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EXAMINING THE ENCEPHALO-ANTIGENIC AND HLA-DR BINDING CAPACITIES OF VARIOUS MYELIN PEPTIDES

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ABSTRACT

Activated CD4+ T-cells targeting specific auto-antigenic peptides of myelin sheath proteins are thought to mediate the pathogenesis of myelin degeneration, axonal and neuronal damage, and glial scaring in multiple sclerosis (MS) patients and experimental autoimmune encephalomyelitis (EAE) mouse models. The disease mechanisms that cause MS remain unknown, but susceptibility maps to specific HLA-DRβ1 alleles (especially 04.01, and 15.01) whose products help present antigens to the immune system. To investigate the genetic predisposition of MS in patients as it pertains to specific peptide/MHC complexes, three recombinant HLA-DRβ1 complexes (01.01 (control), 04.01, and 15.01) were constructed containing 18 different peptides derived from three sheath proteins: myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP). The genes were cloned into the pBAC vector and transfected into SF9 insect cells to evaluate surface expression as it relates to the auto-antigenic activity of various peptide/MHC combinations. FACS analysis was used to evaluate HLA-DR expression in the transfected SF9 cells. Individual myelin peptides were also assayed for the ability to induce EAE in transgenic HLA-DRβ1 04.01 and 15.01 mice. The data showed that two specific sheath peptides (MBP-30-44 and MOG-25-55) induced MS-like paralysis when injected into HLA-DRβ1-15.01 mice, indicating those peptide/HLA-15.01 combinations could play a role in MS induction. Ultimately, the peptide/HLA-DR constructs corresponding to encephalogenic epitopes will be used to make pMHC tetramers to monitor the myelin-specific T cell response in MS patients.

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BACKGROUND

Autoimmune Disorders

Autoimmune disorders are a systemic failure of the body's immune system to recognize antigens of healthy cells and tissues as self (non-foreign). Immune cells normally removed from the body that recognize self-antigens are not eliminated and generate a progressive deterioration of healthy tissue which often leads to debilitating circumstances that range from mild to death. Over 80 different autoimmune disorders have been characterized, and many more diseases are believed to be autoimmune driven. Some of the best known autoimmune diseases are rheumatoid arthritis, vitiligo, psoriasis, type-1 diabetes, and multiple sclerosis. Auto-immune responses are usually thought to be spontaneous or genetically predisposed lymphocyte attacks that occur on specific antigens at the surface of healthy cells and tissues. The differences among the various diseases lie in which peptide becomes auto-antigenic and the type of the immune responses. This project investigated the autoimmune disorder multiple sclerosis.

Multiple Sclerosis

Multiple sclerosis (MS) is the most common autoimmune disorder of the central nervous system (Berer and Krishnamoorthy, 2014). Between 2 and 2.5 million people are thought to be affected globally, with rates varying widely among different populations (Global Burden of Disease Study, 2013). It is an inflammatory disease in which the myelin sheath insulating covers of nerve cells in the brain and spinal cord become damaged. The myelin sheath is a multi-layered membrane, unique to the nervous system, which functions as an insulator to greatly increase the velocity of axonal impulse conduction. Damage to the sheath disrupts nervous system communication, resulting in a wide range of symptoms including physical and mental (Compston and Coles, 2008). There is no known cure. MS has several forms. Symptoms can

either occur in isolated attacks (relapsing MS), or increase over time (progressive MS) (Lublin and Reingold, 1996). Permanent neurological problems often occur as the disease advances.

Symptoms of the disease differ between individuals making a definitive diagnosis difficult without an MRI. The most common symptoms are fatigue, numbness, tingling, weakness, dizziness, vertigo, spasticity (wide-range of muscle spasms), vision problems, bladder problems, bowel problems, emotional changes, depression, body pain, and sexual problems (National Multiple Sclerosis Society). Lesser common MS symptoms include speech problems, tremors, breathing problems, headaches, seizures, itching, hearing loss, and swallowing problems (National Multiple Sclerosis Society). Leaving primary symptoms untreated can cause debilitating secondary and tertiary symptoms such as constant urinary tract infections, decreased bone density, shallow breathing, and immobility (National Multiple Sclerosis Society).

MS Pathophysiology

MS has three main characteristics: 1) the formation of lesions in the central nervous system (also called plaques), 2) inflammation, and 3) the destruction of the myelin sheaths of neurons (Compston and Coles, 2008). The name multiple sclerosis refers to the plaques or lesions (sclerae) that form in the nervous system. The lesions most commonly form in the white matter in the optic nerve, brain stem, basal ganglia, and spinal cord, or white matter tracts close to the lateral ventricles (Compston and Coles, 2008). White matter cells carry signals between grey matter areas (where neuronal processing is done) and the rest of the body. The peripheral nervous system is rarely involved. MS involves the loss of oligodendrocytes, the cells responsible for creating and maintaining the myelin sheath fatty layer that helps the neurons carry electrical signals. The loss of oligodendrocytes results in a thinning of myelin and, as the

disease advances, the breakdown of neuronal axons. Scars eventually result from an ineffective repair process (Chari, 2007).

With respect to the inflammation and destruction of the myelin sheath, the inflammatory process is caused by CD4+ T-cells that gain entry into the brain via disruptions in the blood–brain barrier (BBB). The T-cells recognize myelin as foreign and attack it. As the attack progresses, the BBB breaks down further, causing other damaging effects such as swelling, activation of macrophages, and more activation of cytokines and other destructive proteins. MS has a complex and poorly understood pathogenesis (McFarland and Martin, 2007). The disease is a degenerative process which begins with the formation of acute inflammatory legions at the blood-brain barrier (BBB). This leads to demyelination, axonal and neuronal damage, and glial scaring (McFarland and Martin, 2007). The current model suggests that the breakdown of the BBB is caused by an active T-cell immune response to the central nervous system (CNS). The specific target antigen(s) in multiple sclerosis are not well characterized, but several studies have suggested that the T-cell response targets myelin antigens (McFarland and Martin, 2007), although no specific myelin antigens have been proven to participate in the disease.

MS Genetics and HLA

MS is believed to develop from the interaction of the individual's genetics and as yet unidentified environmental causes. MS is not considered a hereditary disease, but several genetic variations have been shown to increase risk (Dymet et al., 2004). In identical twins, both individuals are affected about 30% of the time, while only 5% are affected for non-identical twins (Hassan-Smith and Douglas, 2011). If both parents are affected, the risk in their children is 10 times that of the general population (Milo and Kahana, 2010).

Genes linked with MS predisposition mostly include mutations in the human leukocyte antigen (HLA) system. This system is a group of genes located on chromosome-6 (**Figure-1**) that serves as the major histocompatibility complex (MHC). Mutations in the HLA region correlating with MS have been known for over 30 years, and the same region has also been implicated in the development of other autoimmune diseases such as diabetes type I and systemic lupus erythematosus (Baranzini, 2011). The strongest correlations between MS and genes occur at the HLA-DRβ1 locus with alleles HLA-DRB1 15.01 and HLA-DQ6 (diagram left side) (Barcellos, 2006; Deluca et al., 2007; Compston and Coles, 2008; Comebella et al, 2008). It has been estimated that HLA mutations account for 20-60% of MS genetic predispositions (Baranzini, 2011; Briggs et al, 2010).

