Structural requirements for initiation of cross-reactivity
and CNS autoimmunity with a PLP$_{139-151}$ mimic peptide
derived from murine hepatitis virus

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MS is an autoimmune CNS demyelinating disease in which infection appears to be an
important pathogenic factor. Molecular mimicry, the cross-activation of autoreactive
T cells by mimic peptides from infectious agents, is a possible explanation for infection-
duced autoimmunity. Infection of mice with a non-pathogenic strain of Theiler's
murine encephalomyelitis virus (TMEV) engineered to express an epitope from
Haemophilus influenzae (HI) sharing 6/13 amino acids with the dominant proteolipid
protein (PLP) epitope, PLP$_{139-151}$, can induce CNS autoimmune disease. Here we
demonstrate that another PLP$_{139-151}$ mimic sequence derived from murine hepatitis
virus (MHV) which shares only 3/13 amino acids with PLP$_{139-151}$ can also induce CNS
autoimmune disease, but only when delivered by genetically engineered TMEV, not by
immunization with the MHV peptide. Further, we demonstrate the importance of
proline at the secondary MHC class II contact residue for effective cross-reactivity, as
addition of this amino acid to the native MHV sequence increases its ability to cross-
activate PLP$_{139-151}$-specific autoreactive T cells, while substitution of proline in the HI
mimic peptide has the opposite effect. This study describes a structural requirement for
potential PLP$_{139-151}$ mimic peptides, and provides further evidence for infection-
duced molecular mimicry in the pathogenesis of autoimmune disease.

Introduction

The mechanism(s) of initiation and perpetuation of
multiple sclerosis (MS), a human immune-mediated
demyelinating disease of the central nervous system
(CNS) are unknown. Clinical and epidemiological
studies suggest that both genetic and environmental
factors are important in the initiation and pathogenesis
of MS [1]. Viral infections have been associated with
other demyelinating CNS diseases, both in humans and
animals [2–5], and various environmental factors have
been associated with the initiation and exacerbation of
MS, including viral infections [6, 7]. MS is believed to be
mediated by myelin-specific CD4$^+$ T cells. Although
myelin-specific autoreactive T cells can be isolated from
the blood of normal healthy individuals, they do not
have an activated phenotype. Therefore, a critical
question is how do these cells become activated in MS
patients? One potential mechanism is molecular mimicry,
whereby autoreactive T cells may be cross-activated

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Abbreviations: DTH: delayed-type hypersensitivity ·
HI: Haemophilus influenzae · MHV: mouse hepatitis virus ·
p9: position 9 · PLP: proteolipid protein · TMEV: Theiler’s
murine encephalomyelitis virus
by epitopes from infectious agents that share structural or sequence homology to self antigens [8, 9]. Direct evidence for molecular mimicry in human autoimmune disease is difficult to obtain and support is limited to demonstration of in vitro reactivation of myelin-specific T cell clones from MS patients by panels of antigens from infectious agents [10]. In this regard, molecular mimicry between the U24 protein of human herpesvirus-6 (HHV-6), a virus that may be associated with MS, and a candidate autoantigenic epitope for MS, MBP 96–102, has recently been demonstrated [11].

Therefore, better understanding of the potential mechanisms of molecular mimicry-induced autoreactive T cell activation is important in elucidating the pathogenesis of MS. Previously, we demonstrated that infection of SJL mice with a non-pathogenic strain of Theiler's murine encephalomyelitis virus (TMEV) encoding a myelin mimic peptide from Haemophilus influenzae (HI) can cross-activate myelin proteolipid protein (PLP) 139–151-specific CD4+ Th1 cells and induce an atypical CNS autoimmune disease [12, 13]. The HI mimic peptide shares 6 of 13 amino acids with the major encephalitogenic peptide in SJL mice, PLP 139–151. Although the sequence identity between the peptides is only 46%, structural identity of amino acids at the primary MHC class II (I-Aα) and TCR contact residues is vital to the mechanism of molecular mimicry [14]. Another potential PLP 139–151 mimic peptide has been identified from murine hepatitis virus (MHV), a single-stranded RNA coronavirus [15]. The MHV 3821–3832 mimic peptide only shares 3 of 13 amino acids with the core encephalitogenic epitope in SJL mice, PLP 139–151 (23% homology), including the primary TCR and MHC class II contact residues, but neither the secondary MHC class II proline residue nor the secondary TCR contact residue. The MHV peptide binds I-Aα and induces limited activation of T cell clones derived from PLP 139–151-primed SJL mice [15] and a proline residue at position 9 (p9) of the MHC binding motif is important for binding to I-Aα [16].

