Summary: Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) that affects about 0.1% of the worldwide population. This deleterious disease is marked by infiltration of myelin-specific T cells that attack the protective myelin sheath that surrounds CNS nerve axons. Upon demyelination, saltatory nerve conduction is disrupted, and patients experience neurologic deficiencies. The exact cause for MS remains unknown, although most evidence supports the hypothesis that both genetic and environmental factors contribute to disease development. Epidemiologic evidence supports a role for environmental pathogens, such as viruses, as potentially key contributors to MS induction. Pathogens can induce autoimmunity via several well-studied mechanisms with the most postulated being molecular mimicry. Molecular mimicry occurs when T cells specific for peptide epitopes derived from pathogens cross-react with self-epitopes, leading to autoimmune tissue destruction. In this review, we discuss an in vivo virus-induced mouse model of MS developed in our laboratory, which has contributed greatly to our understanding of the mechanisms underlying molecular mimicry-induced CNS autoimmunity.

Keywords: cytotoxic T cells, Th1/Th2/Th17 cells, dendritic cells, autoimmunity, T-cell receptors, neuroimmunology

Introduction

The breakdown of peripheral self-tolerance can result in the induction and progression of immune reactivity to self. This phenomenon of autoreactive pathology is the pathological basis for organ-specific autoimmune diseases. Although the downstream effects of this breakdown in tolerance are evidenced by disease pathology, little is understood regarding the initiation of the pathophysiological process in autoimmune disease.

One of the most prevalent neurologic autoimmune diseases is multiple sclerosis (MS) (1). This organ-specific autoimmune disease of the central nervous system (CNS) is characterized by the loss of the protective myelin sheath surrounding the axons of neurons, resulting in ‘plaque’ formation (2, 3). Plaques denote areas in which myelin has been lost. Loss of myelin and plaque formation is mainly attributed...
to myelin-specific CD4+ T cells that cross the blood–brain barrier (BBB) and consequently destroy the myelin sheath (4–6). Upon destruction of myelin, saltatory axonal conduction is disrupted leading to loss of neurologic function. Symptoms include cognitive impairment, muscle weakness, spasms, ataxia, and paralysis (7).

Although the pathophysiology of MS has been extensively studied for many years, the etiology of the disease still remains a mystery. It is clear that genetics contributes significantly to disease susceptibility. Identical twins develop MS with a 25–30% concordance rate, and there is an increased chance of developing disease among siblings as compared to the incidence in the general population (8). Susceptibility is most closely linked to the human leukocyte antigen (HLA) DR [major histocompatibility complex (MHC) class II] locus, and more recently, disease has been more loosely linked to multiple immune-related genes, including interleukin 2 receptor alpha (IL2RA) and IL7RA alleles (9, 10). Thus, it is clear that genetics contributes importantly to MS susceptibility, but there is a large body of epidemiological evidence supporting an equally important role for infections as an inducing stimulus.

Numerous studies have shown a correlation between MS development and latitude, with an increased incidence of disease in temperate and colder climates compared to enhanced resistance in warmer climates closer to the equator (11). Outbreaks of MS have also occurred in isolated populations, for example, in native inhabitants of the Faroe and Shetland-Orkney Islands, after prolonged exposure to non-native individuals during World War II (11–13). This suggests that the possibility that exposure of genetically susceptible individuals to environmental pathogens may be involved in the induction of MS. More direct evidence for this hypothesis comes from reported isolation of viral proteins and RNA from MS plaques. Overall, over two dozen different viruses have been reportedly linked to MS over the past four decades (14, 15). Confirmation of the vast majority of these findings is lacking, and more recent reports have correlated MS with infections by human herpes virus type 6 and Epstein-Barr virus (EBV) (16).

There are multiple theories for how infections may induce autoimmunity. The three major hypotheses include (i) bystander activation, (ii) epitope spreading, and (iii) molecular mimicry (Fig. 1).

Bystander activation is a non-specific mechanism for virus-induced autoimmunity occurring within the inflammatory context generated by virus infection, especially chronic viral infection (16). Autoreactive T cells stimulated directly via pathogen-encoded superantigens or indirectly via tissue-resident antigen presenting cells (APCs) activated via Toll-like receptors (TLRs) and/or other pattern recognition receptors (PRRs) secrete inflammatory mediators, such as interferon-γ (IFN-γ), IL-1β, IL-12, IL-23, tumor necrosis factor-α (TNF-α), etc., which can non-specifically activate autoreactive T cells. These activated T cells can then initiate further tissue damage in the inflammatory milieu (14).