Other than the HLA locus, modern genetic methods (genome-wide association studies) have discovered at least twelve other genes that modestly increase the probability of MS (Baranzini, 2011). Other loci have been shown to have a protective effect, such as HLA-C554 and HLA-DRB1*11. A genome-wide scan of 500,000 single nucleotide polymorphisms (SNPs) identified locus 13q31.3 as being associated with MS (Comabella et al., 2008).

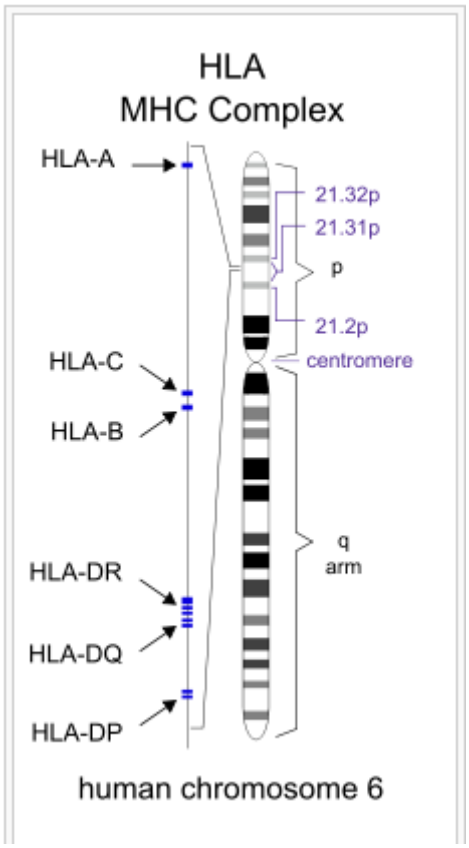

Figure-1: Diagram of Human Chromosome-6 Showing Genes Associated with Multiple Sclerosis. Shown are the genes of the human leukocyte antigen (HLA) system (diagram left side) which serve as a major histocompatibility complex. Strong associations are known for HLA-DR15 and HLA-DQ6. (Compston and Coles, 2008)

This MQP will examine HLA-DRβ1variants 15.01 and 04.01, both have been correlated with MS patients (Weatherby et al., 2001). HLA-β1 variant 15.01 is found more frequently in MS patients and is associated with an early development of MS symptoms and a worsening of symptoms at a later stage (15 years or more) (Weatherby et al., 2001). The heterogeneous presentation of MS in patients suggests that there are multiple antigen/peptide and HLA combinations associated with the pathogenesis of the disease (Disanto et al., 2011). The HLA-DR locus is downstream of the HLA-DQ locus and upstream the class III and class I HLA loci (**Figure-2**). The HLA-DR gene contains two β loci and one α locus. HLA-DRα has no known

functional variation and only encodes 3 alleles, unlike the HLA-DRβ loci (Janeway et al., 2008). The two HLA-DRβ1 loci in particular are ubiquitous and encode an estimated 463 alleles which allows it to comprise the majority of variation in the HLA-DR gene segment (Janeway et al., 2008). Most of the variation occurs in the HLA-DRβ1 peptide-binding groove, enabling the complex to bind with a wide variety of peptides (9 amino acids or longer) and T-cell receptors (Janeway et al., 2008).

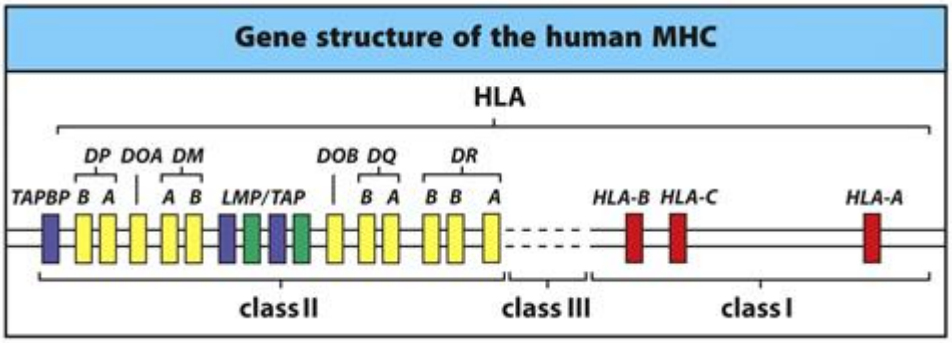

Figure-2: Diagram of the Genetic Organization of the Human Leukocyte Antigen (HLA) Locus. The figure shows three classes of HLA genes: class II (left), class III (middle), and class I (right). Most of the known MS variants map to the HLA-DRβ1 locus (yellow, diagram center). The HLA-DP, HLA-DOA, HLA-DOB, and the HLA-DQ regions (and their corresponding molecules LMP, TAP, and TAPBP) are shown upstream of the HLA-DR regions. (Janeway et al., 2008)

Other Factors Correlating with MS

Environmental factors like vitamin D deficiency from lack of sunlight (Hansdottir et al., 2008; Pereira et al., 2015), viral infections such as Epstein-Barr virus (Ascherio and Munger, 2007; Serafini et al., 2007; Pereira et al., 2015), and smoking (Ramagoplan et al., 2013; Pereira et al., 2015) have also been shown to increase the risk of developing MS, although the mechanisms remain unknown.

EAE Mouse Model

To study the pathogenic mechanisms of MS, an experimental autoimmune encephalomyelitis (EAE) animal model is often used. This model uses the artificial induction of

activated T-cells to cause inflammation, demyelination, axonal loss, and gliosis (Constantinescu et al., 2011) which mimics MS. The model is often used in MS research because of its versatility of induction, and the ability of EAE to accurately mimic MS pathogenesis (Constantinescu et al., 2011).

EAE induction usually uses adjuvants to sensitize the animal's immune system to myelin antigens. The adjuvants commonly contain bacterial components that produce a strong immune reaction, and as a result compel auto-reactive T-cells to the BBB (Constantinescu et al., 2011). Lethal irradiation is sometimes used to activate EAE because it disrupts the BBB artificially allowing activated myelin T-cells to more easily access the myelin proteins. The artificial induction process is unlike the induction of MS, but the model continues to be used to test MS drugs and study the disease. Due to the use of an artificial induction system for inducing EAE, the mouse model is not used for studying MS genetic predispositions. The EAE model only provides researches with a blueprint for creating treatments that can inhibit the immune system and/or inhibit the migration of CD4+ T-cells to the BBB.

T-Cell Development

Because MS is driven by myelin-activated CD4+ T-cells that infiltrate the BBB, this MQP project will focus on the CD4+ T-cell activation response in MS, and the formation of CD4+ T-cells is discussed here. T-cell development is heavily driven by the thymic microenvironment and the binding of the cells to peptide-major histocompatibility complex's (MHCs) at the surface of antigen presenting cells (APCs) (**Figure-3**). T-cell fate begins when lymphoid progenitors derived from hematopoietic stem cells migrate from the bone marrow to the thymus; simultaneously losing their potential for becoming natural killer cells and B-cells (Germain, 2002). T-cell-committed lymphoid progenitors in the thymus lack a T-cell receptor (TCR), and are also CD4 and CD8 negative, so are termed double-negative (DN) T-cells (i.e.