Here, we demonstrate the importance of this MHC class II structural motif in an infection-induced model of molecular mimicry which may be important for recognition of potential pathogen-derived mimic sequences. In addition, we demonstrate that the MHV mimic sequence is a natural immunogenic T cell epitope in SJL mice as it can be processed from a MHV 30-mer peptide by APC. Finally, we demonstrate that autoimmune disease can be initiated in mice infected with replicating virus engineered to express the natural MHV mimic sequence, but not by immunization with the MHV peptide in CFA. Therefore, this paper highlights the structural requirements of pathogen-derived myelin mimic peptides and the critical importance of studying a “live” infection concomitant to presentation of mimic peptides.

**Results**

MHV mimic peptide immunization does not induce demyelinating clinical disease

A previous investigation had identified a potential PLP 139–151 molecular mimic peptide expressed by MHV [15]. We thus asked if this mimic peptide could cross-prime/activate PLP 139–151-specific T cells. Following the immunization of mice with PLP 139–151 or a PLP 30-mer peptide (PLP 130–159) encompassing the core PLP 139–151 encephalitogenic epitope, in CFA, 100% of SJL mice exhibited a typical acute-phase disease course of EAE (Table 1). In contrast, mice immunized with the PLP 139–151 mimic peptide, MHV 3821–3832 (MHV), or an MHV 30-mer peptide (MHV 3813–3842) containing the MHV 3821–3832 minimal epitope did not exhibit clinical disease (Table 1). The ability of MHV immunization to induce the cross-activation of PLP 139–151-specific T cells was measured by in vivo delayed-type hypersensitivity (DTH) and in vitro T cell proliferation assays. DTH

**Table 1.** MHV mimic peptides fail to induce EAE

| Immunizing Aga) | No. EAE | Mean group score ± SEM | Mean day of onset ± SEM |
|-----------------|---------|------------------------|------------------------|
| OVA 323–339     | 0/5     | 0.0 ± 0.0              | 0.0 ± 0.0              |
| PLP 139–151     | 5/5     | 4.0 ± 0.0              | 12.2 ± 0.8             |
| PLP 130–159     | 5/5     | 3.6 ± 0.5              | 13.4 ± 1.5             |
| MHV 3821–3832   | 0/5     | 0.0 ± 0.0              | 0.0 ± 0.0              |
| MHV 3813–3842   | 0/5     | 0.0 ± 0.0              | 0.0 ± 0.0              |

a) Separate groups of mice were immunized s.c. on day 0 with 100 µg of either “self” myelin peptide PLP (PLP 139–151), PLP 30-mer (PLP 130–159), MHV mimic peptide (MHV 3821–3832), MHV 30-mer (MHV 3813–3842) or a control non-self, non-mimic peptide OVA 323–339 and observed for signs of clinical EAE. Results represent the number of mice that developed EAE, the mean group clinical score ± SEM, and the mean day of onset ± SEM.
rechallenge with PLP<sub>139–151</sub> in MHV-immunized mice on day 16 post immunization did not cross-activate PLP<sub>139–151</sub>-specific T cells; however, PLP<sub>139–151</sub> DTH responses were significantly higher in PLP<sub>139–151</sub>-immunized mice compared to MHV- or OVA-immunized mice, or naive mice (§<i>p</i> < 0.05) (Fig. 1A). MHV-immunized mice had significantly greater DTH responses following rechallenge with MHV compared to control groups (*<i>p</i> < 0.05) and, interestingly, PLP<sub>139–151</sub>-immunized mice responded to both PLP<sub>139–151</sub> and MHV rechallenge, suggesting unidirectional molecular mimicry (Fig. 1A). We also tested whether the putative MHV-encoded PLP-mimic sequence was a natural epitope for SJL mice in the context of I-A<sup>b</sup>. Mice immunized to the 30-mer MHV<sub>3813–3842</sub> peptide, encompassing the candidate PLP<sub>139–151</sub> mimic epitope, demonstrated DTH responses in vivo (Fig. 1A) and in vitro proliferative responses (Fig. 1B) upon rechallenge with the MHV short peptide, indicating that the short-length mimic MHV epitope may be processed by APC and induce MHV-specific T cell responses. However, in contrast to the in vivo DTH data, in vitro MHV rechallenge in both PLP-immunized groups of mice did not induce T cell proliferation (Fig. 1B). This result may reflect the differences between in vitro and in vivo antigen processing and the sensitivity of the microenvironment in vivo to peptide rechallenge. In contrast, MHV-immunized mice responded to in vitro rechallenge with the PLP<sub>139–151</sub> peptide, albeit at a significantly lower level than rechallenge of PLP-immunized mice (Fig. 1C). Anti-CD3 antibody stimulation of T cells from peptide-immunized mice was equivalent between all groups (Fig. 1B, C).