A functional consequence of bystander activation and local tissue damage is a phenomenon known as epitope spreading. This often occurs in the setting of persistent infection where a prolonged anti-pathogen specific immune response leads to tissue destruction resulting in release of endogenous or cryptic self-epitopes that are then engulfed and presented by APCs,

**Fig. 1. Mechanisms of viral induced autoimmunity.** (A) Bystander activation. Chronic viral infection leads to activated lymphocytes that secrete inflammatory mediators mediating tissue damage. Once tissue is damaged, self-epitopes are released into the milieu, presented by APCs to autoreactive T cells which can mediate further tissue damage. (B) Epitope spreading. T-cell responses ‘spread’ from virus-specific to self-specific after continuous inflammation in a chronic virus setting. As tissue is destroyed, self-epitopes are released and present to T cells by APCs, which can mediate further damage to the tissue. Upon further tissue destruction, increasing self-epitopes are released and can be presented and activate naïve autoreactive T cells. (C) Molecular mimicry. Tissue damage is mediated by autoreactive T cells that become activated after cross-reacting with viral epitopes presented by APCs.
which activate autoreactive T cells either directly in the target organ or in local lymph nodes draining the target organ (17, 18). Therefore, chronic viral infection and the accompanying inflammatory response poses a significant risk for inducing organ-specific autoimmune disease.

Molecular mimicry is the leading hypothesis for pathogen-induced autoimmunity. Molecular mimicry occurs when T cells bearing receptors (TCR) specific for epitopes derived from foreign pathogens (e.g. viruses) are activated during an infection and cross-react with self-antigens inducing autoimmune disease (19). Mimicry has been postulated as the primary cause of many autoimmune diseases, including rheumatoid arthritis, diabetes, and MS (20–25).

**Studies supporting molecular mimicry-induced autoimmune disease**

Initial studies investigating the potential of foreign antigens to initiate autoimmune disease relied on immunizing animals with mimic peptides sharing varying degrees of sequence homology with self-epitopes and assaying T-cell responses and autoimmune disease symptoms. The initial report of T-cell molecular mimicry involved immunization of rabbits with a peptide from the hepatitis B virus epitope, which shared six amino acids with a sequence from myelin basic protein (MBP), in complete Freund’s adjuvant. Immunization induced autoreactive T-cell responses to MBP; however, the animals failed to develop clinical disease (19). Subsequent studies employed transgenic mice expressing lymphocytic choriomeningitis virus (LCMV), viral antigens expressed in the pancreas under control of the rat insulin promoter. These animals failed to develop spontaneous autoimmune disease, but did develop clinical disease after subsequent LCMV infection (26, 27). Direct evidence for mimicry-induced autoimmunity was demonstrated upon infection with herpes simplex virus type 1 that leads to autoreactive CD4+ T cells specific for corneal antigen and ultimate development of an autoimmune eye disease (28).

Our laboratory took a different approach by determining if infection with a normally avirulent CNS virus engineered to encode molecular mimics of myelin proteolipid protein (PLP) could induce CNS autoimmune disease. We employed a very well characterized virus that is studied as one of the primary animals models for MS, i.e. the use of the natural mouse pathogen Theiler’s murine encephalomyelitis virus (TMEV) (29). TMEV is a single-stranded virus of the picornoviridae family, which is a natural enteric pathogen of mice and rats (30). TMEV is a neurotropic virus that can replicate and persist within the CNS. Intracerebral infection of susceptible inbred mouse strains (e.g. SJL/J) with wildtype TMEV can lead to the induction of a late-onset demyelinating disease, termed TMEV-induced demyelinating disease (TMEV-IDD) which is similar in pathology to MS, via the process of epitope spreading (17, 31). In contrast, disease-resistant mouse strains (e.g. C57BL/6) rapidly clear TMEV infection and develop no apparent CNS clinical manifestations.

Several strains of TMEV exist that differ in their neurovirology. The two most commonly studied are the DA and BeAn strains. The DA strain is less virulent and results in a demyelinating disease approximately 140–180 postinfection (PI) (30). In our current model, we have employed the BeAn strain which results in chronic demyelinating disease with onset of paralytic symptoms approximately 30–40 days PI in SJL/J mice (30).

TMEV is classically characterized by two disease phases: early acute and late chronic demyelinating disease. Infection of both susceptible and resistant mouse strains induces an early acute disease marked by viral replication in the gray matter of the CNS that generally does not lead to clinical signs (30). Anti-virus immunity is marked by CNS infiltration of CD4+ T cells specific to viral proteins, as well as a virus-specific CD8+ cytotoxic T-lymphocyte (CTL) response in both the CNS and periphery. It has not been determined, however, which DCs are responsible for initiating these early adaptive T-cell responses.

In the TMEV-IDD susceptible SJL/J strain, early acute infection results in an ineffectual immune response that limits peripheral virus titers, but does not result in complete virus clearance. Inability to effectively clear the virus results in chronic CNS viral infection, with low viral titers detected for as long as a year or more PI. Typically, replication occurs in CNS-resident antigen-presenting cells and glial cells of the CNS (30, 32).