TCR-negative and CD4-negative) (Germain, 2002). DN T-cells can then differentiate into either γδ or αβ TCR-expressing cells.

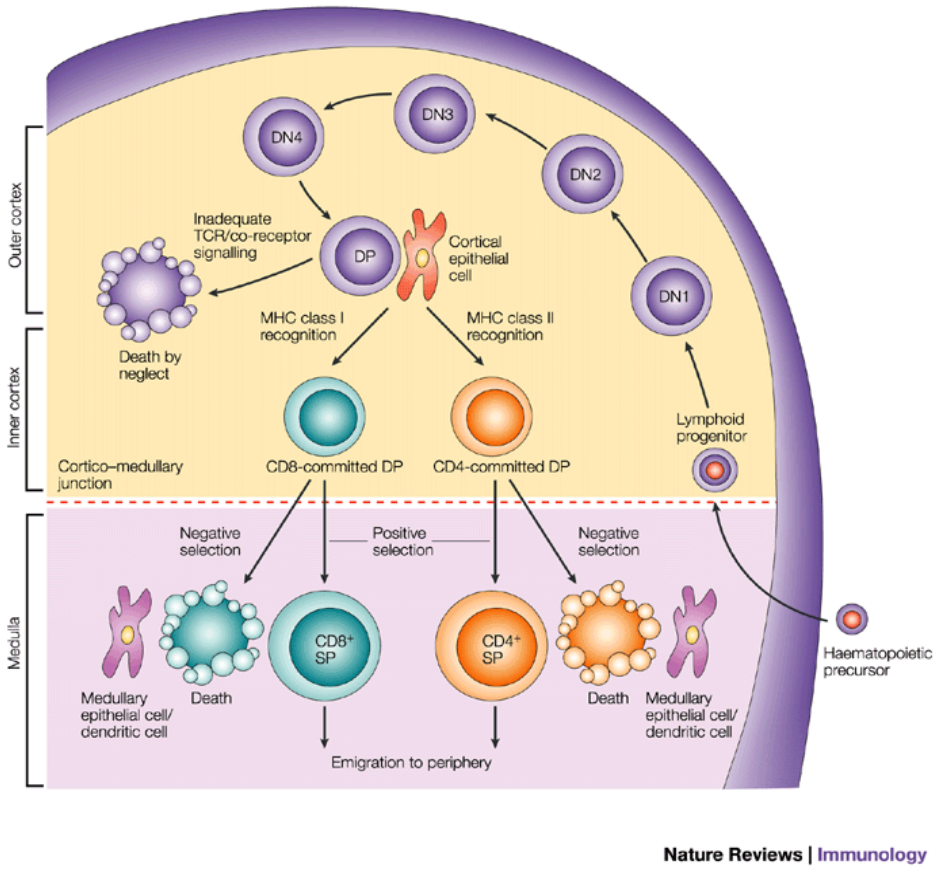

Figure-3: Diagram Outlining T-Cell Development in the Thymus. Shown are the processes of positive selection (whereby CD8+ and CD4+ T-cells are made) (diagram center) and negative selection (diagram lower left) whereby T-cells are destroyed. (Germain, 2002)

Formation of αβ TCR-Expressing CD4+ T-Cells

This project focuses on the αβ TCR-expressing cells which give rise to CD4+ T-cells. DN T-cells first begin to express the pre-TCR-α chain encoded by a non-rearranging locus. The TCR-β chain unlike the TCR-α chain is produced through somatic recombination which creates enormous sequence variability within a structurally conserved framework (Germain, 2002). The TCR-β locus consists of three complementarity determining regions (CDRs) CDR1, CDR2, and

CDR3, respectively. The CDR3 region of the TCR-β locus is the most structurally variable region of the locus, containing V, D, and J segments which produce highly specific CDRs referred to as a V(D)J recombination (Harraway, 2013). The pre-αβ TCR rearrangement creates approximately $1x10^{15}$ unique T-cell receptors. Pre-αβ T-cells undergo TCR-β rearrangement, a maturation process that involves active signaling with a collection of proteins. The active signaling prevents the maturation of pre-αβ T-cells that contain mutations in key T-cell proteins and allow normal pre-αβ T-cells to mature (Harraway, 2013).

Once emerging from β-selection, the T-cells undergo 6-8 cell divisions and TCR α-locus recombination forming mature αβ TCRs. The mature thymocytes also begin to express CD8 and CD4 receptor proteins on their respective cells making them double-positive (DP) T-cells (i.e. TCR+/ CD4+) (Germain, 2002).

T-Cell Selection

Approximately 90% of DP T-cells undergo apoptosis during the first stage of TCR signaling-mediated selection. Cell death is driven by the lack of TCR interaction with available peptide-MHC ligands expressed at the surface of APCs (**Figure-4**). Without adequate binding to peptide-MHC complexes, the cells do not produce essential intracellular signals required to sustain variability (Germain, 2002) resulting in cell death. The remaining 5-10% of the T-cells undergo additional selection processes referred to as positive and negative selection. Negative selection prevents TRCs that bind extremely well with MHC ligands from surviving, which could cause autoimmunity. TCRs that produce a balanced signal (not too weak and not too strong) between MHC ligands begin a single positive (SP) lineage divergence referred to as positive selection.

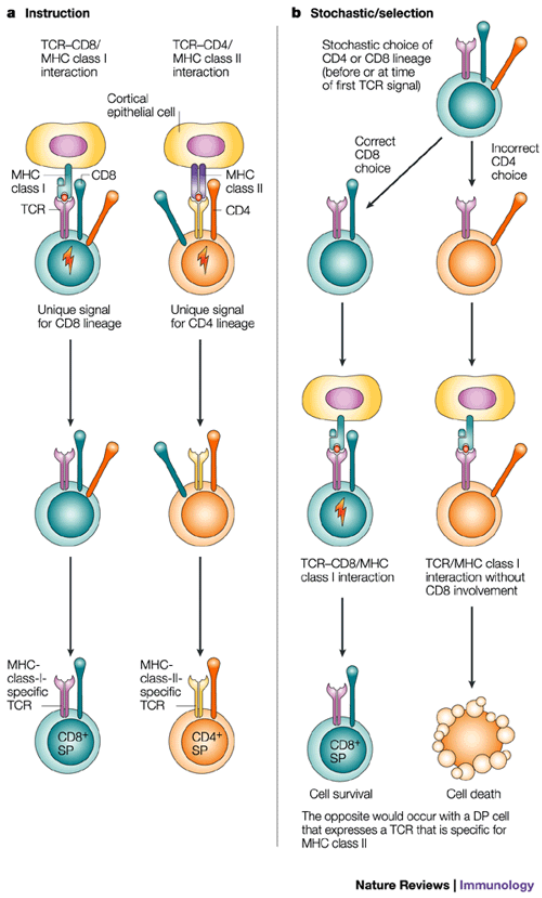

Figure-4: Diagram of T-Cell Selection. Shown are the Instruction Model (left side) and Stochastic Model (right side) of double-positive T-cell lineage divergences. (Germain, 2002)