**Asparagine for proline substitution at p9 effects Th1 responses and clinical disease**

To determine whether the amino acid identity at p9 is important in conferring molecular mimicry to mimic peptides, we produced “altered mimic peptide ligands” by the substitution of asparagine (N) with proline (P) at p9 (P → N) in the MHV peptide (MHV+P; Fig. 2) and immunized mice with either MHV or MHV+P peptides in CFA. Clinical disease was not induced in either group compared with the positive control mice immunized with PLP<sub>139–151</sub> (Table 2). To determine whether MHV+P immunization induced cross-reactive PLP<sub>139–151</sub> proliferative responses, mice were rechallenged in vitro with either the immunizing peptide or PLP<sub>139–151</sub> on day 14 following peptide immunization. Rechallenge with PLP<sub>139–151</sub> in PLP<sub>139–151</sub>-immunized mice induced significant T cell proliferation compared to control groups (Fig. 3A). Interestingly, PLP<sub>139–151</sub> rechallenge in MHV+P-immunized mice also induced a modest T cell proliferative response compared with MHV- or OVA<sub>323–339</sub>-immunized mice (Fig. 3A). In MHV-immunized mice, in vitro rechallenge with MHV induced the greatest proliferative response, as expected, and a
lesser response in MHV+P-immunized mice (Fig. 3B). Responses in PLP139–151- and OVA323–339-immunized mice were similar to those of naive mice (Fig. 3B). Rechallenge with MHV+P induced strong responses in both MHV- and MHV+P-immunized mice (Fig. 3C). In addition, we substituted asparagine at p9 in MHV3821–3832 with proline (MHV3821–3832–P). The I-A\* restricted OVA323–335 peptide used as a negative control does not share sequences with PLP, HI or MHV peptides.

IFN-\(\gamma\) secretion was measured to determine whether MHV+P-induced cross-activation of PLP139–151 T cells could induce Th1 differentiation (Fig. 3F–H). As expected, PLP139–151 rechallenge in PLP139–151-immunized mice induced significant quantities of IFN-\(\gamma\) compared to naive or OVA-immunized mice (Fig. 3F). Interestingly, PLP139–151 rechallenge in MHV+P-immunized mice induced significant IFN-\(\gamma\) production (Fig. 3F) in contrast to MHV-immunized mice. Surprisingly, MHV rechallenge induced a significantly greater IFN-\(\gamma\) response (Fig. 3G) in MHV+P-immunized mice compared to MHV-immunized mice. The populations of cells that respond to MHV or PLP can be thought of as separate populations in which some cells can be cross-activated by mimic peptides. Fig. 3A, B shows that restimulation of MHV-immunized mice with MHV induces greater proliferation than PLP rechallenge of PLP-immunized mice. However, PLP rechallenge of PLP-immunized spleenocytes can induce significantly larger IFN-\(\gamma\) secretion than MHV rechallenge of MHV-immunized mice (Fig. 3F, G). Therefore, the MHV+P peptide may “target” more “PLP-specific” cells (high IFN-\(\gamma\), moderate proliferation), rather than the MHV-specific cells (high proliferation, low IFN-\(\gamma\) secretion). Differences between the populations may be due to relative binding affinities of the respective peptides for I-A\* [15]. Although rechallenge with the MHV+P peptide induced similar quantities of IFN-\(\gamma\) in MHV- and MHV+P-immunized mice, rechallenge with MHV+P, but not MHV, induced IFN-\(\gamma\) responses in PLP139–151-immunized mice (Fig. 3H). Anti-CD3 antibody responses in all groups were equal (data not shown).

