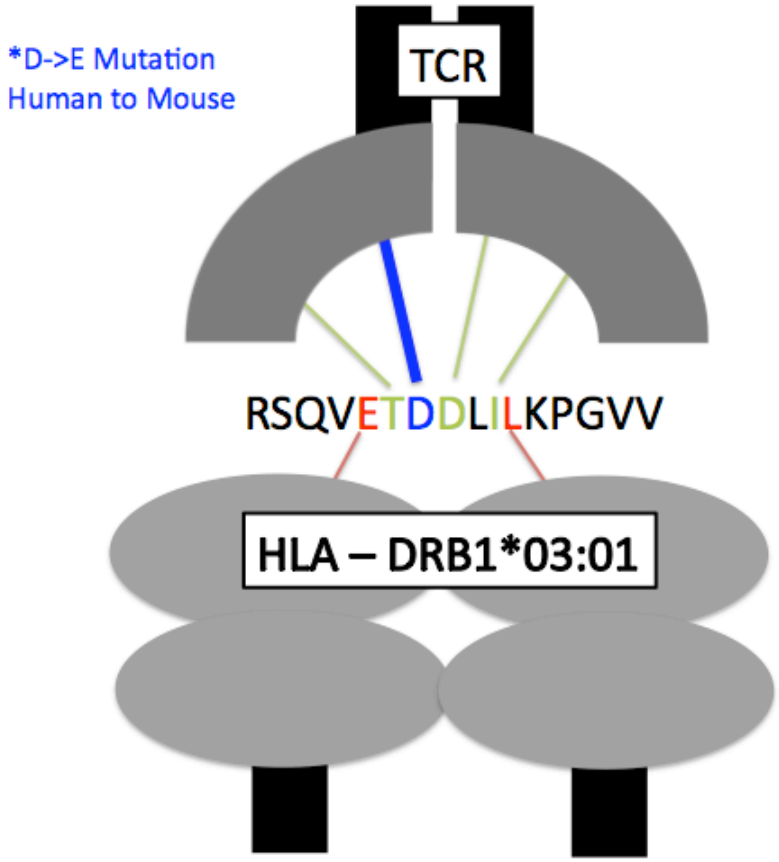

Figure 4.9 – Critical residues for presentation and activation of T cells in the context of *HLA-DRB1*03:01.*

Critic anchor and B.10.Ab^{KO} TCR contact amino acids are specified in diagram. E_{288} and L_{294} are required DR17 MHCII anchor residues, while T_{289}, D_{290}, D_{291}, and I_{293} are critical B.10.Ab^{KO} TCR interacting residues.

*Immunization with hAQP4$_{281-300}$ leads to an Ig isotype switch in HLA-DRB1*03:01 transgenic mice*

Knowing that Th_1 and Th_{17} may play an important role in activating AQP4-specific B cells to secrete pathogenic NMO-IgG , I next examined whether these hAQP4 specific T cells were capable of causing IgM to IgG isotype switching in the transgenic mice. Taking serum from whole protein hAQP4 or hAQP4$_{281-300}$ immunized mice, I quantified the concentration of whole protein and peptide specific antibodies generated over time (Figure 4.10). Since the NMO-IgG is a human IgG1 isotype, I examined both the murine IgG2a and IgG2b isotype due to their similarities in binding complement and the Fcγ receptor in mice. I saw a shift from IgM specific antibodies to IgG2b specific antibodies in mice immunized with either the immunogenic hAQP4$_{281-300}$ peptide or whole protein AQP4 (Figure 4.10). This suggests that while hAQP4$_{281-300}$ specific T cells were unable to generate any form of clinical disease, they are capable of driving isotype switching to occur in autoreactive B cells specific for AQP4. These B cells within the transgenic mice are capable of taking up the peptide or whole protein via their antigen specific BCR and presenting the hAQP4$_{281-300}$ peptide on the *HLA-DRB1*03:01* MHC II molecule. Post interactions with hAQP4$_{281-300}$ specific T cells, the B cells begin to secrete IgG2b-hAQP4 specific autoantibodies. This data does not imply that the discovered autoantibodies are pathogenic since they are most likely specific to the intracellular region of AQP4, especially the antibodies found in peptide immunized mice. These findings may be more critical in the context of human patients rather than our mouse

model seeing as antibodies specific to the intracellular region of AQP4 are found in NMO patients (246). Since previous data showed that $mAQP4_{281-300}$ could not generate a strong cellular immune response, I further examined whether isotype switching was still capable of occurring in $mAQP4_{281-300}$ immunized mice. While IgM antibodies specific for hAQP4 could be found in the serum, it was delayed and no IgG2b isotype switching had occurred in these mice (Figure 4.11). This data suggests that the cellular immune response against the immunogenic hAQP4 peptide is capable of causing isotype switching to occur in AQP4-specific B cells and activated $hAQP4_{281-300}$-specific T cells may play a critical role in the generation of NMO-IgG antibodies in patients.