Role of MHC I and II in T-Cell Selection

Lineage divergence of double-positive thymocytes (i.e. TCR+/CD4+) is mediated by MHC class I and class II complexes. The major histocompatibity complex (MHC) is a heterodimeric glycoprotein that serves as APC surface receptor that binds to TCRs (Zacharias et al., 2004). MHC molecules are subdivided into class I and class II surface molecules. Class-I MHC molecules present intracellular-derived antigen fragments to CD8+ T-cells, while class-II MHC molecules present exogenously-derived antigenic peptides to CD4+ T cells (**Figure-4**). Differentiation of DP T-cells to SP cells involves the silencing of transcription factors of either the CD4 or CD8 co-receptor locus. It is suggested that the silencing of one lineage locus is due to

affective signaling to either MHC class I or II molecules, or that the silencing occurs randomly, the latter is more supported (**Figure-4**).

Autoimmunity and T-Cell Selection

Cross-reactivity results from a failure of the T-cell negative selection process to eliminate self-peptides during T-cell maturation. During autoimmunity, self-peptides are recognized as foreign antigens, T-cells reactive against them are not eliminated, and they mount an attack against tissues expressing the self-antigen. Because MHCs express the antigens in APC's, they play a significant role in T-cell development and cross-reactivity. As previously discussed in the MS genetics section, some HLA alleles are associated with autoimmune disorders, including MS. The strongest correlations are for the HLA-DR locus (Deluca et al., 2007). The specific HLA-DR peptide sequence that triggers myelin-autoreactive T-cells in MS is unknown, and is the subject of this project. Variants in the HLA-DR β1 locus appear to be involved, but not HLA-DRα (Deluca et al., 2007).

PROJECT PURPOSE

As stated in the Background section, activated CD4+ T-cells targeting specific auto-antigenic peptides of myelin-sheath proteins are thought to mediate MS pathogenesis, but the mechanism remains unknown. MS susceptibility maps to specific HLA-DRβ1 alleles whose products help present antigens to the immune system. MS susceptibility increases with the expression of the HLA-DRβ1 locus. HLA-presented antigens at the surface of antigen presenting cells (APCs) are crucial for the development of T-cells and are also involved in T-cell auto-reactivity.

Current mouse EAE research models are limited in their usefulness. The current model uses an artificial induction of activated T-cells to induce inflammation, demyelination, axonal loss, and gliosis that mimic some aspects of MS, but it cannot account for the heterogeneous symptoms observed in MS patients, nor can the model accurately demonstrate how genetic factors (such as the HLA-DRβ1 alleles) and environmental factors activate CD4+ T-cells to generate an immune response at the BBB against self-antigens.

In an effort to determine the combination of peptide / HLA-DRβ1 molecule(s) responsible for the auto-reactivity of myelin reactive T-cells, we constructed over 45 soluble HLA-DR β1 molecules presenting 18 different myelin peptides. Two MHC variants HLA-DR β1 04.01, and HLA-DR β1 15.01 are known to increase the risk of autoimmune disorders such as MS (Barcellos et al, 2006; Deluca et al, 2007). These two MS MHC variants, and a control HLA-DR β1 01.01 (not know to increase the risk of MS) were constructed by recombinant DNA cloning, and combined with the genes encoding 18 different myelin peptides, and expressed at the surface of Sf-9 insect cells to determine the auto-antigenic capacity of each pMHC complex.

The myelin peptides tested were selected via MS patient mapping and the IEDB epitope prediction database (see Methods). Each peptide represents a 12-19 amino acid portion of full-length proteins located at the surface of myelin sheaths. The three sheath proteins tested were myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP). PLP is essential for the compaction of myelin sheaths and may help stabilize and maintain myelin (Nadon et al., 1998). MBP is the most abundant protein on myelin sheaths, and is commonly used as a marker for the breakdown of sheaths (Deber and Reynolds, 1991). The role of MBP is scarcely understood, but it may also interact with lipids to stabilize and maintain the correct structure of myelin. Myelin oligodendrocyte glycoprotein (MOG) has been found to have no phenotype when knocked-out in mice (Delarasse et al., 2003), so its normal function remains unknown. But MOG T-cell activation appears to be important in EAE models (Delarasse et al., 2003).

In order to identify the encephelo-antigenic properties of each MHC and peptide combination, transgenic HLA-DR β1 15.01 and HLA-DR β1 04.01 mice were challenged with the full-length peptides identical to the ones used to construct the pMHC soluble molecules. The challenged mice were then scored using the EAE scoring system to phenotypically identify whether the mice had EAE.

METHODS

Synthetic Peptides and Oligonucleotides

DNAs encoding various peptides to myelin sheath proteins were amplified by PCR from plasmids in our lab. The reverse-oligonucleotides used were developed using a combination of template overlapping region, peptide sequence, a linker, and one SpeI enzyme site, respectively. The oligonucleotides developed enabled us to PCR-in (clone the peptide-encoding sequence as part of the HLA PCR) each peptide sequence with corresponding HLA-DR β1 variants. The forward-oligonucleotides were designed previously in our lab, and primed the HLA-DR β1chain. 18 peptides corresponding to different myelin sheath protein epitopes were designed (**Table-1**), and each were combined with the three HLA-DR β1 variants tested (01.01 (control), 04.01, and 15.01).

Myelin Peptides Amino Acid Sequences	
PLP 190-204	SKTSASIGSLCADARMY
PLP 175-192	YIYFNTWTTCQSIAFPSK
PLP 172-195	TWTTCQSIAFPSKTSA
PLP 39-53	LTGTEKLIETYFSKN
PLP 40-58	TGTEKLIETYFSKNYQDYE
PLP 181-195	TTCQSIAFPSKTSAS
PLP 91-110	YTTGAVRQIFGDYKTTICGK
PLP 97-108	RQIFGDYKTTIC
MOG 33-47	TGMEVGWYRSPFSRV
MOG 38-51	VGWYRSPFSRVVHLY
MOG 97-108	TCFFRDHSYQEE
MOG 31-49	NATGMEVGWYRSPFSRVVH
MOG 41-55	RPPFSRVVHLYRNGK
MBP 85-100	ENPVVHFFKNIVTPRT
MBP 30-44	PRHRDTGILDSIGRF
MBP 85-99	ENPVVHFFKNITPR

MBP 131-145	ASDYKSAHKGFKGVD
MBP 140-154	GFKGVDAQGTLSKIF

Table-1: Amino Acid Sequences of Myelin Sheath Peptides. Shown are the 18 different peptide sequences chosen for testing in this project, and their corresponding amino acid sequence used to produce the various peptide-MHC combinations. The three sheath proteins tested were myelin proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG), and myelin basic protein (MBP).

The peptides used in the EAE injection experiments were synthesized at the University of Massachusetts Medical School. In order to minimize frameshifts in the pMHC constructs which would disrupt the TCR-binding region of the MHC complex, truncated versions of the peptides were used. Full-length peptides were utilized in the EAE induction experiments.