TMEV-IDD is thought to be initiated by the bystander CNS damage mediated by pathogenic proinflammatory virus-specific CD4+ T cells, as evidenced by the fact that susceptibility is correlated with high class-II-restricted TMEV-specific delayed type hypersensitivity (DTH) (33). In addition, in vivo depletion of CD4+ T cells in SJL mice resulted in decreased incidence of demyelinating disease and slower progression (34). In the SJL/J strain, immunodominant CD4+ T cells are specific for the TMEV epitope VP270–86 (35). Sub-immunodominant epitopes include VP1233–250 (36) and VP324–37 (37). Relevant to a potential role for molecular mimicry in inducing TMEV-IDD following infection with the wildtype BeAn virus strain in SJL/J mice, there appears to be no
cross-reactivity between the immunodominant TMEV CD4 epitopes and the immunodominant epitopes on PLP or other myelin proteins, including MBP and MOG (31), thus mimicry appears not to be involved in disease initiation in wildtype virus infection.

Disease progression is exacerbated by epitope spreading to self-myelin epitopes. When tissue damage reaches a critical mass, autoreactive CD4+ T cells against the immunodominant PLP$_{139-151}$ epitope initially arise and mediate further myelin damage (38). CD4+ T-cell responses against subdominant self-epitopes sequentially arise with further disease progression. We have shown that epitope spreading in susceptible SJL mice is initiated by activation of T cells in the CNS target organ by activation of naive T cells by peripherally derived myeloid dendritic cells (39, 40). Demyelination in the CNS results in chronic progressive hind limb paralysis, which can eventually lead to death.

TMEV-induced model of molecular mimicry-induced CNS autoimmunity

As detailed above, infection of SJL/J mice with wildtype TMEV leads to CNS autoimmunity via epitope spreading. To determine if natural virus infection can induce autoimmunity by molecular mimicry, we engineered a non-pathogenic strain of TMEV-BeAn to express self-peptide, peptide mimics, and eventually pathogen-derived self-mimics. This unique and direct model allowed us to examine the requirements for infection-induced cross-reactive activation of autoreactive T cells and in vivo autoimmune disease development.

We employed a mutant non-pathogenic BeAn strain of TMEV which had a ClaI restriction site inserted into the leader sequence of the virus resulting in a 23 amino acid deletion (ΔClaI-TMEV) (29). SJL/J mice infected with the ΔClaI-TMEV strain of TMEV did not develop demyelinating disease (Fig. 2B,
Group 1). The non-pathologic nature is due to the fact that the virus is not able to persist in the CNS of the infected animals. We then constructed additional viruses by inserting sequences encoding the native encephalitogenic self-myelin epitope [PLP130–159 (PLP139-TMEV)], which encompasses the immunodominant encephalitogenic PLP139–151 epitope, or altered peptide ligands of this epitope into the ClaI restriction site. The altered peptide ligands were generated by making point mutations at the primary and secondary TCR recognition sites, W144A and H147A, respectively (Fig. 2A). As a control, we also constructed encoding the non-self ovalbumin OVA317–346 epitope (OVA323-TMEV). This model is unique because it allowed us to examine the processing of either native or APL sequences in the context of the larger VP2 protein (29).

Mice infected with the PLP139-TMEV (Fig. 2B, Group 2) developed clinical symptoms of disease (loss of tail tone and flaccid hind limb paralysis) rapidly (days 7–10 PI compared with normal delayed disease onset 30–40 days PI for wildtype TMEV) and with increased severity (clinical scores of three compared to two for wildtype). Inflammation and demyelination were observed in the lower lumbar spinal cord of these animals. This increased pathology was quite striking and was the first evidence that a self-epitope processed from whole virus could be presented to autoreactive T cells resulting in initiation of demyelinating disease. Significantly, it should be noted that innate signals provided by TMEV, a positive strand RNA virus, were sufficient to induce the early onset 'EAE-like' disease and did not require exogenous priming with other TLR agonists [e.g., complete Freund’s adjuvant (CFA) containing Mycobacteria]. Mice infected with a TMEV variant encoding a 30-mer expressing the non-self OVA323–339 epitope (OVA323-TMEV) failed to develop early onset disease due to molecular mimicry (Fig. 2, Group 3). However, these mice developed delayed onset spastic hind limb paralysis similar to SJL/J mice infected with wildtype TMEV. It was found that insertion of the OVA 30-mer into the leader deleted ΔClaI virus restored CNS viral persistence consistent with this clinical finding (29). One potential explanation for restoration of virus persistence is a potential conformational change in ΔClaI-TMEV upon insertion of the OVA 30-mer which promotes viral replication and CNS persistence. In fact, it was found that all strains which received insertions of 30-mer peptides were able to persist in the CNS and lead to late stage disease.