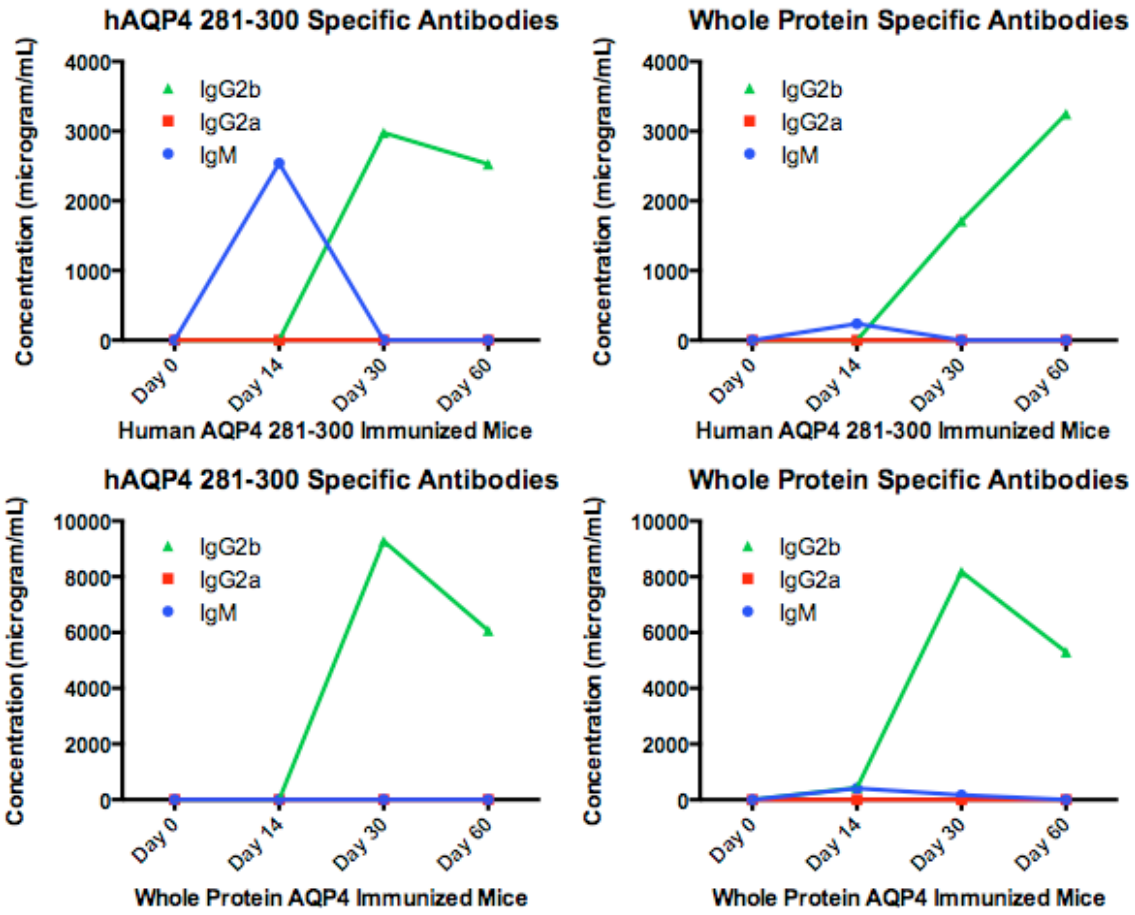

Figure 4.10 – hAQP4$_{281-300}$ specific T cells are Capable of Causing IgG Switching to Occur.

HLA-DRB1*03:01 **transgenic mice were immunized with hAQP4$_{281-300}$ (top) or whole protein hAQP4 (bottom). On day 0, 14, 30, and 60, serum was taken from mice via submandibular bleed. ELISA plates were coated with either hAQP4$_{281-300}$ (left) or whole protein hAQP4 (right) to quantify antigen specific antibodies (IgM (blue), IgG2a (red), and IgG2b (green).**

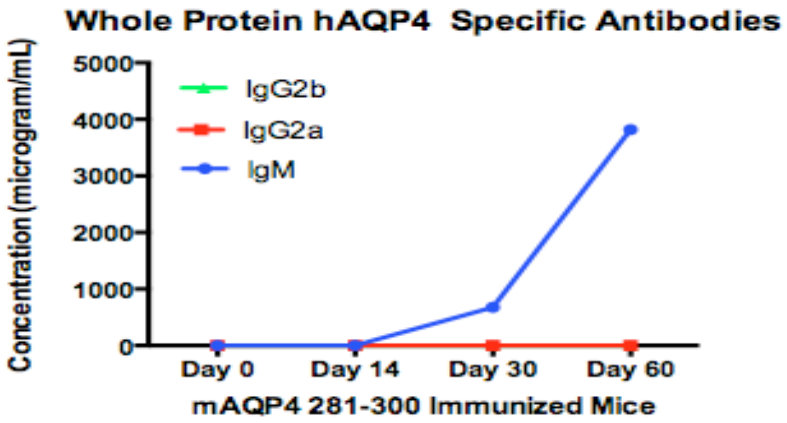

Figure 4.11 – $mAQP4_{281-300}$ specific T cells are incapable of causing IgG switching to occur.

HLA-DRB1*03:01 **transgenic mice were immunized with $mAQP4_{281-300}$. On day 0, 14, 30, and 60, serum was taken from mice via submandibular bleed. ELISA plates were coated with whole protein hAQP4 were utilized to quantify antigen specific antibodies (IgM (blue), IgG2a (red), and IgG2b (green).**

*Residues of hAQP4$_{281-300}$ that mediate binding to HLA-DRB1*03:01 and* the B.10.Ab^{KO} *TCR have sequence homology with Hepatitis C virus subtype 5a polyprotein (HCV)$_{24-30}$*

The *molecular mimicry* concept proposes a structural or partial amino-acid sequence homology between the inoculated pathogen and endogenous proteins of the host (247). This structural homology is not sufficient for a pathogen to be recognized as "self", which would result in immune-tolerance. APC, such as B cells or DC, process the pathogen at the site of inoculation, leading to T cell activation. The pathogen and the homologous autoantigen should ideally be highly expressed in the same compartment. Activated T cells may in turn cross-activate antigen specific B cells. Both, activated T cells and B cells are quite capable of entering the tissues for routine immune surveillance. Thus, even after clearance of the pathogen, these antigen-specific cells may encounter the homologue endogenous protein during their physiologic surveillance. They may become reactivated by tissue APCs, causing an inflammatory immune reaction against the presumed foreign antigen; thus, the initially physiological immune response leads to detrimental autoimmunity.

Utilizing the NCBI BLAST website, I investigated possible pathogenic antigens that have sequence homology with hAQP4$_{288-294}$ (ETDDLIL), the critical region of the hAQP4 peptide that contained the *HLA-DRB1*03:01* MHC II anchor residues and the B.10.Ab^{KO} TCR contact residues (Table 4.2). Hepatitis C virus subtype 5a polyprotein (HCV)$_{24-30}$ (CBF58687.1) showed 100% positive sequence homology with 0% gaps when compared to hAQP4$_{288-294}$. Because of the identical

sequence homology in such a critical region of the immunogenic peptide, it is possible that the HCV_{24-30} may be presented within the context of HLA-DRB1*03:01, and possibly be recognized by $hAQP4_{281-300}$-specific $CD4^+$ T cells.

Human AQP4 281-300 sequence homology

Protein	Sequence Homology
AQP4 281-300 (*Homo sapiens*)	**EDNRSQVETDDLILKPGVVH**
AQP4 281-300 (*Mus musculus*)	**EDNRSQVETDDLILKPGVVH** **EDNRSQVETEDLILKPGVVH**
Polyprotein 24-30 (*Hepatitis C subtype 5a*)	**EDNRSQVETDDLILKPGVVH** ---------- **ETDDLIL**----------

Table 4.2 – $hAQP4_{281-300}$ BLAST Results

Identifying the cross-reactive potential between hAQP4$_{281-300}$ and HVC Polyprotein$_{19-34}$

With HVC polyprotein$_{19-34}$ (HVC$_{19-34}$) containing the critically immunogenic region of hAQP4$_{281-300}$, it is conceivable that T cells specific for either peptide are capable of recognizing the other when bound to the *HLA-DRB1*03:01* MHC II molecule. To test this possibility, *HLA-DRB1*03:01* transgenic mice were immunized with hAQP4$_{281-300}$ or HVC$_{19-34}$. Ten days post immunization I performed CellTrace proliferation assays on LN cells isolated from each group to examine whether CD3$^+$ CD4$^+$ T cells were capable of expanding in response to the cross-reactive peptide that the cell had not been exposed to previously. I found that both hAQP4$_{281-300}$ and HVC$_{19-34}$ elicited a CD4$^+$ T response against the antigen the mice were immunized against as well as the cross-reactive peptide (Figure 4.12). Additionally, ELISpot assays showed that these cross-reactive cells produced IL-17 in response to the cross-reactive peptide. (Figure 4.13). Together, this data strongly suggests that if HVC$_{19-34}$ specific T cells are present in a *HLA-DRB1*03:01* positive individual, it could potentially generate a Th$_{17}$ immune response against hAQP4, the same cellular immune response associated with NMO. In future studies, it would be of interest to examine whether HVC$_{19-34}$ specific T cells are capable of activating AQP4-specific B cells and cause isotype switching to occur. The results of that study would show the pathological potential of the cross-reactive T cells in the context of initiating early NMO pathogenesis.