Proline at p9 is required for induction of myelin-specific Th1 responses

As substitution of asparagine by proline at p9 of the MHV peptide increased its potential to cross-activate PLP-specific T cells, we investigated the consequences of substitution of proline by asparagine (P\(\rightarrow\)N) at p9 in the HI574–586 peptide, a known PLP139–151 mimic peptide [12, 13, 15]. No clinical disease was induced in SJL mice immunized with either HI or the HI peptide with the proline at p9 substituted with asparagine (HI–P) (Table 2). Mice immunized to HI demonstrated significant proliferative responses to rechallenge with HI, and modest responses to PLP139–151, HI–P, MHV+P

### Table 2. Altered mimic peptides fail to induce EAE

| Immunizing Ag\(^a\) | No. EAE | Mean group score ± SEM | Mean day of onset ± SEM |
|---------------------|---------|------------------------|-------------------------|
| OVA | 0/5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| PLP | 5/5 | 3.4 ± 0.5 | 13.8 ± 1.3 |
| HI | 0/5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| HI–P | 0/5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| MHV | 0/5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| MHV+P | 0/5 | 0.0 ± 0.0 | 0.0 ± 0.0 |

\(^a\) Separate groups of mice were immunized s.c. on day 0 with 100 \(\mu\)g of either “self” myelin peptide PLP139–151 (PLP), viral mimic peptides MHV3821–3832 (MHV) or MHV3821–3832+P (MHV+P), bacterial mimic peptides HI574–586 (HI) or HI574–586–P (HI–P), or a control non-self, non-mimic peptide OVA323–339 (OVA) and observed for signs of clinical EAE. Results represent the number of mice that developed EAE, the mean group clinical score ± SEM, and the mean day of onset ± SEM.
and MHV peptide rechallenge compared to OVA-immunized or naive mice (Fig. 3D). In contrast, HI–P immunized mice responded significantly to rechallenge with HI–P, and modestly to HI but not to other peptides, including PLP139–151 (Fig. 3E).

IFN-γ secretion was analyzed to determine whether the HI–P peptide was less active than HI in inducing Th1 PLP139–151 cross-reactive responses. Significant Th1 responses were observed in HI-immunized mice rechallenged in descending order with HI > PLP > HI–P >> MHV+P compared to control groups (Fig. 3F–J). However, HI–P-immunized mice responded to HI–P >> HI >> MHV > MHV+P but not to PLP (Fig. 3F–J). Interestingly, MHV+P-immunized mice also demonstrated significant Th1 cross-reactivity following HI rechallenge, compared to MHV peptide-immunized mice (Fig. 3I).

Proline residue at p9 is critical for tolerogenic inhibition of PLP139–151-induced EAE

To further determine the mimic potential of “altered mimic peptide ligands”, SJL mice (n = 8) were pretolerized to MHV+P on day −7/+3 and immunized to PLP139–151, to determine whether the altered MHV+P peptide could deliver a cross-reactive tolerogenic signal to PLP139–151-specific T cells and inhibit the onset of EAE. Mice tolerized with PLP139–151, HI574–586 or MHV+P all exhibited a significant amelioration of clinical disease (*p < 0.05) compared with untolerized mice and OVA peptide-tolerized controls (Table 3). As anticipated, tolerance with the native MHV peptide did not inhibit EAE. Furthermore, unlike HI574–586-tolerized mice, HI–P tolerance failed to protect against PLP139–151-induced EAE.

In vivo DTH and in vitro T cell proliferative responses were measured to determine the effect of the altered mimic peptide tolerogenic signal on the activation of PLP139–151-specific T cells. Mice tolerized to either PLP139–151, HI574–586 or MHV+P all showed significantly reduced DTH and proliferative responses following rechallenge with PLP139–151 compared to control groups or groups tolerized to MHV (*p < 0.05; Fig. 4A, B). The groups tolerized with the altered peptide mimics, MHV+P and HI–P, demonstrated significantly lower or higher DTH and proliferative responses, respectively, compared to groups tolerized with HI.
with the native peptide sequences, following rechallenge with PLP139–151.