Similar disease kinetics were seen when mice were infected with the ΔClaI-TMEV expressing the PLP peptide with H147A mutation (H147A-TMEV). These mice developed early onset EAE-like disease similar to mice infected with PLP139-TMEV (Fig. 2B, Group 4). However, mice infected with the W144A-TMEV virus strain (Fig. 2B, Group 5) failed to develop early onset disease (29). These results corresponded with previous studies in which SJL/J mice primed with the W144A peptide in CFA exhibited peptide (W144A)-specific CD4+ T-cell response in vitro, but these mice did not develop EAE. However, mice primed with the H147A APL in CFA exhibited T-cell cross-reactivity to PLP139–151 and develop clinical EAE (41–43). These results indicate that maintenance of the wildtype tryptophan residue at the primary TCR contact site at position 144 was critical for induction of disease via molecular mimicry, but that the residue at the secondary TCR contact site was not critical.

To determine if the initial virus infection had to occur in the eventual target organ of the autoimmune disease, the CNS in our model, we tested if the mice could be infected by different routes. Importantly, we determined that route of infection with these particular mutant viruses did not affect clinical disease outcome, in that infection by either the intravenous or intraperitoneal routes resulted in similar disease induction (29, 44). This is important because it suggests that infections of the periphery (and activation of peripheral T cells) can lead to autoimmunity within the CNS—a site distal from the initial infection. To determine whether infection of the CNS target organ was required for induction of infection-induced molecular mimicry disease, we inserted PLP130–159 into Coxsackie virus B3 (PLP139-CVB3), which infects the heart and pancreas, but not the CNS. SJL/J mice infected with PLP139-CVB3 develop early onset mild demyelinating disease (Fig. 2B, Group 6) and concomitant activation of PLP139–151-specific CD4+ T-cell responses (unpublished), indicating that virus infection distal to the CNS target organ was sufficient to induce activation of myelin peptide-specific T cells via molecular mimicry, which can home to the CNS.

These results demonstrated the potential to induce CNS autoimmune disease as a consequence of a natural viral infection. Prior to the development of this model, studies had largely focused on immunization in adjuvant to examine cross-reactive T cells responses to potential mimic epitopes. The use of a non-pathogenic strain of TMEV as a vector for presenting the mimic epitopes supports the hypothesis that autoimmunity can be triggered by infection-induced molecular mimicry and enables the investigation of the underlying mechanisms by which a virus may present self-mimics and induce T-cell cross-reactivity and subsequent disease.
Infection with PLP-mimic viruses induces autoreactive Th1 responses

The induction of early onset EAE-like disease in SJL/J mice infected with PLP139-TMEV led us to investigate the immune mechanism(s) underlying disease induction. It was critical to determine if the disease was dependent on the activation of PLP<sub>139–151</sub>-specific CD4<sup>+</sup> T cells subsequent to virus infection.

Responses to myelin epitopes in SJL/J mice infected with wildtype TMEV only appear 40–50 days PI following epitope spreading. In contrast, PLP<sub>139–151</sub>-specific T-cell responses were triggered in PLP139-TMEV infected mice within 7–14 days PI, consistent with a major role for autoimmune pathology in disease induction (29, 44). These PLP peptide-specific T cells produced large amount of IFN-γ and IL-2. T-cell responses from mice infected with the PLP<sub>139–151</sub> mimics (W144A and H147A) had differential outcomes in cross-reactivity. T cells from H147A-TMEV activated cross-reactive PLP<sub>139–151</sub>-specific CD4<sup>+</sup> T cells, whereas T cells from W144A-TMEV did not (29, 44). Thus, TCR contact residues of potential mimics are crucial for initiating self-reactive CD4<sup>+</sup> T-cell responses, an important consideration to consider when analyzing potential naturally occurring mimics.

To further determine the encephalitogenic capacity of the myelin peptide-specific T cells, we examined their ability to transfer disease, as well as the effects of PLP<sub>139–151</sub>-specific tolerance in preventing disease onset in PLP139-TMEV infected mice. To examine their pathogenicity, we harvested spleens from animals infected with PLP139-TMEV. After reactivation in vitro with PLP<sub>139–151</sub> peptide, cells were transferred to naive SJL/J mice. Mice receiving either bulk splenocytes or purified CD4<sup>+</sup> T cells developed clinical disease by day 10 post-transfer. This is clear evidence that highly pathogenic autoreactive T cells are induced early after infection with a virus expressing a self-epitope (44). The effector role of PLP<sub>139–151</sub>-specific CD4<sup>+</sup> T cells was further shown using antigen-specific tolerance induced by the i.v. injection of ethylncarbodiimide (ECDI)-fixed, peptide-coupled splenocytes. This tolerance induction protocol is effective in inducing tolerance for prevention and treatment of various autoimmune disease models, e.g. the EAE of MS and the non-obese diabetic (NOD) mouse model of type 1 diabetes, as well as inducing tolerance to allogeneic transplants (45–47). In SJL/J mice with TMEV-IDD induced by infection with wildtype TMEV, tolerance induced with intact TMEV virions, which anergizes virus-specific CD4<sup>+</sup> T cells (48, 49), but not tolerance induced with splenocytes coupled with mouse spinal cord homogenate (containing a crude mixture of myelin epitopes) or PLP<sub>139–151</sub> (50), was able to inhibit disease induction indicating that virus-specific T-cell initiated disease. In contrast, in PLP139-TMEV-infected mice, previous tolerization with PLP<sub>139–151</sub>-coupled splenocytes was able to significantly inhibit development of early onset demyelinating disease (44), clearly indicating that PLP<sub>139–151</sub>-specific effector CD4<sup>+</sup> T cells were responsible for disease initiation. Taken together with the demonstration of induction of potent PLP<sub>139–151</sub>-specific CD4<sup>+</sup> Th1 responses in PLP139-TMEV and H147A-TMEV-infected mice (44), these data clearly indicate that early onset clinical disease is due to the activation of myelin peptide-specific autoreactive T cells following infection with TMEV-expressing myelin peptides and myelin mimics.