Recombinant HLA-DRβ1 pBAC Plasmid Constructs

HLA variant cDNA genes 01.01 (control), 04.01, and 15.01 were amplified by PCR from plasmids prepared in our lab. To display the pMHC combinations at the surface of baculovirus infected cells, modifications were made by flanking the HLA-DR β1variants with Acc651 and BsiWI restriction sites, and subcloning the fragments into pBAC vectors to enable the surface expression of the complex. The site encoding the HLA-DR β1 peptide-binding region was then disrupted by cutting with EcoRI and SpeI enzymes, and then ligated with similarly cut amplicons encoding various sheath peptides. This enabled the HLA-DR β1variants to express the previously designed peptides (Table-1) at the surface of the MHC molecules.

Plasmid DNA Isolation

The pBAC vector constructs containing recombinant HLA-DR/peptide sequences were transformed into XL1-Blue *E. coli* (Stratagene, La Jolla, California, United States). Promega DNA purification kits were used to isolate and purify plasmid DNA from *E. coli* cultures.

DNA Sequencing

DNA sequencing was used to verify the nucleotide sequences of the cloned constructs and to ensure that no mutations, frameshifts, and/or other unwanted alterations had occurred during the DNA construction process. The HLA-DR β1 region with corresponding peptide-encoding and transmembrane anchoring region were sequenced. pMHC pBAC construct was sequenced using Genewiz (Cambridge, MA), a contract research organization that specializes in DNA sequencing. Sequences were then analyzed using the pDRAW software (*PDRAW.* Sunnyvale, CA).

Transfection of SF9 Cells

The transfection protocol used was as previously outlined in Invitrogen expression protocols (ViraPowe BacMam Expression System and BacMam pCMV-DEST Vector Kit). $1x10^6$ Sf-9 cells were plated for each construct in supplemented Grace's medium. The cells were allowed to incubate for 45 minutes at 27^{o}C and then washed with unsupplemented Grace's medium and plated on 0.8ml of unsupplemented Grace's medium. A mixture of 20ng of purified baculovirus DNA, 500ng of plasmid DNA, 4ul of Cellfectin, and 100ul of unsupplemented Grace's medium was made and incubated for 45 minutes at room temperature. The mixture was then added to the Sf-9 cells and incubated at 27^{o}C for 4 hours. After the incubation, the cells were washed once with unsupplemented Grace's medium and plated on 3ml of Supplemented

Grace's medium. Cells were then incubated at 27^{o}C for 7 days, and viruses were harvested on day 14.

mAbs and Flow Cytometry

FACS analysis was used to assay the expression of various peptide/HLA-DR complexes on the surface of transfected SF9 cells. Transfected Sf-9 cells expressing peptide/HLA-DRB1 variants were harvested on day 7. 200ul of cells were collected from each Sf-9 transfection and spun down for 4 minutes at 1,400rpm. Cells were then stained with HLA-DR mAbs (Becton Dickinson Bioscience, Concord, MA) containing an allophycocyanin (APC) fluorophore to mark HLA-DR expression with the color blue. A balanced salt solution (BSS) buffer was then added to the stained cells and allowed to incubate at room temperature for 45 minutes. Once stained, cells were spun down for 4 minutes at 1,400rpm and washed with BSS, and once again spun down under the same conditions. After this wash, the stained cells were re-suspended in BSS wash buffer and analyzed by FACS using both a SORP and LSR machine.

EAE Induction/EAE Mice Scoring

In order to test the encephalo-antigenic nature of each peptide-MHC combination, NSG (NOD, SCID, IL2 common gamma chain deficient) mice with MHC II knockout and transgenic for either HLA-DRB1 15.01, or 04.01 were challenged with antigen/peptides previously described in Table-1. On day zero, NSG transgenic mice were injected subcutaneously with an emulsion consisting of a 4mg/ml concentration of complete Freud's adjuvant (CFA) (Miller et al., 2007) combined with 533ug of mycobacterium tuberculosis peptide and 400ug of a specific antigen/peptide. This injection was then followed by an intraperitoneal (IP) injection of 200ng of pertussis toxin, and two days following the first immunization a second IP injection of 200ng of

pertussis toxin was given (Miller et al., 2007) (**Figure-5**). Following the injections, the mice were weighed and scored daily for 27 days after the first emulsion and IP injection were given. A classical EAE scoring system was adopted for the study to systemically determine EAE symptoms (Hooke Laboratories, Lawrence, Massachusetts, United States). Mice were scored on a scale from 0 to 5, with zero being normal and 5 being morbid. A score of zero equates no apparent paralysis, a score of one equates to paralysis of the tail or an apparent limp tail, a score of two indicates the mice had a limp tail and one paralyzed hind limb, a score of three is associated with a limp tail and two both paralyzed hind limbs. Scores four and five are the most severe; a score of four indicates that the mouse has a limp tail, two paralyzed hind limbs, and partial front leg paralysis, and finally a scored of five equates to a mouse found completely paralyzed, or dead due to paralysis (**Figure-5**). Mice who appeared on the border of one score or another were scored with an additional 0.5 to indicate their possible transition to a higher score.

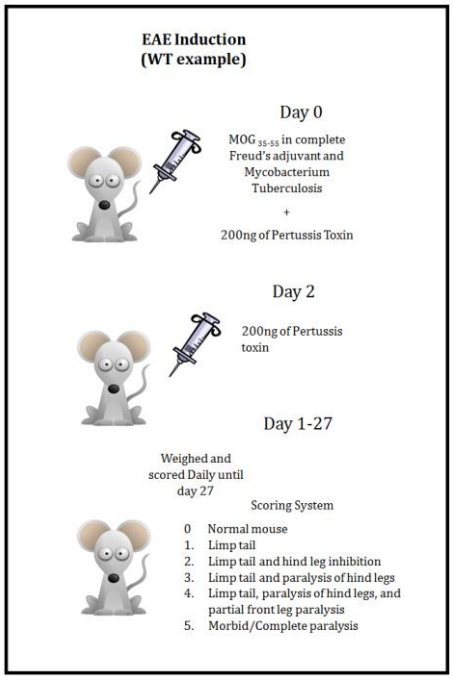

Figure-5: Diagram of Injections for EAE Induction and Scoring Procedure. The figure illustrates the EAE induction process of NSG HLA-DR transgenic mice induced with WT peptide/antigen MOG 35-55. The classical EAE scoring scale used to identify the disease in mice is also shown.

RESULTS

Current mouse EAE research models are limited in their representation of multiple sclerosis (MS) pathology. The current model uses an artificial induction of activated T-cells to induce inflammation, demyelination, axonal loss, and gliosis that mimic some aspects of MS, but it cannot account for the heterogeneous symptom presentation commonly seen in MS patients, nor can it accurately demonstrate how genetic factors (such as the HLA-DRβ1 alleles) and environmental factors activate CD4+ T-cells to generate an immune response at the BBB against self-antigens.

