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

J. Ludovic Croxford, Anne M. Ercolini, Mathew DeGutes and Stephen D. Miller*

Department of Microbiology-Immunology and the Interdepartmental Immunobiology Center, Northwestern University Feinberg School of Medicine, Chicago, USA

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-induced 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-induced molecular mimicry in the pathogenesis of autoimmune disease.

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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

Correspondence: Dr. Stephen D. Miller, Dept. of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, 303 E. Chicago Avenue, Chicago, IL 60611, USA
Fax: +1-312-5031154
e-mail: s-d-miller@northwestern.edu

Abbreviations: DTH: delayed-type hypersensitivity · **HI:** *Haemophilus influenzae* · **MHV:** mouse hepatitis virus · **p9:** position 9 · **PLP:** proteolipid protein · **TMEV:** Theiler's murine encephalomyelitis virus

* **Current address:** Department of Immunology, National Institute of Neuroscience, National Centre of Neurology & Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187–8502, Japan

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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-A^s) 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 $PLP_{139-151}$ (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-A^s 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-A^s [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 Ag[a)]	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.

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rechallenge with $PLP_{139–151}$ in MHV-immunized mice on day 16 post immunization did not cross-activate $PLP_{139–151}$-specific T cells; however, $PLP_{139–151}$ DTH responses were significantly higher in $PLP_{139–151}$-immunized mice compared to MHV- or OVA-immunized mice, or naive mice ($^{\S}p <0.05$) (Fig. 1A). MHV-immunized mice had significantly greater DTH responses following rechallenge with MHV compared to control groups ($^{*}p <0.05$) and, interestingly, $PLP_{139–151}$-immunized mice responded to both $PLP_{139–151}$ 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^s. Mice immunized to the 30-mer $MHV_{3813–3842}$ peptide, encompassing the candidate $PLP_{139–151}$ 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 $PLP_{139–151}$ 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).

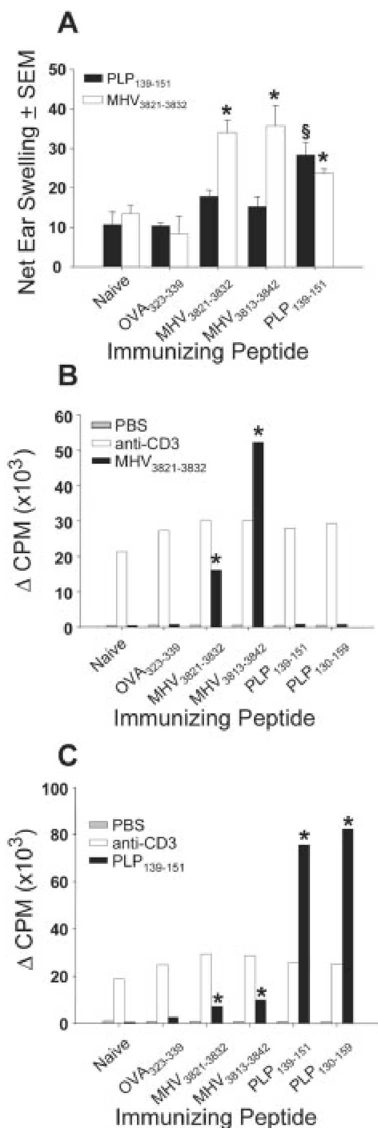

Figure 1. Cross-reactivity between PLP and MHV peptides *in vivo*. (A) Separate groups of mice were immunized s.c. on day 0 with 100 μg of either "self" myelin peptide $PLP_{139–151}$, $PLP_{130–159}$, mimic foreign peptide $MHV_{3821–3832}$, $MHV_{3813–3842}$ or a control non-self, non-mimic peptide $OVA_{323–339}$. *In vivo* differentiation of $CD4^+$ Th1 cells was assessed by DTH ear swelling assays on day 16 post priming upon challenge with 10 μg of either $PLP_{139–151}$ or $MHV_{3821–3832}$. Results are expressed as mean 24 h ear swelling ± SEM in groups of four to five mice. *Values significantly above control levels in naive or $OVA_{323–339}$-immunized mice, $p <0.05$. §Values significantly above levels in $OVA_{323–339}$-, $MHV_{3821–3832}$- or $MHV_{3813–3842}$-immunized or naive mice, $p <0.05$. $CD4^+$ T cell proliferative responses were assessed on day 18 in response to restimulation with 50 μM $MHV_{3821–3832}$ (B) or $PLP_{139–151}$ (C). T cell proliferation was determined at 96 h and results are expressed as cpm × 10^3 from triplicate cultures. *Values significantly above control levels in naive or $OVA_{323–339}$-immunized mice, $p <0.05$.