Induction of CNS autoimmunity using viruses encoding natural PLP<sub>139–151</sub> mimic epitopes

Haemophilus influenzae mimic

Our data show that TMEV encoding a 30-mer encompassing either the immunodominant encephalitogenic PLP<sub>139–151</sub> epitope or the altered peptide ligand (H147A) containing a conservative amino acid substitution in the primary TCR contact residue can trigger virus-induced CNS autoimmunity. However, to address the question of mimicry-induced autoimmunity in a more physiological manner, it was critical to determine if PLP<sub>139–151</sub> mimics encoded by natural pathogens could induce autoimmune disease. We thus used the same non-pathogenic strain of TMEV (ΔClaI-TMEV) engineered to express a sequence from the H. influenza serine protease IV protein (HI<sub>574–586</sub>). This protein is responsible for the degradation of proteins, peptides, and glycopeptides. Interestingly, this sequence is a naturally occurring mimic of PLP<sub>139–151</sub>, as it shares 6 of 13 amino acids, including the primary TCR (position 144) and primary and secondary MHC (positions 145 and 148) sites (42) (Fig. 2A). However, it does not share the secondary TCR contact residue (position 147). Previous work had demonstrated that HI<sub>574–586</sub> was able to bind to I-A<sup>3</sup> and induce T-cell responses cross-reactive with PLP<sub>139–151</sub> in adjuvant primed SJL/J mice (42).

Mice infected i.c. with the PLP139-TMEV virus into which HI<sub>574–586</sub> was substituted for the core PLP<sub>139–151</sub> sequence (HI574-TMEV) were shown to develop early onset demyelinating disease, although with less severity compared to mice infected with PLP139-TMEV (Fig. 2B, Group 7). This disease was atypical compared to PLP139-TMEV disease. The clinical symptoms observed were ruffled fur, hunched back, and waddling gait, and these symptoms did not progress over time (51). Although the disease was milder, this was the first
demonstration that molecular mimics naturally expressed in an infectious pathogen could indeed induce clinical disease. Upon histologic examination of HI574-TMEV-infected mice, we observed infiltration of CD4+ T cells into the thoracic region of the spinal cord; however, infiltration was mainly perivascular with limited infiltration into the parenchyma. This could potentially explain the limited disease seen in infected animals, i.e., T cells were unable to properly traffic to areas of the spinal cords to cause sufficient damage to induce progressive disease (51).

We also determined the nature of the T-cell responses induced by infection with HI574-TMEV. We found the temporal appearance of cross-reactive PLP139–151-specific T cells which proliferated and produced IFN-γ in response to in vitro restimulation with PLP139–151 peptide. In vitro responses to both TMEV peptide (VP270–286) and HI574–586 were also observed (51). This is the first model to directly demonstrate that a virus encoding a mimic of a self-myelin epitope can induce autoreactive CD4+ T cells. To verify the pathologic contribution of myelin-specific (cross-reactive) T cells in disease induction in HI574-TMEV infected animals, we tolerized these animals to PLP139–151 peptide before infection. Tolerance to the self-myelin peptide significantly reduced clinical disease in mice infected with the H. influenzae mimic-encoding virus concomitant with a reduction in PLP139–151-specific DTH and proliferative responses (52). Thus, CNS autoimmune disease in these animals was the result of cross-reactive induction of myelin peptide-specific T cells responses by molecular mimicry.

To determine if the H. influenzae-derived mimic epitope could be processed and presented from the native protease IV protein and induce CNS autoimmune disease, we tested the disease-inducing ability of a ΔCIAI-TMEV variant expressing a 39-mer sequence (HI566–595, HI566-TMEV) derived entirely from the bacterial protein (52). This provides a more stringent test of molecular mimicry, in that the minimal mimic peptide epitope would have to be processed from its own flanking sequences in order, to be presented by SJL/J APCs to cross-activate self-specific pathogenic PLP139–151-specific T cells. Interestingly, SJL/J mice infected with HI566-TMEV (Fig. 2B, Group 8) developed an early onset demyelinating disease (day 7–10 PI) similar to mice infected with HI574-TMEV. HI566-TMEV infected mice also displayed cross-reactive responses to PLP139–151 as measure by DTH, proliferation, and cytokine production (52). Therefore, these results indicate that it is possible for mimic epitopes to be processed and presented in the context of the native protein sequence to lead to pathogen-induced autoimmunity.