Surface Expression Experiments

In an effort to facilitate the development of a new model system, HLA-DRβ1 alleles 04.01 and 15.01 that correlate with an increased risk of developing MS were combined with 18 different peptide epitopes of three proteins found within myelin sheaths. Recombinant MHC variants HLA-DRβ1 15.01 and 04.01 were modified as described in the Methods to anchor the molecule on the surface of insect cells. The variant MHC genes were then combined with DNAs encoding 18 different myelin protein epitopes and sequenced for accuracy (Methods, **Table-1**). The MHC variant HLA-DRβ1 01.01 not associated with MS or any other autoimmune disorder was also constructed and combined with the same antigen/epitopes as a negative control. The diverse combinations of peptide/MHCs were developed to mimic the MHC restriction concept (**Figure-6**) which states that T-cell receptors must recognize (match) the specific MHC and peptide combination, to enable CD4+ T-cells to be specified for that specific MHC/peptide combination. Developing different peptide/MHC combinations not only allows the potential identification of specific antigens related to the initiation of MS, but also verification of which

component most likely increases the risk of MS (the peptide sequence or the MHC variant), and why the specific variant or peptide by-passes the negative selection process during T-cell development.

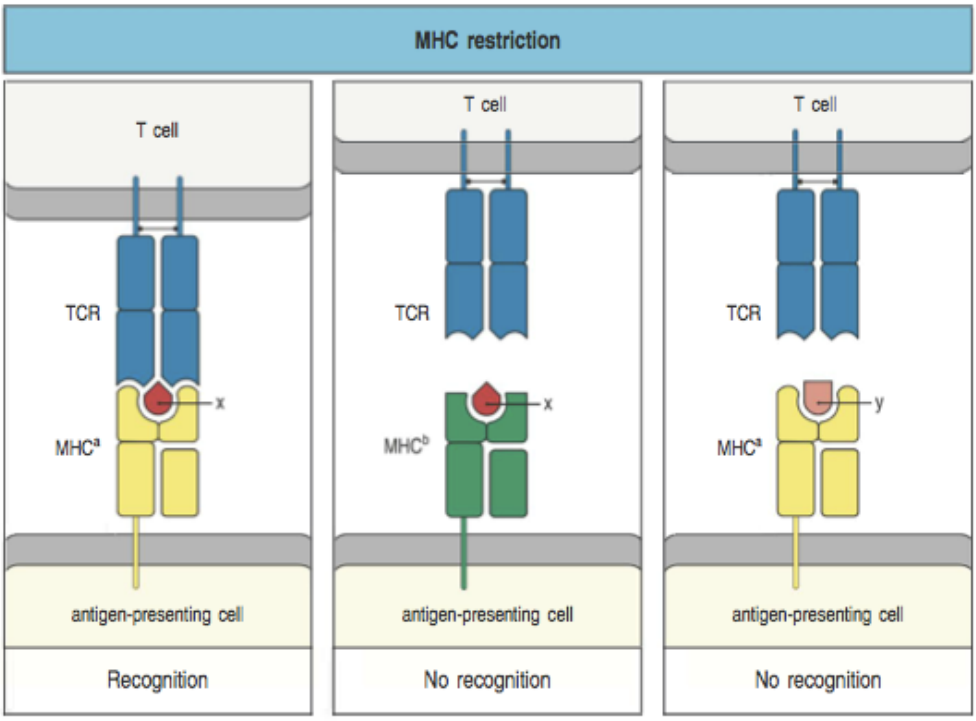

Figure-6: Diagram of the MHC Restriction Concept. The left panel illustrates how the T-cell receptor (TCR) (blue) recognizes specific MHC (yellow) and ligand peptide (brown) combinations on an antigen presenting cell (light yellow). If the MHC does not match the TCR specificity (diagram center) there is no recognition/binding of the ligand complex. Similarly, if the peptide ligand does not match the TCR specificity (diagram right) the T-cell will not recognize the peptide/MHC complex. (Janeway et al., 2008)

Recombinant HLA-DRβ1 gene variants and their combined myelin protein gene epitopes were cloned into an expression plasmid, and expressed at the surface of Sf-9 insect cells as described in Methods. The expression levels of the HLA-DR molecules were analyzed by FACS using HLA-DR mAbs with an APC fluorophore (**Figure-7**). The hypothesis tested was that MS-associated variants HLA-DRβ1 15.01 and 04.01 would have higher levels of expression than

control variant HLA-DRβ1 01.01. However, the data showed no statistical difference between the various types of HLA-DR expression levels. All peptide-MHC combinations (red curves) showed increased surface expression relative to the antibody omission non-expression control (blue curves). Differences were observed for specific peptide/MHC combinations, which could affect differences in T-cell binding. The highest level of expression was observed for HLA-DRB1 15.01 combined with peptide MOG 33-47.