Infection of mice with MHV30-BeAn induces a flaccid paralytic disease

Although P at p9 of the MHV peptide is critical for the effective cross-activation of PLP-specific T cells, we previously demonstrated that a weak stimulation of PLP-specific T cells by immunization with the mimic peptide in CFA could be enhanced by delivery of the mimic peptide encoded in an infectious virus [12, 13]. Therefore, we tested whether intracerebral infection of mice with recombinant TMEV engineered to express the MHV 30-mer peptide containing the putative PLP139–151 mimic peptide (MHV30-BeAn) could induce activation of PLP139–151-specific T cell responses and ultimately clinical disease. SJL mice infected with MHV30-BeAn exhibited a mild disease with pronounced gait abnormality, similar in severity and early onset to mice infected with TMEV expressing the self PLP139–151 epitope (PLP-BeAn) (Fig. 5A). This was in contrast to mice infected with OVA-BeAn. In vitro rechallenge with PLP 139–151 peptide demonstrated PLP139–151 reactive CD4+ T cells present at day 20 post infection in PLP-BeAn- and MHV30-BeAn-infected mice as measured by IFN-γ secretion (Fig. 5B), in contrast to mice infected with OVA-BeAn. Furthermore, significant responses to MHV3821–3832 peptide rechallenge in vitro (IFN-γ secretion; Fig. 5C) demonstrated that the MHV 30-mer peptide is a likely natural epitope in SJL mice.

Table 3. Altered mimic peptide tolerance of PLP139–151-induced EAE

| Tol Ag(1) | No. EAE | Mean group score ± SEM | Mean EAE score ± SEM | Mean day of onset ± SEM |
|-----------|---------|------------------------|----------------------|-------------------------|
| No tol    | 5/5     | 3.8 ± 0.2              | 3.8 ± 0.2            | 12.0 ± 0.9              |
| OVA       | 5/5     | 3.6 ± 0.2              | 3.6 ± 0.2            | 11.6 ± 0.8              |
| PLP       | 2/5     | 0.4 ± 0.2(2)           | 1.0 ± 0.1(2)         | 14.5 ± 1.5              |
| HI        | 3/5     | 0.8 ± 0.4(2)           | 1.3 ± 0.3(2)         | 13.7 ± 1.9              |
| HI–P      | 5/5     | 3.0 ± 0.5              | 3.0 ± 0.5            | 11.8 ± 0.6              |
| MHV       | 5/5     | 3.4 ± 0.2              | 3.4 ± 0.2            | 11.8 ± 0.9              |
| MHV+P     | 5/5     | 2.0 ± 0.4(2)           | 2.0 ± 0.4(2)         | 11.4 ± 0.4              |

(1) Separate groups of mice were immunized s.c. on day 0 with 100 μg of PLP139–151 and were tolerized to OVA323–339 (OVA), PLP139–151 (PLP), HI574–586 (HI), HI574–586–P (HI–P), MHV3821–3832 (MHV), or MHV3821–3832+P (MHV+P) at 7 days pre and 3 days post immunization with PLP139–151. Results represent the number of mice that developed EAE, the mean group clinical score ± SEM, the mean EAE score ± SEM (excluding mice not exhibiting disease), and the mean day of onset ± SEM.

(2) p < 0.05 compared to control OVA-tolerized or non-tolerized mice.

Figure 4. Proline at p9 of the mimic MHV3821–3832 or HI574–586 peptides is critical for induction of cross-reactivity to PLP139–151 as assessed by peripheral tolerance. (A) Separate groups of SJL mice were tolerized to OVA323–339 (OVA), PLP139–151 (PLP), HI574–586 (HI), HI574–586–P (HI–P), MHV3821–3832 (MHV), or MHV3821–3832+P (MHV+P) coupled to syngeneic, ECDI-fixed splenocytes at 7 days pre and 3 days post immunization with PLP139–151. CD4+ T cell proliferative responses were measured in response to PLP139–151 rechallenge. *Values significantly reduced compared to control levels in OVA-tolerized or non-tolerized mice, p < 0.05. (B) In vivo DTH ear swelling assays on day 14 post priming to PLP139–151. Mice tolerized to PLP, HI or MHV+P exhibited reduced DTH responses in contrast to OVA-tolerized or MHV3821–3832-tolerized mice or non-tolerized mice, p < 0.05.
rarely observed in the same region from OVA-BeAn-infected mice (Fig. 5E).