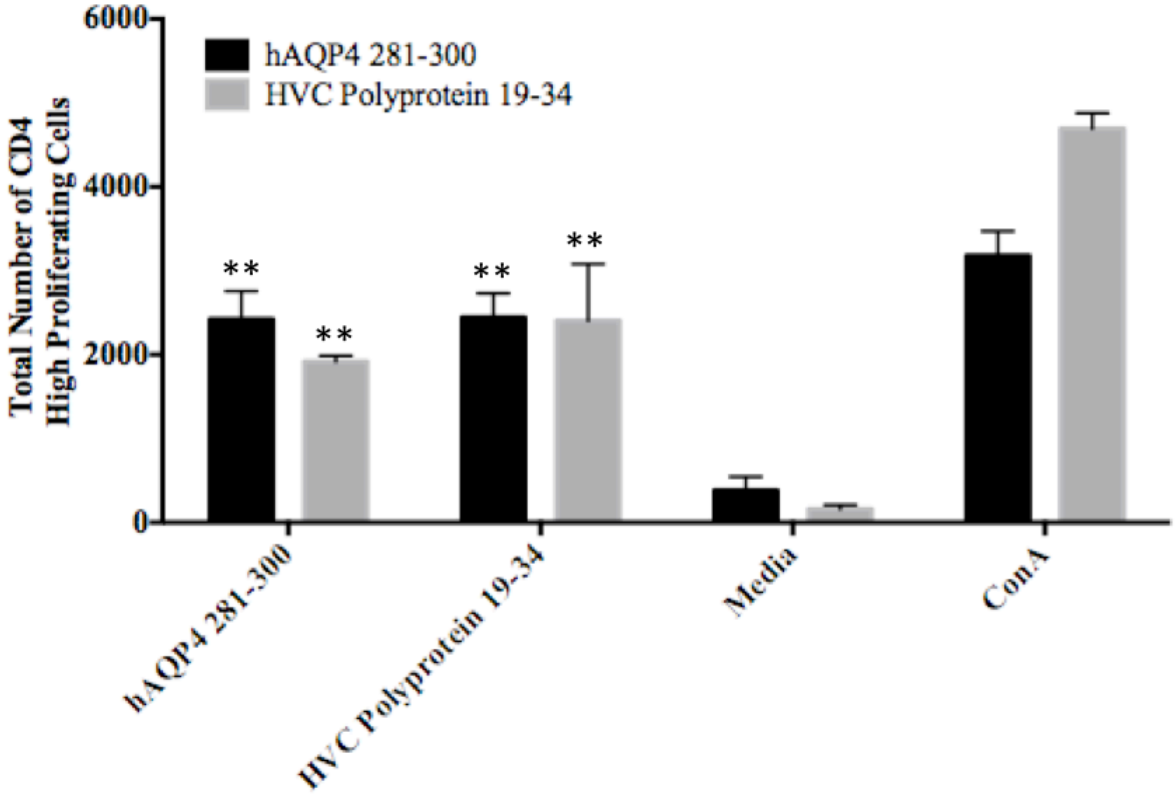

Figure 4.12 – $CD4^+$ T cells specific for $hAQP4_{281-300}$ or HVC $Polyprotein_{19-34}$ are cross-reactive against one another.

$hAQP4_{281-300}$ (black) or HVC $Polyprotein_{19-34}$ (grey), lymph node cells taken from transgenic mice, stained with CellTrace Violet then restimulated for 96 hours with $hAQP4_{281-300}$ (25μg/mL), HVC $Polyprotein_{19-34}$ (25μg/mL), media, or ConA (1μg/mL). Data represents the total number of $CD3^+$ $CD4^+$ high T cells (T effectors. (*=P-value <0.05 and **=P-value <0.01).

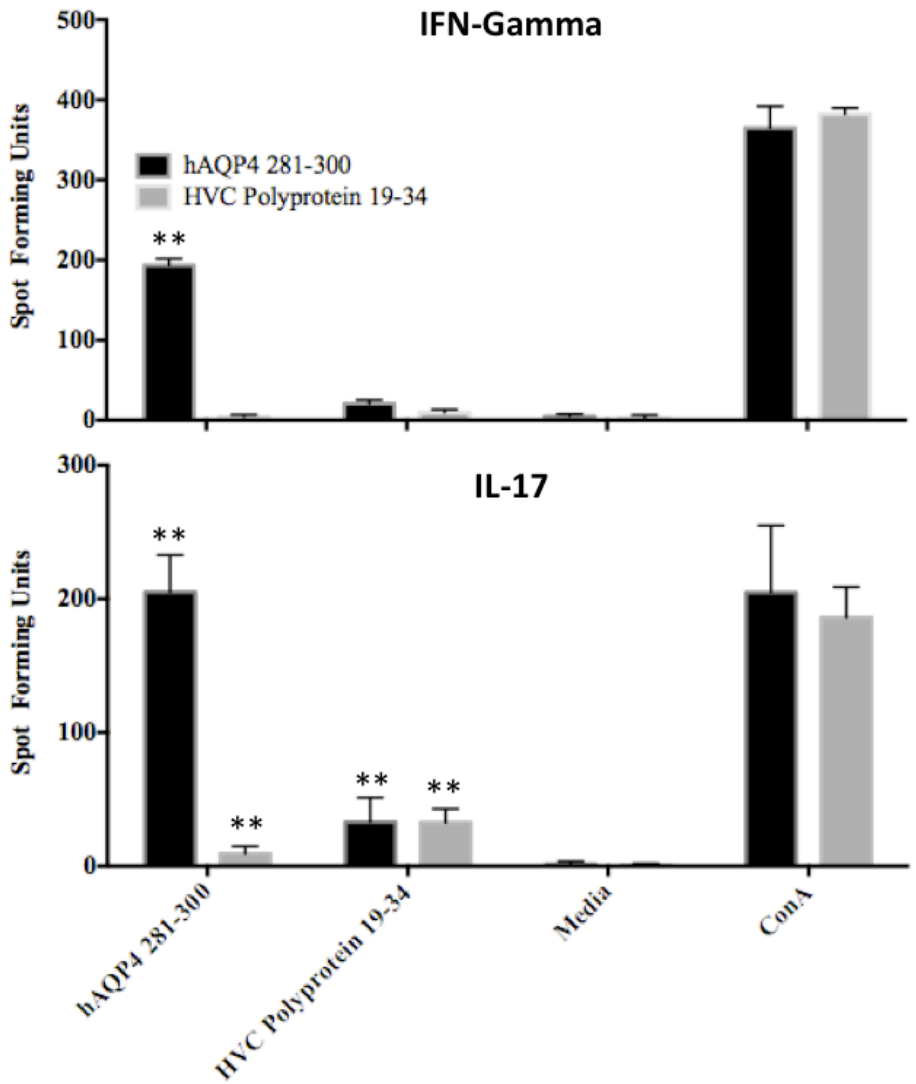

Figure 4.13 – Cross-reactive cells generate a greater Th_{17} immune response against the HVC Polyprotein$_{19-34}$.