Other explanations could explain the limited disease observed in SJL/J mice infected with either HI574-TMEV or HI566-TMEV. A discrepancy exists between the appearance of cross-reactive PLP139–151 CD4+ T cells and disease severity. Although cross-reactive T cells exist, the disease is mild and non-progressing. Potential explanations for this phenomenon include binding efficiency of the HI peptide, as well as differences in cross-reactive populations of T cells. First, it has been described that the I-Aβ molecule has lower affinity for the HI574–586 peptide than for PLP139–151. Therefore, in vivo processing, presentation of this peptide from the larger virus protein by endogenous APCs may induce suboptimal priming of cross-reactive CD4+ T cells. A secondary hypothesis could be explained by differences in the PLP139–151 and HI574–586-specific CD4+ T-cell populations. The PLP139–151 T-cell population cross-activated in HI-TMEV infected mice may be less skewed toward a pathologic Th1/17 phenotype and/or include a regulatory phenotype. Therefore, the balance between harmful and protective T cells may be tipped such that disease progression is controlled. Further exploration of this secondary hypothesis would be interesting to determine if such a relationship exists.

**Mouse hepatitis virus mimic**

To complement our studies examining the potential of H. influenzae as a naturally occurring PLP139–151 mimic, we also examined the pathologic capacity of a mimic sequence derived from murine hepatitis virus (MHV). MHV is a single-strand RNA coronavirus consisting of multiple strains. Infection with the JHM strain of MHV can cause a chronic demyelinating disease similar in pathology to MS (53). In humans, there are reports showing coronavirus antigens within MS plaques (54, 55).

It has been previously described that MHV expressed a potential PLP139–151 molecular mimic (42). MHV3821–3832 is located in the ORF1a gene which encodes for the replicative polyprotein PL1-PRO, which is involved in transcription of RNA and cleaving the polyprotein into function products. Unlike the HI mimic that shares 46% (6/13 amino acid residues) homology, the MHV expressing mimic only shares 23% (3/13 amino acid residues) identity (Fig. 2A), but includes homology at the primary TCR (position 144) and primary MHC class II (position 145) contact residues.

We again engineered the ΔCIAI-TMEV strain to express a 30-mer peptide encompassing the potential myelin mimic (MHV3813-TMEV). Mice infected with MHV3813-TMEV developed an early onset (day 20 PI) demyelinating disease similar in severity to mice infected with PLP-TMEV (56).
endogenous PLP139–151 CD4+ T cells after myelin damage by a severe waddling gait and mild flaccid hind limb paralysis. (Chastain & Miller) With TMEV expressing the HI 574–586 sequence developed a model, animals are primed with PLP139–151 emulsified in CFA. An important aspect of the studies was the comparison of infection with HI574-TMEV to priming with HI574–586 in CFA. As described, mice infected with TMEV expressing the HI574-586 sequence developed a reproducible early onset yet mild clinical disease; however, mice primed with HI/CFA did not develop clinical disease (51, 52, 56). This indicates that innate immune events associated with viral replication (cytokine production, activation of APCs, TLR/PKR signaling) were required for disease initiation, while priming with this mimic in CFA is insufficient in generating disease. Interestingly, although HI574–586/CFA priming failed to invoke clinical disease, PLP139–151-specific T-cell proliferation was observed, as well as a DTH response 14 days PI. However, the self-peptide-specific T cells produced significantly less IFN-γ than animals primed with PLP139–151/CFA (51, 52). Perhaps ‘lack of skewing’ toward pathogenic Th1 or perhaps Th17 cells could explain the inability to develop disease. It will be interesting in the future to examine other T-helper phenotypes including Th17 and Treg cells.

We also investigated the possibility that the route of antigen exposure could explain the difference seen in priming versus infection with the HI mimic peptide, i.e. it is possible that innate activation of CNS cells is a prerequisite for disease development as CNS-resident cells (microglia) are activated following either i.c. or i.p. infection within the mimic peptide-expressing viruses, which is not true following s.c. peptide/CFA priming.

To address this question, SJL/J mice were infected with either the HI-TMEV or control OVA-TMEV strain of TMEV and subsequently primed with HI574–586, OVA323–339 or PBS in CFA. Interestingly, only mice preinfected with HI-TMEV and then primed with HI574–586/CFA developed a severe rapid-onset disease (52). Mice preinfected with H1574-TMEV but then primed with OVA323–339 developed clinical disease similar to H1574-TMEV infection alone (52). Therefore, this indicates that secondary challenge with the original mimic peptide is imperative to induce exacerbated clinical disease. We also determined that persistent infection is a prerequisite for disease development, as mice infected with the ΔCid-TMEV, which does not replicate/persist, did not result in severe disease in mice, subsequently primed with HI574–586/CFA. Enhanced disease was accompanied by increased PLP139–151-specific proliferation and IFN-γ production and increased CNS pathology (52).