**Discussion**

MHV is a single-stranded RNA coronavirus. Multiple strains exist that can induce a variety of diseases including hepatitis and enteritis. Interestingly, infection of mice with the JHM strain can induce acute encephalitis and chronic demyelination of the CNS with some similarities to MS pathology [17]. Although coronavirus infection of humans is usually associated with upper respiratory tract infections, coronavirus antigens have been identified in demyelinating plaques from the CNS of MS patients; however, it is not clear if these viruses have a role in MS pathology [18, 19].

Recently, we demonstrated that infection of mice with a recombinant TMEV virus encoding a myelin mimic peptide from HI, HI574–586, but not priming with the core HI574–586 epitope in CFA, could induce a non-classical form of CNS inflammatory autoimmune disease, by the cross-activation of PLP139–151-specific Th1 cells [12, 13]. MHV3821–3832 is another potential PLP139–151 mimic peptide with 23% homology to the major encephalitogenic peptide in SJL mice, PLP139–151 [15]. Although the majority of the amino acid sequence is different between the natural PLP and viral MHV peptides, they share similar primary TCR (W at p5) and MHC class II (K at p4) contact residues. MHV3821–3832 is located in the ORF1a gene which codes for the replicative polyprotein PL1-PRO (Papain-like proteases), a multifunctional protein involved in the transcription of negative-stranded RNA, leader RNA, subgenomic mRNA and progeny virion RNA, as well as
cleaving the polyprotein into functional products located at the N terminus of the replicase polyprotein. We and others have demonstrated that immunization of SJL mice with HI574–586 can induce expansion of PLP139–151-specific T cells, although Th1 differentiation is minimal and mice do not exhibit clinical signs of CNS autoimmune disease even after multiple immunizations in CFA ± pertussis toxin and/or LPS as additional innate immune stimuli [12, 13, 15]. It has been proposed that the sequence of mimic peptides need not be homologous to self peptides; rather, homology at the key structural TCR and MHC residues is important for mimicry. Our study of the properties of the MHV mimic peptide confirms that of Carrizosa et al. [15], as although immunization of SJL mice with the MHV mimic epitope induced a robust recall T cell response to MHV peptide, we observed only minor PLP139–151-induced recall responses, particularly IFN-γ production. Nevertheless, these data demonstrated the potential for cross-activation of PLP139–151 T cells by the MHV peptide. In PLP139–151-immunized mice, we also observed a trend towards cross-reactivity following recall with the MHV mimic peptide. To further confirm these initial observations, we demonstrated that despite limited cross-reactivity between the MHV and PLP peptides, MHV3821–3832 peptide-coupled splenocyte tolerance of PLP139–151-induced EAE was ineffective at inhibiting disease and T cell recall responses, in contrast to PLP139–151 or HI574–586-induced tolerance.

Previously, it was shown that the critical anchor residues in the I-Aβ ligand binding motif were p4, p7, and especially p9 [16]. It was postulated that the residue at p9 may be a critical susceptibility factor in I-Aβ-associated autoimmune disease with regard to presentation of self peptides [16]. To take this observation further and to attempt to understand the differences in PLP139–151-specific T cell cross-activating potential between the HI574–586 and MHV3821–3832 mimic peptides, we sought to determine the influence of the p9 MHC class II proline anchor residue in molecular mimicry between the “self” PLP139–151 epitope and foreign viral antigens. Secondly, we wanted to establish whether viral delivery of the MHV mimic peptide could overcome the threshold necessary for cross-reactive activation of pathogenic, autoreactive PLP-specific Th1 cells similar to what we had previously reported for disease induction by the HI574–586 mimic peptide.

Both PLP139–151 and HI574–586 have a proline residue (P, aliphatic) at p9 in the MHC class II ligand motif, whereas MHV3821–3832 contains asparagine (N, amideic). Interestingly, substitution of the secondary MHC class II contact residue at p9 (N→P) conferred an ability on the MHV peptide to induce stronger cross-reactive PLP139–151-specific T cell responses, as measured by T cell proliferation or IFN-γ ELISA assays. In contrast, substitution of P→N at p9 of the HI mimic peptide (HI–P) abrogated the ability of that peptide to induce cross-activation of PLP139–151-specific T cells. In each case, mice made the most robust response to the immunizing peptide, as expected, but the gain or loss of proline at p9 either induced or negated the “mimic potential” of the peptide, respectively. Interestingly, mice immunized to MHV responded more strongly to recall with HI–P than HI, probably as neither peptide contained P at p9. Conversely, only MHV+P and not MHV peptide recall challenge induced IFN-γ production by T cells from HI574–586-immunized mice, likely due to the presence of P at p9 in both peptides. Although mice responded with robust IFN-γ responses to their initial immunizing peptide, only mice with a strong PLP139–151 response (i.e., PLP139–151-immunized mice) exhibited clinical EAE. As the mimic peptide-specific T cells were shown to be competent IFN-γ producers this suggests that they do not recognize or are not sufficiently reactivated in vivo in the CNS by endogenously presented PLP peptides and therefore do not induce clinical EAE [20].