Ten days post immunization of *HLA-DRB1*03:01* transgenic mice with either hAQP4$_{281-300}$ (black) or HVC Polyprotein$_{19-34}$ (grey), lymph node cells taken and restimulated with hAQP4$_{281-300}$ (25μg/mL), HVC Polyprotein$_{19-34}$, media, or ConA (1μg/mL) for 48 hours in IFNγ, or IL-17. (*=P-value <0.05 and **=P-value <0.01).

Summary of Results

In this specific aim, I show that immunization with the immunogenic, $hAQP4_{281-300}$ determinant in *HLA-DRB1*03:01* transgenic mice, while leading to a robust Th_1 and Th_{17} immune response, does not lead to a clinical disease phenotype via active immunization or passive transfer of cells. My data indicates that a single amino acid substitution between hAQP4 and mAQP4 will not allow us to establish an animal model of NMO in *HLA-DRB1*03:01* transgenic mice. Within all of the EAE animal models, it is critical that the proper autoantigen is utilized during immunization. For some, there is sequence homology between mouse and human myelin proteins, such as with MOG_{35-55}. However, it has been shown that for EAE to occur, the autoantigen must be specific to endogenous peptide(239). Within the pathogenic $AQP4_{281-300}$, the glutamic acid (D) to aspartic acid (E) mutation results in the addition of a methyl group within the side chain of the $AQP4_{287}$ residue in the mouse peptide. Despite being the same polarity, the $hAQP4_{281-300}$-specific B.10.Ab^{KO} TCR can differentiate between the human and the mouse determinants. This was further corroborated with my data showing that the $AQP4_{287}$ residue was important for contact with the B.10.Ab^{KO} TCR rather than being a MHC II anchor residue. As a result, antigen recall with $mAQP4_{281-300}$ in *HLA-DRB1*03:01* mice immunized with $mAQP4_{281-300}$ does not result in proliferation of $CD4^+$ T cells. Likely, negative thymic selection for $mAQP4_{281-300}$ specific $CD4^+$ T cells occurs in these mice, and prevents active disease induction.

Despite the negative results, I was able to further characterize the role of the cellular immune response against $hAQP4_{281-300}$. I showed that T cells specific for $hAQP4_{281-300}$ were capable of driving an isotype switch from IgM to IgG2b in transgenic mouse. The isotype switch is peptide specific since it did not occur in $mAQP4_{281-300}$ immunized mice most likely due to the lack of $CD4^+$ T cell activation. With these T cells clearly capable of driving the generation of AQP4 specific IgG antibodies, the data suggests that hAQP4-specific T cells may play a pathogenic role in NMO patients. Perhaps most intriguing was my discovery that there is sequence homology between the critical residues of $hAQP4_{281-300}$ and the HVC_{19-34}. Seeing that preliminary data showed cross-reactivity between $hAQP4_{281-300}$ specific T cells and the HVC_{19-34} in the context of HLA-DRB1*03:01, I believe that there may be a critical role HVC infections play in potentially driving NMO pathogenesis.

DISCUSSION AND FUTURE DIRECTIONS

Discussion

Generating a NMO Animal Model for further studying of the disease.

The identification of immunodominant determinants of human AQP4 may have important implications for understanding the etiology of NMO, and monitoring disease activity in patients afflicted with this disorder. Immunodominant determinants are stretches of AQP4 protein that can activate T cells. In different wild-type mouse strains, including C57BL/6 (H-2b) and SJL/J (H-2s) mice, these determinants of the M1 isoform of AQP4 were recently identified (220),(231).

Nelson et al and Kalluri et al. were the first groups to identify critical linear determinants that were capable of stimulating a cellular immune response in mice (220, 231). By screening overlapping peptides of human and mouse AQP4 in C57BL/6 or SJL/J mice, $AQP4_{21-40}$ was identified to be an immunogenic peptide capable of stimulating a cellular immune response in both mouse strains. Specifically, this linear determinant was found to lead to an increase in thymidine incorporation in proliferating T cells in culture as well as production of T cell production of IL-2, IFNγ, GM-CSF, IL-10, and IL-17a (220, 231).

Despite $hAQP4_{21-40}$ generating a Th_1 and Th_{17} immune response, active immunization with this immunodominant determinants could not generate an NMO animal model similar to the animal model for multiple sclerosis, experimental

autoimmune encephalomyelitis (EAE). It was not determined whether this peptide was capable of inducing anti-AQP4 antibodies. While human and mouse AQP4 have a 93% sequence homology, it is important to note that the immunogenic region of $AQP4_{21-40}$, $AQP4_{22-36}$, has 100% sequence homology between human AQP4 in the M1 isoform and murine AQP4 in the M1 isoform (220). Despite this sequence homology, immunization of $AQP4_{22-36}$ did not lead to ascending paralysis similar to EAE. Spinal cord and optic nerve T cell infiltration was not observed in AQP4 immunized mice, suggesting that while this peptide is immunogenic in culture, it is not encephalogenic.

Though this data in C57BL/6 and SJL/J mice identified AQP4 linear determinants that stimulate a cellular immune response in H-2, the C57BL/6 genetic background does not express H-2-IE, the equivalent gene of the human class II MHC molecule HLA-DR. Therefore, I utilized transgenic mice expressing the *HLA-DRB1*03:01* gene to identify linear dominant determinants of hAQP4. This study specifically aimed to identify immunodominant linear determinants of AQP4 in the context of *HLA-DRB1*03:01*, as this HLA haplotype was recently associated with NMO in several patient cohorts (141, 207, 208). Utilizing HLA-DRB1*0301 transgenic mice, I discovered that $hAQP4_{281-300}$, was capable of leading to a Th1 and Th17 recall response.