We believe that studying molecular mimicry within the context of a replicating virus infection is critical for determining the capacity for a particular mimic to induce disease. Indeed, responses induced by mimic peptides in the ‘artificial’ context of CFA priming are not an accurate depiction of whether a particular peptide is able to act as a disease-inducing mimic. It is also likely that type of virus and its cell tropism are important factors for initiating disease. For instance, infection of APCs may be an important prerequisite for ability to induce clinical autoimmune disease.

### Mimic peptides and cross-reactivity

Although our studies to this point mainly addressed our work with H. influenzae peptides, we have also explored the potential of other PLP139–151 peptide mimics to induce in vitro T-cell responses and examined the minimum requirement to induce cross-reactivity with PLP139–151. We therefore used mimics derived from Candida albicans (CAN), E. coli (ECO), Salmonella typhimurium (SAL), and MHV that had been previously described by Carrizosa et al. (42).
Examination of the potential of these mimics to induce T-cell cross-reactivity revealed differential requirements for activating naive PLP<sub>139–151</sub>-specific T-cell precursors versus reactivating PLP<sub>139–151</sub>-primed T cells. CD<sup>4+</sup> T cells from mice primed with the MHV mimic peptide proliferated in response to PLP<sub>139–151</sub>; however, the reverse was not true (58), i.e. T cells from PLP<sub>139–151</sub>-primed mice did not proliferate in response to the MHV peptide. In contrast, mice primed with ECO did not proliferate when restimulated with PLP<sub>139–151</sub>, whereas PLP<sub>139–151</sub>-primed T cells cross-reacted and proliferated in response to the ECO peptide (58). We found that peptides best recognized by PLP<sub>139–151</sub>-primed T cells, all contained a proline residue at the 10th position which is the secondary I-A<sup>b</sup> binding site.

One of the observations that we made was the degree to which mimic-primed T cells cross-reacted to other mimics in vitro. In other words, disease appearing to be attributed to one mimic (based on in vitro response) may in fact have been induced by a different mimic. Thus, this system is somewhat 'leaky', and disease causation cannot strictly be attributed to a T-cell response to a given pathogen. In addition, autoreactive cells may also cross-react with pathogens (in vitro), although they were induced by another means (such as autoimmune disease).

We found somewhat different results when we infected the animals with TMEV expressing the peptide mimics, instead of priming with CFA. Again, we generated ΔCl-Al-TMEV strains that expressed three of the mimics (ECO-TMEV, CAN-TMEV, and SAL-TMEV). Increased cross-reactivity (determined by proliferation) was seen to PLP<sub>139–151</sub> when mice were infected with the mimic strains (58), and all three strains were able to induce significantly more severe clinical signs of disease than animals infected with OVA323-TMEV-infected animals. However, we did observe the cross-reactivity to other pathogen mimics. For example, mice infected with MHV-TMEV (which induces an early onset demyelinating disease) proliferated poorly to MHV<sub>3821–3832</sub> yet responded more vigorously to both HI<sub>574–586</sub> and PLP<sub>139–151</sub> (58).

Again addressing the idea of cross-reactivity and cross-specificity, we made an interesting observation with animals’ tolerized with HI<sub>574–586</sub> peptide. Using ECDI splenocytes coupled to HI<sub>574–586</sub> peptide, we tolerized animals and then primed them with PLP<sub>139–151</sub> in CFA. Interestingly, tolerization with the mimic epitope was able to significantly suppress EAE compared to OVA coupled splenocytes (52). HI<sub>574–586</sub> tolerization was as efficient as PLP<sub>139–151</sub> tolerization at suppressing disease, indicating bi-directional cross-reactivity between these two epitopes.

### Molecular mimicry in a humanized mouse model

Many studies have been conducted to identify the specificity of autoreactive T-cell clones isolated from in MS patients. Whereas PLP<sub>139–151</sub> is the immunodominant myelin protein epitope in SJL/J mice, MBP<sub>85–99</sub> is the CD4<sup>+</sup> immunodominant epitope in humans. Double transgenic mice expressing a humanized MBP<sub>85–99</sub>-specific TCR and human HLA-DR2 (MHC class II) on the C57BL/6 mouse background can be induced to develop a demyelinating disease similar to MS when primed with MBP<sub>85–99</sub>/CFA (59).

We employed two different strains of humanized mice to examine the ability of bacterial MBP mimics to induce EAE. The two strains we used were on genetically different backgrounds, which interestingly resulted in differential outcomes in disease. The first strain was generated on the C57BL/6 background with a null mutation for mouse MHC class II (H2-A) (dTgH2A<sup>neg</sup>) (60). When primed with MBP<sub>85–99</sub>/CFA [plus pertussis toxin (PT)], these mice developed a severe monophasic disease. In contrast, humanized mice on the mixed DBA/2xC57BL/6 (dTgH2A<sup>pos</sup>) background with intact mouse MHC class II developed a relapsing disease course when primed with MBP<sub>85–99</sub>/CFA (61).