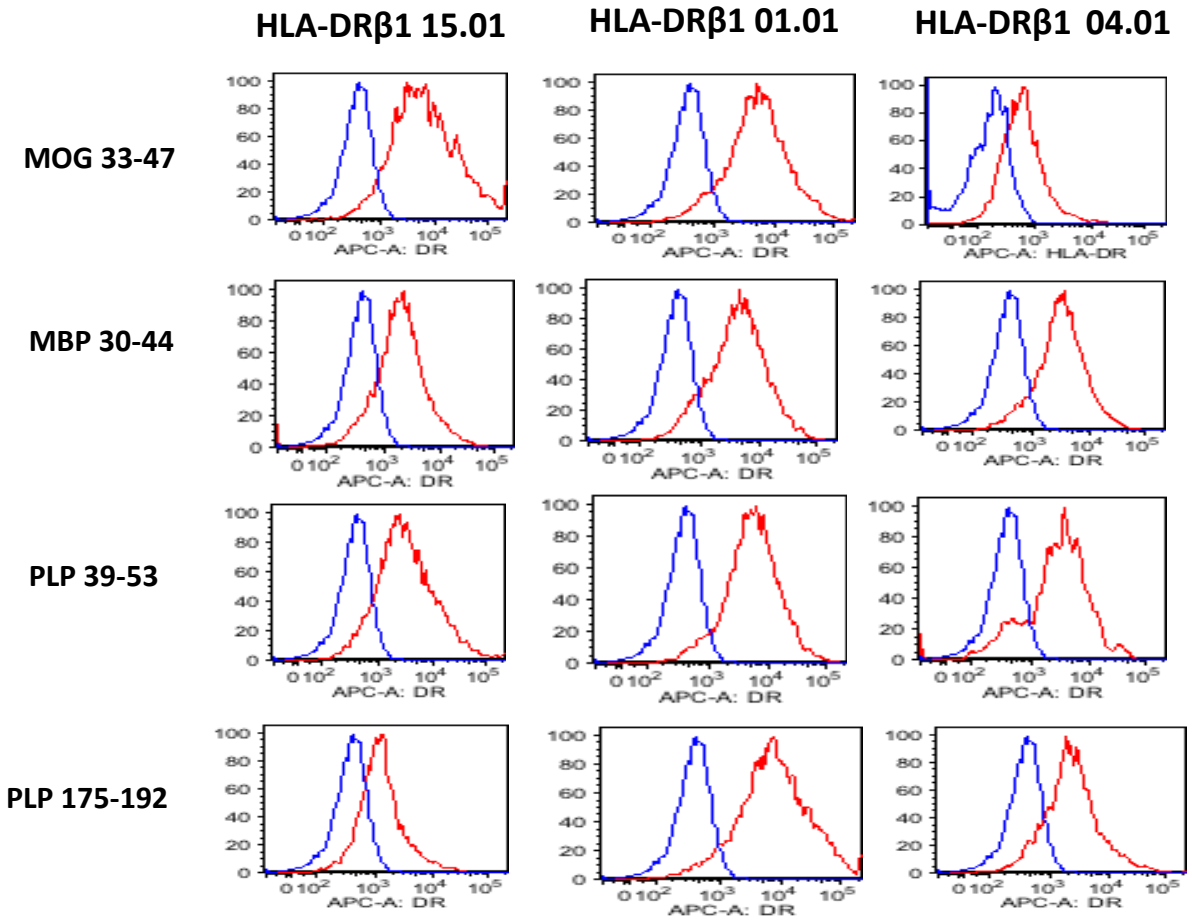

Figure-7: FACs Analysis of the Sf-9 Cell Surface Expression of Various Peptide and HLA-DRβ1 Complexes. HLA variants tested were 01.01 (control), and 04.01 and 15.01 (MS-associated). The peptides that were combined with the HLA variants are listed on the left side of the figure. Blue represents antibody omission negative controls, and red the HLA-specific antibody staining.

These surface expression findings have relevance to the mechanisms previously discussed in the Background stating that surface expression levels are important for inducing T-cell activation. The T-cell must recognize the MHC-expressed self-antigen. Because all the peptide-HLA combinations showed some surface expression, a lack of expression likely is not responsible for inactivity *in vivo*. In the future, T-cell binding assays could be conducted to determine whether the highest surface expressed combination (15.01 with MOG 33-47) is the most antigenic. *In vivo* studies are also crucial to perform because of the wide variety of signals that affect CD4+ cell maturation and migration.

The peptide/MHC combinations shown on **Figure-7** are of peptides shown to develop classical EAE symptoms in our in vivo study, and that showed atypical symptoms or MS like symptoms. The peptides MOG 33-47 and PLP 175-192 are truncated versions of MOG 25-55 and PLP 174-206 respectively. Truncated versions of these peptides were used to develop peptide/MHC constructs to prevent frameshifts at the peptide binding site of the MHC molecule that would in effect disrupt proper TRC ligand binding and recognition.

Peptide Injection Experiments

In order to assay the encephalo-antigenic properties of various HLA-DRβ1 15.01 and 04.01 and myelin sheath peptides, NSG mice (lacking murine HLAs) transgenic for human HLA-DRβ1 15.01 or HLA-DRβ1 04.01 were injected with elongated versions of the peptides tested in the previous figure. These *in vivo* studies enabled us to test which specific peptide/MHC combination might induce MS-like symptoms or EAE in mice expressing human MHC 15.01 or 04.01 molecules. The injected mice were weighed and scored daily as indicated in

the Methods **Figure-5**. The injected mice were scored for classic classical EAE symptoms such as progressive paralysis.

Interestingly, HLA-DRβ1 variant 04.01 transgenic mice (thought to correlate with MS patients) did not develop any EAE or MS symptoms when challenged with any of the myelin peptides (data not shown). The 04.01 mice gained weight normally throughout the 27 day scoring period, further suggesting that variant 04.01 and the myelin peptides did not activate CD4+ T-cells against myelin proteins at the BBB.

Variant HLA-DRβ1 15.01 transgenic mice showed classic EAE symptoms in mice challenged with peptides MOG 25-55 and MBP 30-44 (**Figure-8, top two panels**). One of 7 15.01 mice challenged with MOG 25-55 showed a classical EAE score of 1.0 or 1.5 in days 10-27 post-injection (**Figure-8, top left panel**). In this case, the EAE score of 1.0 was determined through the apparent tail paralysis of the mouse, and a score of 1.5 was given when the mouse appeared to have weakening of one hind limb (that eventually relapsed three days later). However, the reaction was not consistent, and the other seven 15.01 mice challenged with MOG 25-55 did not develop classical EAE symptoms. One 15.01 mouse (B950) challenged with MBP 30-44 developed a severe case of EAE on day 15 resulting in death (**Figure-8, top right panel**). Mouse B950 did not progressively develop EAE, but the disease reached morbid levels over the course of 24 hours with a classical EAE score of 5.0. This mouse suggests that peptide MBP 30-44 in 15.01 mice is able to activate CD4+ T-cells to MBP protein epitopes and elicit an immunogenic response at the BBB. The death of the mouse is not believed to be due to the induction EAE toxins, but due to the activation of CD4+ T-cells against the MBP epitope because no other mice given MBP 30-44 elicited severe classical EAE symptoms (**Figure-8, top right panel**). Transgenic HLA-DRβ1 15.01 mice challenged with peptide/antigens PLP 39-53 (lower left panel) and PLP 174-206 (lower right panel) did not elicit classical EAE symptoms,

but were found to have atypical EAE symptoms and symptoms resembling MS patient symptoms (**Table 2**).