Similar to studies performed in C57BL/6 and SJL/J mice, while $hAQP4_{281-300}$ was able to generate a strong Th_1 and Th_{17}, immune response, no active EAE or passive EAE protocol resulted in any form of a clinical disease phenotype. I show

that despite there being only an aspartic acid (D) to glutamatamic acid (E) mutation at the $AQP4_{290}$ residue, $hAQP4_{281-300}$-specific T cells from B10 mice are incapable of being reactived against $mAQP4_{281-300}$ despite the peptide being able to be presented on the *HLA-DRB1*03:01* MHC II molecule. This result suggests that the immunodominant Th_1 and Th_{17} immune response cannot be reactived in the CNS due to the failure to recognize endogenous $mAQP4_{281-300}$. It can be inferred that within the thymus, negative selection against $mAQP4_{281-300}$-specific T cells occurs. In order to overcome central tolerance against immunogenic mAQP4 peptides, I would have to generate $mAQP4^{-/-}$ HLA-DRB1*03:01 mice. This mouse would allow the identification of additional cryptic-turned-dominant determinants of mAQP4 in *HLA-DRB1*03:01*, which could be more encephalitogenic than my previously discovered $hAQP4_{281-300}$. Subsequent adoptive transfer of mAQP4-specific inflammatory T cells would then lead to clinical disease by driving an inflammatory response against highly regulated, dominant mAQP4 epitopes. Additionally, transferring autoreactive B cells in addition to the autoreactive T cells may have a synergistic effect that could be necessary for an animal model that fully embodies the entire disease phenotype of NMO.

Despite all studies being capable of stimulating a cellular immune response against linear determinants of hAQP4 or mAQP4, it appears that a critical step in generating the NMO phenotype is the activation of the cellular immune response against endogenous AQP4 peptides that results in the activation of AQP4-specific B cells.

Molecular Mimicry in Initiating NMO

The most enigmatic aspect of autoimmune diseases is the event that leads to break-down of peripheral tolerance against self antigen. Molecular mimicry is one putative mechanism by which auto-immunogenicity occurs. This concept proposes that pathogenic proteins contain sequence and structural homology with endogenous proteins. The original clearance of the pathogen results in the generation of pathogen-specific $CD4^+$ T cells capable of recognizing endogenous antigens and perceiving them as foreign. After the clearance of the pathogen, cross-reactive memory $CD4^+$ T cells patrol tissues during regular immune surveillance. At the site where the homologous, endogenous protein is encountered, in the context of MHC II, an inflammatory immune response against the presumed foreign antigen is initiated.

Human AQP4 shares sequence similarities with foreign peptides, suggesting a role for molecular mimicry in the initiation of the adaptive immune response against endogenous hAQP4 in the context of NMO. Ren *et al* found that hAQP4 peptides showed sequence homology to the bacterial AQPz protein and generated a strong cellular and humoral immune response against hAQP4 in wild-type SJLJ mice (248). In a small cohort of NMO patients, Varrin-Doyer *et al* showed sequence homology between $hAQP4_{63-76}$ and an adenosine triphosphate-binding cassette transporter permease found in *Clostridium perferingens* (249). Within my own study, a BLAST search of the residues of $hAQP4_{281-300}$ that mediate the interactions between *HLA-DRB1*03:01* and the $B.10.Ab^{KO}$ TCR found multiple analogous peptides that shared

identical peptide sequences to the region. Most notably, the HCV_{19-34} had a 100% sequence homology to this region. The sequence homology between $hAQP4_{281-300}$ and HCV_{19-34} may suggest a causal connection between the two diseases (This is shown in Figure 5.1).

HCV has long been associated with autoimmune diseases (250, 251). It is of note that independent case studies have shown a possible correlation between hepatitis C and immune mediated transverse myelitis and optic neuritis(252-255). The *HLA-DRB1*03:01* haplotype is also positively associated with chronic HCV infections (256-258). Preliminary studies in *HLA-DRB1*03:01* transgenic mice show that $hAQP4_{281-300}$ specific T cells also proliferate against the HCV_{19-34} and that, these $hAQP4_{281-300}$ specific T cells generate a strong Th_{17} immune response to the HCV_{19-34}. It is also interesting that both hepatitis C and NMO have been associated with an elevated expression of IL-6 (226, 259) and IL-21 (226, 260). Further investigations will be required to ascertain whether a causal link is present between Hepatitis C viral exposure and the initiation of NMO disease activity.

It is conceivable that a HCV infection in an *HLA-DRB1*03:01* positive individual may result in two scenarios that leave the patient with a greater susceptibility to develop NMO. (1) A vigorous immune response to HCV leads to the clearance of the virus but results in the generation of cross-reactive $hAQP4_{281-300}$-specific memory T cells in the periphery, or (2) the HCV infection is not cleared, leading to chronic reactivation of $hAQP4_{281-300}$-specific T cells, and initiating CNS autoimmunity. In one case, it was

shown that a chronic HCV patient developed optic neuritis due to the presence of anti-AQP4 antibodies (261). Optic neuritis occurred in this patient after being placed on interferon-β (IFN-β) treatment. This is intriguing in light of small cohort studies examining the treatment options for NMO patients found that the response to IFN-β treatment was highly variable with some patients experiencing exacerbations in disease progression (262-264). In contrast, IFN-β treatment has been shown to be highly effective in aiding patients with chronic HCV infections (258). I theorize that IFN-β treatment may result in the clearance of the virus through the up regulation of MHC I molecules and $CD8^+$ T cell targeting HCV infected cells. Clearance of lysed, HCV infected cells would inevitably result in pathogenic antigens being taken up by APCs and presented to $CD4^+$ T cells in *HLA-DRB1*03:01* . As a result, cross-reactive $hAQP4_{281-300}$-specific $CD4^+$ T cells would be generated. Some of these antigen-specific $CD4^+$ T cells may subsequently enter the CNS and initiate NMO relapses. By taking these findings into account, future studies will determine whether there is a conclusive connection between certain NMO patients and HCV infection, therefore aiding in the determination of how to best treat patients in a case by case manner.