To determine the ability of bacterial mimics to induce CNS autoimmune demyelinating disease in the humanized mice, we primed both strains with five bacterial peptide-mimics — Staphylococcus aureus (SAU), Mycobacterium avium (MBA), Mycobacterium tuberculosis (MBT), Bacillus subtilis (BACS), and Haemophilus influenza (HI) (Table 1). Homology to MBP ranged from 20 to 33% (3–5/15 AA residues), and all mimics shared at least two of three primary TCR contact residues. This appears to be important for disease induction, whereas fidelity between the primary MHC contact residues was variable. Disease was induced by all mimics in dTgH2A<sup>neg</sup> mice, with the exception of HI and MBT. Similar to MBP<sub>85–99</sub>-induced EAE, the mice exhibited a monophasic disease, although the clinical severity was milder. In dTgH2A<sup>pos</sup> mice, all mimics, including HI, induced R-EAE; however, HI disease was extremely mild (62).

We examined the infiltrate in the brains and spinal cords of infected mice. In both transgenic strains, CD4<sup>+</sup> T cells were seen in the cerebellum, albeit around the periphery, in mice primed with the mimics (62). The degree of infiltration correlated with disease; mice primed with the HI mimic peptide (in the dTgH2A<sup>neg</sup>) did not have any infiltration in either the cerebellum or lower lumbar spinal cord. There was a slight difference between strains, in that more F4/80<sup>+</sup> infiltrates...
were found in the cerebellum of dTgH2A\textsuperscript{pos} compared to dTgH2A\textsuperscript{neg} (62). Perhaps this could partially explain this difference in disease phenotype seen between the two strains. However, in both strains, the immune infiltration was observed mainly in the lower lumbar spinal cord. We also observed demyelination in clinically affected mice of both strains.

We also compared T-cell responses between naive and primed animals to determine the degree of cross-reactivity among the mimic peptides. A second goal was to determine whether the degree of response of naive transgenic T cells would be indicative of later disease outcome. In vitro assays were employed to examine proliferation and cytokine production. In both strains, the greatest response was seen when naive T cells were cultured with MBP\textsubscript{85–99}; however, both naive and primed T cells proliferated (cross-reacted) with all mimics. Cytokine production, however, was not consistent with proliferation. While low levels of IFN-\(\gamma\), IL-17, and IL-2 were seen with most mimics, SAU induced large amounts of IFN-\(\gamma\) (even from naive T cells) and high levels of IL-17 (in primed dTgH2A\textsuperscript{neg} T cells). MBT also skewed toward a Th1 response with production of IFN-\(\gamma\) in naive dTgH2A\textsuperscript{neg} T cells (62). Overall, this model is extremely relevant to human disease, as the mice express humanized receptors and the pathogen-derived mimics employed are routinely encountered by humans.

Conclusions

Our studies indicate that molecular mimicry is a viable explanation for the induction of a wide variety of autoimmune diseases that have been shown to have both a genetic predisposition and are associated with environmental influences. Given the wide genetic diversity of individuals displaying a particular autoimmune disease, it is highly unlikely that any one infectious agent will be found to be exclusively associated with a disease. Depending on the genetics of the individual, it is possible that an autoimmune disease could be triggered by a variety of infectious agents contingent on many variables. These variables could include the number of potential cross-reactive epitopes encoded by the pathogen, the ability of the infectious agent to activate the innate immune system via stimulation of pattern recognition receptors, such as TLRs, the cellular/tissue tropism of the agent, the route of infection, and the age and immune repertoire of the affected individual at the time of infection. Significantly, progression of mimicry-induced autoimmune disease can be mediated by additional mechanisms, including epitope spreading, thus these two potential mechanisms for initiation of infection-induced autoimmune disease are not mutually exclusive. In conclusion, much still remains to be learned about the interaction of genetics, environment, and immune effector function/regulation in the induction and progression of autoimmune disease.

Table 1. Amino acid sequences of MBP\textsubscript{85–99} and its bacterial mimics

| Peptide source (abbreviation) | Amino acid residue/sequence |
|-----------------------------|----------------------------|
| Myelin Basic Protein (MBP)  | E\(^*\) N P V V H F F K N I V T P R |
| Staphylococcus aureus (SAU) | V L A R L H F Y R N D V H K E |
| Mycobacterium avium (MBA)   | Q R C R V H F L R N V L A Q V |
| Mycobacterium tuberculosis (MBT) | Q R C R V H F M R N L Y T A V |
| Bacillus subtilis (BACS)     | A L A V L H F Y P D K G A K N |
| Haemophilus influenzae (HI)  | D F A R V H F I S A L H G S G |

Boldface indicates residues shared with the self-human myelin basic protein epitope. Residues 5 and 8 are primary MHC contact residues; residues 6, 7, and 9 are primary TCR contact residues (63).

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