Although immunization with the MHV+P peptide did not induce clinical disease, we further demonstrated its increased cross-reactivity to PLP compared to MHV, by peptide-coupled splenocyte tolerance studies. In contrast to MHV or HI–P peptide-coupled splenocytes which were not protective, MHV+P induced inhibition of PLP-induced clinical disease and tolerance of PLP-specific T cells similar to tolerance induced with HI or PLP. The cross-reactive effect of MHV+P could also be measured by suppressed DTH and T cell proliferative responses to PLP rechallenge in MHV+P-tolerized, PLP-induced EAE mice.

Although the presence of proline at the secondary MHC class II contact residue is a critical factor in MHV-induced cross-reactivity, it is insufficient to induce EAE upon priming of SJL mice with the peptide. Previously, we demonstrated the importance of studying PLP-mimic peptides in the context of a concomitant infection [12, 13]. Here, we corroborate this hypothesis as infection of mice with TMEV engineered to express the 30-mer MHV peptide induces a chronic autoimmune disease characterized by atypical clinical symptoms, similar in severity and time course to disease initiated with PLP- or HI-expressing TMEV [12, 13, 21]. In contrast MHV/CFA-immunized mice do not develop disease. This is likely due to the fact that MHV30-BeAn-infected mice develop a robust IFN-γ-secreting PLP139–151-specific T cell population, unlike MHV/CFA-immunized mice. It is likely that activation of peripheral and central immune cells by TMEV infection provides innate immune stimuli that can overcome the threshold for activation of PLP139–151-specific T cells by the MHV peptide. Interestingly, Th1 recall responses, particularly
IFN-γ, to the self PLP139–151 epitope were significantly higher in MHV30-BeAn-infected mice than were responses to the MHV mimic peptide itself. This likely reflects prolonged restimulation of high-avidity PLP139–151-specific CD4+ T cells by self peptide endogenously released as a consequence of chronic myelin damage presented locally in the CNS by infiltering dendritic cells [22], and the increased affinity of PLP139–151 (IC50 87 nM) for I-Aα compared to MHV3821–3832 (IC50 727 nM) [15]. We also observed modest numbers of CD4+ T cells in the brain of MHV30-BeAn mice on day 40 post infection, in contrast to OVA-BeAn-infected mice, suggesting that the presence of MHV-specific T cells correlates with the early onset of disease observed in MHV30-BeAn mice. Furthermore, we demonstrate that the MHV mimic peptide is a natural epitope for the SJL mouse as infection with MHV30-BeAn encoding the MHV 30-mer mimic sequence could be processed and presented by APC as measured by in vivo DTH responses.

In conclusion, this study demonstrates that the secondary MHC class II (I-Aα) anchor residue at p9 plays a critical role in the ability of PLP139–151 Mimic peptides to induce molecular mimicry leading to induction of CNS autoimmune disease and may be an important consideration when identifying candidate mimic peptides encoded by infectious pathogens. Our model of mimic peptide-expressing TMEV allows us to test other potential infectious agent-encoded PLP-mimic peptides with reproducible immunological readouts. This study also highlights an important structural feature of candidate mimic peptides which may be relevant to the pathogenesis of MS and also the necessity of studying mimicry in an infectious setting.

Materials and methods

Mice

Female SJL mice at 5–6 wk of age were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Mice were housed under barrier conditions at the Northwestern University Medical School Center for Comparative Medicine. All protocols were approved by the Northwestern University Animal Care and Use Committee. Paralyzed mice were afforded easier access to food and water.