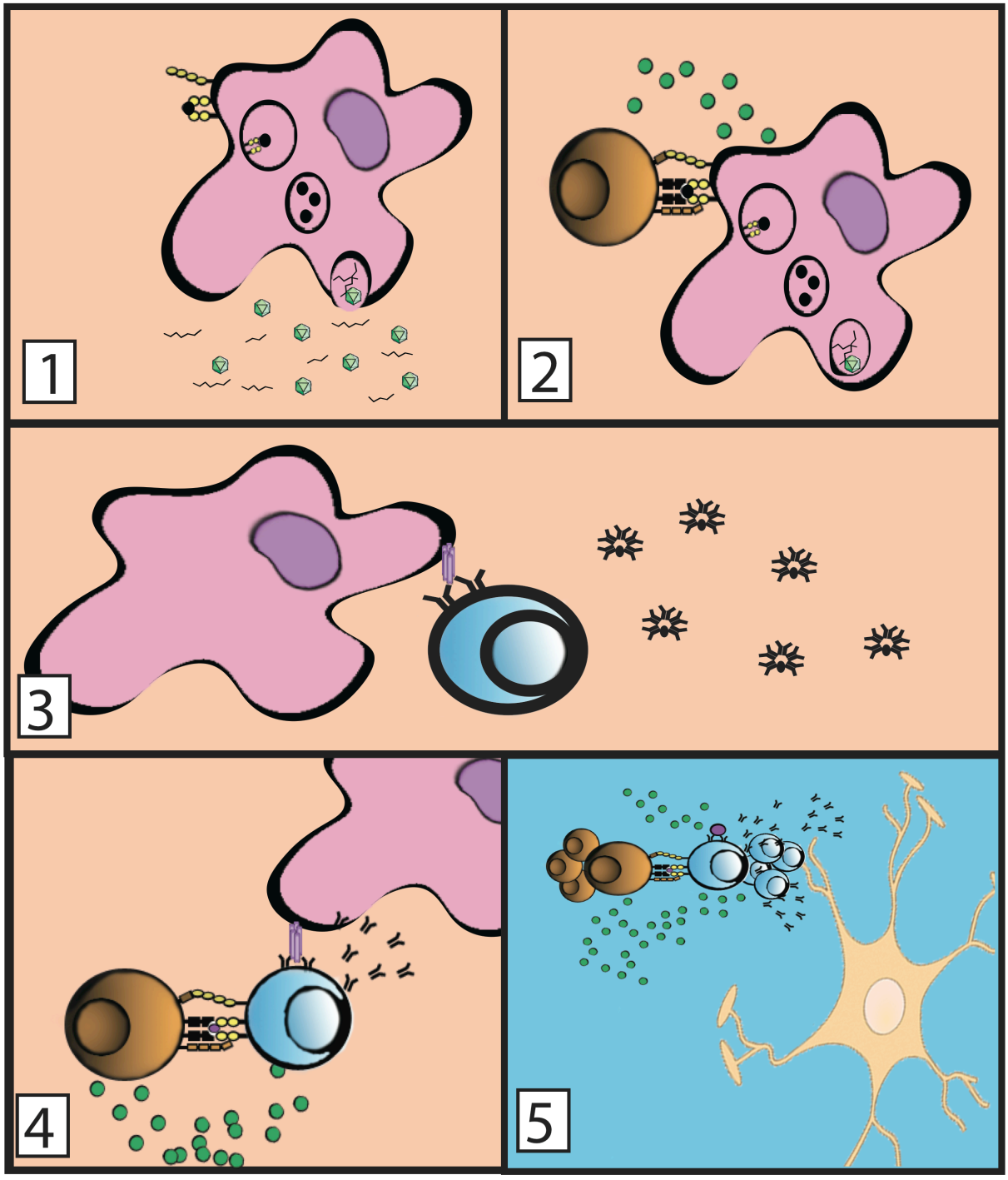
1
2
3
4
5

Figure 5.1 – Molecular mimicry between $hAQP4_{281-300}$ and HCV_{19-34} in NMO

A sequential group of physiological and aberrant immunological events in the periphery (red background) may eventually lead to the generation of NMO IgG, and the onset of neuromyelitis optica (NMO).

1) **Hepatitis C virus (HCV) infection: Following an infection of an *HLA-DRB1*03:01*-positive individual with Hepatitis C virus (HCV), the virus is phagocytosed by a myeloid derived dendritic cells (mDC) and brought to the draining lymph node.**
2) **$CD4^+$ T cell memory against HVC_{19-34} and $hAQP4_{281-300}$ is generated. In the draining lymph node, HVC_{19-34} is presented on *HLA-DRB1*03:01* to $CD4^+$ T cells. Due to molecular mimicry between HVC_{19-34} and $hAQP4_{281-300}$, an immunological $CD4^+$ T cell memory against both antigens is generated.**
3) **AQP4-specific B cells are generated. In a separate event, B cell encounter conformational epitopes of AQP4 as an antigenic target. This may occur secondary to injury of any tissue where AQP4 is expressed. 2 and 3 may occur in a reverse temporal sequence.**
4) **T cell - B cell cross-activation occurs, and B cell memory is generated. In secondary lymphoid tissue, HVC_{19-34} -$hAQP4_{281-300}$ – reactive $CD4^+$ memory T cells and AQP-specific B cells have spatial proximity. . The HVC_{19-34} - $hAQP4_{281-300}$ – reactive $CD4^+$ memory T cells activate the naïve, AQP4-specific B cell, leading to an Ig isotype class switch to IgG (bottom left).**

5) **Amplification of a humeral immune response in the CNS occurs. It has to be assumed that there is constant physiological turnover of astrocytes within some areas of the brain and spinal cord. Also, CNS tissue injury due to trauma, hypoxia, or infection occurs. Immune surveillance of the CNS by AQP4-specific B cells would result in recognition of conformational epitopes of AQP4, and destruction of astrocytes.**

The Role of hAQP4$_{281-300}$-Specific T cells in NMO Pathogenesis

The identification of the immunodominant determinant hAQP4$_{281-300}$ has important implications for understanding the etiology of NMO, and in monitoring disease activity in patients afflicted with this disorder. NMO is a complex disease in which the precise pathogenic role of $CD4^+$ T cells is currently not fully understood. With other CNS autoimmune diseases, such as MS, $CD4^+$ T cells mediate the demyelination that occurs within the disease. This is perhaps best reflected in the EAE animal model, in which autoreactive Th_1 or Th_{17} cells are generated in healthy, naïve mice. Autoreactive T cells localize to the CNS and become reactivated by microglia cells presenting myelin antigens as part of physiological tissue repair, which initiates an inflammatory immune response. Activated T cells recruit infiltrating macrophages, monocytes, and DCs to the CNS via cytokine and chemokine production. The activated macrophages and DCs then strip myelin from axons surround the site of infiltration, which then results in the paralysis that correlates with the EAE animal model (265). In the context of NMO, $CD4^+$ T cells may play a similar role in the localization of inflammatory cells to the CNS, but primarily drive disease pathogenesis via activation of NMO-Ig secreting B cells.

Because NMO-IgGs are clearly pathogenic, understanding the interactions between T cells and B cells in the context of AQP4 and the disease associated *HLA-DRB1*03:01* is critical for inhibiting the generation of the disease driving autoantibody. This interaction is crucial at many stages of the disease. For naïve AQP4-specific B cells, signals from MHC II molecule, co-stimulatory molecules, and Th_{17} secreted

proinflammatory cytokines initiate activation and isotype switching from IgM to IgG specific for AQP4.autoantibodies. Once these activated B cells become plasma cells, they continuously secrete the autoantibody for the life of the cell from the bone marrow of the patient. Long-term survival of plasma cells has been shown to require T cell help for long term survival. Elucidation of AQP4 as the autoantigen has been important in beginning to understand the T cell role in the context of NMO.