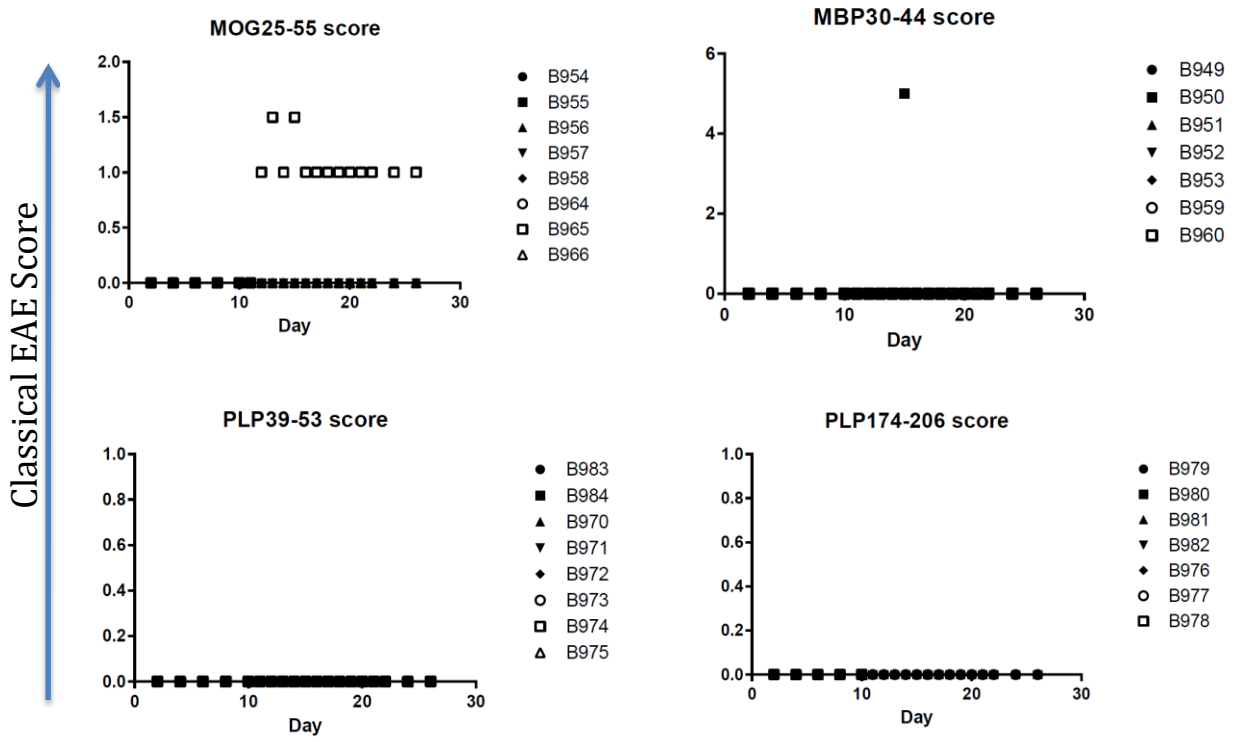

Figure-8: Classic EAE Scores for HLA-DRβ1 15.01 Transgenic Mice Injected with Different Myelin Sheath Peptides. The peptide injected is shown in the upper center of each panel. Y-axis represents the classic EAE score determined as described in the Methods.

The obvious symptoms of HLA-DRβ1 15.01 transgenic mice injected with peptides MOG 25-55, MBP 30-44, PLP 174-206, or PLP 39-53 were recorded to identify patterns and symptoms otherwise ignored by the EAE scoring system (**Table-2**). Peptide MBP 30-44 was injected into two 15.01 mice (B950 and B952). Both died, but 950 showed classical EAE symptoms prior to death on day-15, while 952 dies on day-17 with no apparent EAE symptoms. The lack of illness in the days prior to 952's death suggests that the mouse may have become morbid from EAE symptoms and died before a score could be recorded. 950's death also

occurred quickly, but an EAE score was obtained. Two 15.01 mice (B955 and B964) injected with peptide MOG 25-55 also died, with 955 showing classical EAE symptoms prior to death. Mouse B955 had an apparent front limb paralysis on day 14, and died two days later with no apparent remission increased severity of symptoms (**Table-2**). Four 15.01 mice (B982, B981, B978, and B979) were injected with peptide PLP 174-206. Two of the mice were found dead (B982 and 981), B978 had an enlarged intestine (perhaps due to constipation), and B979 had a large bladder (lack of urination). These symptoms are not characteristic of EAE, but are symptoms commonly seen in MS patients. Finally, four 15.01 mice (B984, B983, B975, and B973) were injected with peptide PLP 39-53. B984, B983, and B975 were found dead on day-10, but B973 developed atypical EAE characterized by a lack of natural reflexes and balance which are indications of possible neuronal damage elicited by CD4+ T-cells. B973 had an atypical score of 3.0 on day 11, with improper limb movement and no front limb reflexes (**Table-2**).

HLA-DRβ1	Peptide	Mouse	Day of Death/ Euthanasia	Description
15.01	MBP 30-44	B950	Day 15	Completely paralyzed/Died before samples were harvested
15.01	MBP 30-44	B952	Day 17	Found dead
15.01	MOG 25-55	B955	Day 14	Apparent front left limb paralysis two days prior to death
15.01	MOG 25-55	B964	Day 10	Euthanized/Found Dead
15.01	PLP 174-206	B982	Day 10	Euthanized/Found Dead
15.01	PLP 174-206	B981	Day 10	Euthanized/Found Dead
15.01	PLP 174-206	B978	Day 11	Enlarged Large Intestine due to apparent constipation
15.01	PLP 174-206	B979	Day 11	Enlarged bladder due to lack of urine secretion
15.01	PLP 39-53	B984	Day 10	Euthanized/Found Dead
15.01	PLP 39-53	B983	Day 10	Euthanized/Found Dead
15.01	PLP 39-53	B975	Day 10	Euthanized/Found Dead
15.01	PLP 39-53	B973	Day 11	Atypical score of 3 on Day 11

Table-2: Symptoms of Mice Injected with Various Myelin Peptides.

Thus, some of the symptoms observed are not characteristic of classical EAE, but coincide with symptoms commonly seen in MS patients. This suggests that the heterogeneity of the MS disease creates different MS subtypes, each activating specific sets of CD4+ T-cells against different peptide/MHC complexes and eliciting different symptoms. This heterogeneity theory has been proposed previously (Barcellos, 2006; Disanto et al., 2011), but the current EAE model limits researchers from investigating the genetic aspects of MS.

DISCUSSION

Multiple sclerosis (MS) is a complex auto-immune disease with no effective cure despite years of extensive research. Researchers often depend upon the experimental autoimmune encephalomyelitis (EAE) mouse models, however, they do not accurately incorporate the clinical, radiological, pathological, and genetic features of MS (Denic et al., 2011). In this project, our EAE induction experiments showed that multiple peptides were capable of inducing EAE, which suggests there is not a specific protein target, but multiple targets that can produce the same demyelination and neuronal damage. From the EAE induction experiment we showed that variant HLA-DRB1 15.01, which correlates with a higher risk of developing MS in humans (Weatherby et al., 2001), is also the more encephalo-antigenic of the two HLA alleles (15.01 and 04.01) tested. The encephalo-antigenic nature of the 15.01 variant seems to correlate more with the induction of EAE than the specific myelin protein epitope presented by 15.01. This opens the question of why negative selection during T-cell development does not destroy these myelin self-antigens, and how variant 15.01 may be contributing to the self-antigen's survival or presentation.

In 15.01 mice, peptide/antigens MOG 25-55 and MBP 30-44 were shown to induce classical EAE symptoms, while peptides PLP 39-53 and PLP 174-206 induced the development of atypical EAE and MS patient-like symptoms. These findings suggest that the EAE model may elicit different symptoms depending on which myelin antigen is presented, and suggest that the heterogeneous presentation of MS in patients may correlate with the presence of different antigen-HLAs. Nonetheless, in order to establish more definitive conclusions, the EAE induction experiment should be repeated several times.

Because the 15.01 allele appears to be more encephalo-antigenic, the data show that the EAE model (that uses an artificial induction of activated T-cells to cause inflammation, demyelination, axonal loss, and gliosis) is not appropriate for studying the genetic features of MS. The EAE model scoring system in particular does not fully represent the heterogeneous clinical syndromes observed in MS patients, and does not take into consideration other neuronal damage symptoms besides paralysis. As indicated by our PLP peptide/antigen EAE induction experiments, symptoms much more related to MS than paralysis can be observed in these mice, but can go un-classified if using the EAE scoring system to characterize disease.

In the future, in an effort to produce a genetically based MS animal model, recombinant HLA-DRβ1 constructs (with their corresponding peptides) used in our transfection experiments will be used to make tetramers. These tetramers will be used to challenge/activate MS patient T-cells, which we hope will identify patient-specific peptide-MHC combinations responsible for the pathogenesis of MS. These constructs and patient T-cells studied will then be used to make a more genetically-driven MS animal model.

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