Production of MHV30-BeAn

MHV30-BeAn virus was produced as follows. Two 110-bp oligonucleotides (Sigma-Genosys, The Woodlands, TX) were annealed together to form double-stranded DNA encoding a 30-amino acid piece of MHV (TTMLSLATAKVIKWALNVLYFTDVPQIK – encompassing the core 12-amino acid CD4+ T cell epitope) with a Cla I restriction site on each end. To anneal, the oligonucleotides were resuspended in buffer (50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 10 mM dithiothreitol, 25 μg/mL BSA pH 7.5), heated to 95°C for 2 min, then ramp-cooled to 25°C over a period of 45 min. Annealed DNA was then digested with Cla I and inserted into the △Clai-BeAn TMEV genome [21]. Viral RNA was transfected into BHK-21 cells in serum-free DMEM medium to produce an infecting virus stock. Mice were infected by intracerebral injection with 9 × 10^6 PFU of virus and scored at weekly intervals on a clinical scale of 0–5: 0, no signs of disease; 1, mild gait abnormalities; 2, severe gait abnormalities; 3, paralysis in one limb; 4, more than one paralyzed limb; 5, moribund. The data are plotted as the mean clinical score for each group of animals.

Induction of active EAE

For actively induced R-EAE, mice (n = 8–10) were immunized s.c. with 100 μL of an emulsion of CFA containing 400 μg Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI) and 50 μg PLP139–151 distributed over three sites on the lateral and dorsal hind flanks. For H1745–86 or OVA323–339 peptide priming, the same protocol was used with 50 μg peptide per animal. Clinical scores were assessed on a 0–5 scale as follows: 1, lack of tail tone; 2, impaired righting reflex; 3, partial hind limb paralysis; 4, total hind limb paralysis; and 5, moribund.

Peptides

The peptides were purchased from Peptides International (Louisville, KY): PLP130–159 30-mer (QAHSLERVCHCLGKWLGHPDKFGVT), PLP139–151 (HSLGKWLGHPKDF), HI574–586 (EQLVKWLGLPAPI), HI-P (EQLVKWLGLNAPI), MHV3821–3832, MHV3813–3842, and OVA323–339 (ISQAVHAAHAEINAGR). The amino acid composition was verified by mass spectrometry, and purity was assessed by HPLC.

Delayed-type hypersensitivity

DTH responses were elicited by injecting mice s.c. with 5–10 μg of the challenge peptides, PLP139–151 or MHV, into alternate ears following measurement of ear thickness using a Mitutoyo model 7326 engineer’s micrometer (Schlesinger’s Tools, Brooklyn, NY). At 24 h following peptide challenge, the ears were re-measured and differences in ear swelling over pre-challenge thickness were expressed in units of 10^4 inches ± SEM.

T cell proliferation and cytokine analysis

Spleens were removed from infected mice (n = 2) at various times following infection. T cell proliferation and cytokine analysis were performed as described [23]. Proliferation was determined from triplicate wells for each peptide concentration and then expressed as counts per minute (CPM) or as CPM, background subtracted. For IFN-γ cytokine analysis, a duplicate set of proliferation wells were used to collect supernatants at 48 and 72 h, and cytokine concentrations were
Tolerance was induced by i.v. injection of 5 × 10⁷ ethylene carbodiimide-treated peptide-pulsed syngeneic splenocytes, as described [23–25].

Immunohistochemistry

Immunohistochemistry was performed as described [13]. Briefly, two mice per experimental group were anesthetized and perfused with 1 × PBS on day 40 post infection. Brains were immediately frozen in OCT (Miles Laboratories; Elkhart, IN) in liquid nitrogen. Cross-sections (10 μm thick) from longitudinal sections of brain were sectioned. Nonspecific staining was blocked using anti-CD16/CD32 antibody (Fc receptor III/II, 2.4G2; BD PharMingen) and an avidin/biotin blocking kit (Vector Laboratories). Tissues were stained with biotin-conjugated antibody to mouse CD4 (BD PharMingen, San Diego, CA) and positive staining visualized by a Tyramide Signal Amplification (TSA) Direct kit (NEN, Boston, MA) according to the manufacturer’s instructions. Sections were counterstained with 4’,6’-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and then coverslipped with Vectashield mounting medium (Vector Laboratories). Slides were examined and images were acquired via epifluorescence using the SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI). Sections from each group were analyzed at 160 × magnification.

Statistics

Analysis of clinical severity was presented as the mean group clinical score, and the statistical difference was calculated by one-way ANOVA.

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