My studies show that hAQP4 peptides can be presented on the *HLA-DRB1*03:01* MHC II molecule and lead to the activation of $CD4^+$ T cells specific for $hAQP4_{281-300}$ in *HLA-DRB1*03:01* transgenic mice. $hAQP4_{281-300}$-specific T cells are capable of causing class switching in AQP4-specific B cells resulting in IgM isotype switching to the IgG2b isotype in *HLA-DRB1*03:01* transgenic mice. The murine IgG2b isotype is similar to the human IgG isotype in its capability of causing complement-dependent cytotoxicity and antibody dependent cellular cytotoxicity. With my studies showing that $hAQP4_{281-300}$-specific T cells drive isotype switching, these autoreactive cells could play a major role in disease pathogenesis in the context of NMO via activating AQP4-specific B cells that secrete the pathogenic NMO-IgG. Additionally, the Th_1 and Th_{17} inflammatory response scene in $hAQP4_{281-300}$-specific T cells are able to play an important role in localizing and activating the lesion infiltrating eosinophils, neutrophils, and other pathogenic cells as well as complement. While NMO-IgG is critical for the astrocyte death, the $CD4^+$ T cells play an essential role in initiating and prolonging the inflammatory environment that leads to pathogenesis. All of these critical

events support the idea that targeting AQP4 specific T cells will be crucial for the generation of NMO specific therapeutics.

There are currently several therapies that are effective in ameliorating the disease course of NMO. Recently, several open label trials with the anti-CD20 chimeric monoclonal antibody rituximab have been conducted (266-268). The rationale for utilizing rituximab as a pharmacotherapy in patients with NMO was derived from histopathological evaluations of biopsy and autopsy material, where IgM and IgG deposition were abundantly present (158). As stated above, data from relatively small, uncontrolled retrospective and prospective studies show that rituximab appears to have a substantial effect on the relapse rates in NMO, which has been used as a clinical outcome. To date, no class I evidence from placebo controlled treatment trials support the use or rituximab or other B cell depleting agents in patients with NMO. Anti-CD20 therapy is potentially immunosuppressive. The U.S. Food and Drug Administration (FDA) recently approved changes to the prescribing information of ofatumumab and rituximab to add new Boxed Warning information about the risk of reactivation of hepatitis B virus (HBV) infection (269). The FDA also issued a warning that two patients have died after being treated with rituximab for systemic lupus erythematosus (SLE) (270). While these patients had previously been treated with immunosuppressant agents and glucocorticosteroids, these observations strongly suggest that depletion of $CD20^+$ cells may have an immunosuppressive effect in some patients**.** Eculizumab, a therapeutic monoclonal IgG that neutralizes the complement protein C5 was recently

tested in 14 patients with NMO spectrum disorders (271). While most patients experienced symptomatic improvement, one patient had meningococcal sepsis and sterile meningitis about 2 months after the first eculizumab infusion. These two examples show that B cell and complement-directed therapies may very well be effective. However, they are broad immunosuppressants and they carry with them relatively high risks of potentially fatal infections.

Modulating or depleting pro-inflammatory T cells has been the basis of various therapies in MS, and might also benefits patients with NMO. Fingolimod is an agent with a relatively novel mode of action that is currently approved in the United States of America to treat patients with RRMS (272), and in Europe to treat patients with RRMS who failed first line treatment, or who display an evolving severe RRMS clinical phenotype (273). Fingolimod engages the sphingosine-1-phospate (S1P) receptors mostly on $CD4^+$ T cells. Consequently, these $CD4^+$ T cells are unable to egress from lymphatic tissues(274). Lymphocytes that reside within secondary lymphoid organs account for approximately 2% of the entire lymphocyte pool. The biological functions of circulating lymphocytes do not seem to be affected by fingolimod. While there is reason to believe that fingolond therapy may benefit patients with NMO, there are currently no controlled studies to show this.

The poster child for pharmacotherapies that prevent the migration of leukocytes into the CNS is natalizumab, a monoclonal recombinant humanized IgG_4 mAb targeting the α4-chain of α4β1 integrin and other α4-integrin-containing adhesion molecules. The

development of natalizumab is the result of rational drug design based on the knowledge of cell migration and adhesion molecules that was accumulated over the past three decades. Migration of leukocytes from the blood into the CNS involves multiple steps (275). It is believed that slow rolling on endothelial walls allows leukocytes to identify proper arrays of chemoattractants and integrin ligands. Prolonged selectin-mediated rolling of neutrophils and lymphocytes may also lead to integrin activation (276-278). Once firmly arrested, integrins facilitate the binding of leukocytes to other leukocytes and platelets. Activated T cells and B cell blasts express highly adhesive integrins (279). All other circulating leukocytes maintain their integrins in mostly inactive states and must undergo *in situ* modulation to develop high avidity for their specific ligands (280). Following rolling adhesion, the arrest of lymphocytes and myeloid cells in venules is mediated by the *in situ* activation of at least one of the four main integrins: α4β1, LFA-1, Mac-1, or VLA-4 (275). Integrins of the β1 subfamily, specifically VLA-1, VLA-2, VLA-4, VLA-5 and VLA-6, have been shown to facilitate leukocyte migration across the basement membrane of blood vessels (281), and across extracellular matrix (ECM). There is considerable redundancy: Multiple ligands have been identified for a single receptor, and multiple receptors bind a single ligand (281). The proadhesive properties of integrins are overlapping and additive, and depend on specific cytoskeletal and transmembrane associations with cytoskeletal adaptor molecules (282). Integrins are activated bidirectionally: (1) Cytoplasmic rearrangements of their subunit tail, and (2) extracellular binding by their ligands (283). A specific combination of chemokines

and G protein–coupled receptors (GPCRs) is required for activation of integrin-dependent arrest under shear flow. Engagement of α4-integrin by natalizumab impairs its ability to bind to vascular cell adhesion molecule-1 (VCAM-1) and its other ligands, including fibronectin. As a consequence, leukocytes are diminished in their ability to adhere to the inner lining of cerebral vascular walls, and to subsequently migrate through the blood-brain-barrier (BBB) into the CNS (284-287). Due to the broad targeting of all T cells with these therapies, patients are left immune compromised. Lethal infections such as progressive multifocal leukoencephalopathy are known to occur in a small subset of patients given these therapies. In addition, while natalizumab is very effective in preventing disease activity in MS, it appears to not have beneficial effects in patients with NMO. On the contrary; most patients series suggest that natalizumab therapy may lead to NMO disease exacerbation (165, 288, 289). Rather than broadly targeting all $CD4^+$ T cells, new therapies must target antigen specific T cells to allow for normal immune surveillance for patients post treatment.

There are currently only a few theorized therapeutic methods that target antigen-specific T cells. The first method utilizes altered peptide ligands (APL) to cause antigen specific $CD4^+$ T cells to become anergic (290). APLs are peptides that share sequence homology to autoantigens, but due to single or multiple mutations in key residues that bind the TCR of autoreactive T cells, the cellular immune response is down regulated with some theories suggesting that the T cells become anergic (291). Within the EAE animal model, this method has shown to be effective when the altered peptide ligand is

a derivative of the autoantigen that is driving the disease phenotype (292)(293-295). Early trials in MS patients showed little to no change in patient EDSS score; however, there was a correlation between clinical outcomes and the frequency of MBP_{83-99} (the APL target antigen in these trials) specific T cells. Since multiple autoantigens may be driving the pathogenesis in MS, knowing the autoantigen of NMO allows for APL therapies to be more effective. The second method was developed by Stephen Miller's group at Northwestern, in which they utilize either biodegradable nanoparticles or apoptotic cells that are cross-linked to autoantigens on their surface and injected intravenously into patients (296, 297). In mice, beads or apoptotic splenocytes cross-linked with EAE driving myelin antigens lead to T cell unresponsiveness in a peptide-dependent manner (298). Either method could be utilized with the immunogenic peptide identified in my studies in order to effectively treat NMO patients with a high frequency of $hAQP4_{281-300}$-specific $CD4^+$ T cells in the periphery. Though NMO pathogenesis is thought to be primarily driven by the NMO-IgG, characterizing and inhibiting AQP4-specific T cells from activating AQP4-specific B cells should prove beneficial in reducing disease progression in patients. Targeting T cells as a treatment option in NMO would only be effective in combination with a B cell depleting therapy due to memory B cells that secrete anti-AQP4 antibodies. And while Rituximab depletes a large portion of those autoantibody secreting B cells, it does not deplete $CD20^-$ plasma cells in the bone marrow (299). Future treatment options would require an anti-CD19 therapy to target all

memory B cells secreting NMO-IgG, while an additional therapy concurrently targeted AQP4-specific T cells to block the activation of future NMO-IgG secreting plasmablasts.

My preliminary studies utilizing peripheral blood mononuclear cells from NMO patients who are either positive or negative for *HLA-DRB1*03:01* showed that there are clearly hAQP4 peptide specific cells capable of secreting IFNγ circulating through the periphery of both experimental groups (data not shown). Studies performed in other labs have also shown that there is a clear cellular immune response against hAQP4 peptides that results in the proliferation of $CD4^+$ T cell (249). The presence of hAQP4-specific cytokine secreting cells in the periphery could be used as a biomarker for diagnosing NMO patients. In preliminary studies with a small subset of NMO spectrum disorder (NMOSD) patients (n=4), I discovered that half of these patients had memory cells proliferating against a multitude of hAQP4 peptides sometimes even against $hAQP4_{281\text{-}300}$, while the others did not (data not shown). An ELISpot assay or a tetramer assay could detect the frequency of $hAQP4_{281\text{-}300}$-specific T cells in a disease specific and HLA haplotype-specific manner. Once patients are found to have these cells present in the periphery, targeting these cells would most likely aid in slowing down the pathogenesis of the disease primarily through inhibiting the reactivation of AQP4 specific B cells.

Future Directions

*Examining HLA-DRB1*03:01 NMO Patients*

In spite of the insight obtained from my studies in the *HLA-DRB1*03:01:* transgenic mouse, my results must still be verified within the context of NMO patients. It is very possible that $hAQP4_{281-300}$ is bound to the *HLA-DRB1*03:01* MHC II molecule results in, negative selection and the deletion of all autoreactive T cells in most individuals. The frequency of $hAQP4_{281-300}$-specific $CD4^+$ T cells in NMO patients could be determined via tetramer enrichment. Briefly, fluorochrome-labeled beads are covalently linked to four recombinant MHC II molecules with a peptide loaded into each of the binding grooves. Incubation of PBMCs with the tetramer allows for the labeling of T cells with TCRs that bind to the specific MHC II-peptide complex. Because the frequency of antigen specific T cells is extremely low, enrichment of these cells can occur via magnetic separation through targeting of the flourochrome linked to the tetramer bead. This allows for the enrichment of tetramer binding T cells when PMBCs are passed through a magnetic column. From this point, cells could be sorted or stained for a number of variables that allow for a greater characterization of these low frequency antigen-specific T cells.

Our lab has been collaborating with Gerald Nepom at the Benaroya Research Institute to generate $hAQP4_{281-300}$-*HLA-DRB1*03:01* tetramers that would allow for the comparison of *HLA-DRB1*03:01* positive NMO patients versus healthy *HLA-*

*DRB1*03:01* positive healthy controls. As the peptide was determined in an HLA specific manner, it is pertinent to the study that all individuals be positive for the HLA-DRB1*03:01 haplotype. It may also be of interest to perform serum antibody ELISAs to titer the $hAQP4_{281-300}$-specific IgG in *HLA-DRB1*03:01* patients. If a positive correlation is found between the frequency of $hAQP4_{281-300}$-specific T cells and $hAQP4_{281-300}$ IgG titer levels, this ELISA could be utilized by clinicians as a test to determine whether specific treatments options are viable on a case to case basis.

Examining the role of HVC in the generation of $hAQP4_{281-300}$ specific T cells.

Once a positive association is confirmed, it would be pertinent to examine the cross-reactivity of the peripheral hAQP4-specific T cells to the HVC_{19-34} peptide. I would begin to study an association between these two entities by examining *HLA-DRB1*03:01* HVC patients to quantify the frequency of HVC_{19-34} specific T cells in the periphery via tetramer enrichment. Initially, I would perform ELISpots to quantify the number of antigen specific cells that secrete Th_{17} associated cytokines (IL-17, IL-6, and IL-21) in response to HVC_{19-34} and $hAQP4_{281-300}$. Once I have verified that $hAQP4_{281-300}$ specific T cells are present in NMO negative HVC patients, I would then incubate PBMCs taken from said patients with either HVC_{19-34} or $hAQP4_{281-300}$ *in vitro* for 48-72 hours, then enrich for antigen specific cells utilizing either a DRB1*03:01-HVC or a DRB1*03:01-hAQP4 tetramer. Cells could either be stained for Th_{17} markers or sorted for and utilized in low cell number qPCR to examine IL-17 and RORγT mRNA levels.

I would expect to see that PBMCs incubated with either the HVC_{19-34} or the $hAQP4_{281-300}$ peptide generate a strong Th_{17} immune response. Control experiments would utilize alternative hAQP4 peptides that are found to be strong binders to the *HLA-DRB1*03:01* MHC II molecule, but show no sequence homology to HVC.

It may be of use to also examine *HLA-DRB1*03:01* positive NMO patients with latent or chronic HCV infections. To demonstrate that molecular mimicry between HCV_{19-34} and $hAQP4_{281-300}$ drives NMO disease relapses, it is necessary to perform longitudinal studies to show that HCV relapse infections result in the reactivation of $hAQP4_{281-300}$ specific T cells prior to NMO disease symptoms.

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