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 $PLP_{139–151}$ (Table 2). To determine whether MHV+P immunization induced cross-reactive $PLP_{139–151}$ proliferative responses, mice were rechallenged *in vitro* with either the immunizing peptide or $PLP_{139–151}$ on day 14 following peptide immunization. Rechallenge with $PLP_{139–151}$ in $PLP_{139–151}$-immunized mice induced significant T cell proliferation compared to control groups (Fig. 3A). Interestingly, $PLP_{139–151}$ rechallenge in MHV+P-immunized mice also induced a modest T cell proliferative response compared with MHV- or $OVA_{323–339}$-immunized mice (Fig. 3A). In MHV-immunized mice, *in vitro* rechallenge with MHV induced the greatest proliferative response, as expected, and a

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Peptide	Sequence
	p1 p2 p3 p4 p5 p6 p7 p8 p9
$PLP_{139-151}$	- H S L G K W L G H P D K F -
$MHV_{3821-3832}$	- K V I A K W L A V N V L -
$MHV_{3821-3832}$ + P	- K V I A K W L A V P V L -
$HI_{574-586}$	- E Q L V K W L G L P A P I -
$HI_{574-586}$ - P	- E Q L V K W L G L N A P I -
$OVA_{323-339}$	- I S Q A V H A A H A E I N E A G R -

Figure 2. Peptide sequences of myelin and myelin mimic peptides. The mimic $MHV_{3821-3832}$ and $HI_{574-586}$ epitopes share 3 and 6 of 13 amino acids, respectively, with the native $PLP_{139-151}$ epitope (in bold type), including the primary I-A^s binding residue (L145, p6) and the primary TCR contact residue (W144, p4). However, only the HI mimic peptide shares the (P148) I-A^s binding residue. To determine the role of proline in PLP molecular mimicry we substituted proline at p9 for asparagine ($HI_{574-586}$–P). In addition, we substituted asparagine at p9 in $MHV_{3821-3832}$ with proline ($MHV_{3821-3832}$+P). The I-A^s-restricted $OVA_{323-339}$ peptide used as a negative control does not share sequences with PLP, HI or MHV peptides.

lesser response in MHV+P-immunized mice (Fig. 3B). Responses in $PLP_{139-151}$- and $OVA_{323-339}$-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, MHV+P rechallenge induced T cell proliferative responses in $PLP_{139-151}$-immunized mice (Fig. 3C) in contrast to MHV rechallenge (Fig. 3B).

IFN-γ secretion was measured to determine whether MHV+P-induced cross-activation of $PLP_{139-151}$ T cells could induce Th1 differentiation (Fig. 3F–H). As expected, $PLP_{139-151}$ rechallenge in $PLP_{139-151}$-immunized mice induced significant quantities of IFN-γ compared to naive or OVA-immunized mice (Fig. 3F). Interestingly, $PLP_{139-151}$ rechallenge in MHV+P-immunized mice induced significant IFN-γ production (Fig. 3F) in contrast to MHV-immunized mice. Surprisingly, MHV rechallenge induced a significantly greater IFN-γ 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 splenocytes with MHV induces greater proliferation than PLP rechallenge of PLP-immunized mice. However, PLP rechallenge of PLP-immunized splenocytes can induce significantly larger IFN-γ secretion than MHV rechallenge of MHV-immunized mice (Fig. 3F, G). Therefore, the MHV+P peptide may "target" more "PLP-specific" cells (high IFN-γ, moderate proliferation), rather than the MHV-specific cells (high proliferation, low IFN-γ secretion). Differences between the populations may be due to relative binding affinities of the respective peptides for I-A^s [15]. Although rechallenge with the MHV+P peptide induced similar quantities of IFN-γ in MHV- and MHV+P-immunized mice, rechallenge with MHV+P, but not MHV, induced IFN-γ responses in $PLP_{139-151}$-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→N) at p9 in the $HI_{574-586}$ peptide, a known $PLP_{139-151}$ 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 $PLP_{139-151}$, 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 μg of either "self" myelin peptide $PLP_{139-151}$ (PLP), viral mimic peptides $MHV_{3821-3832}$ (MHV) or $MHV_{3821-3832}$+P (MHV+P), bacterial mimic peptides $HI_{574-586}$ (HI) or $HI_{574-586}$–P (HI–P), or a control non-self, non-mimic peptide $OVA_{323-339}$ (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.

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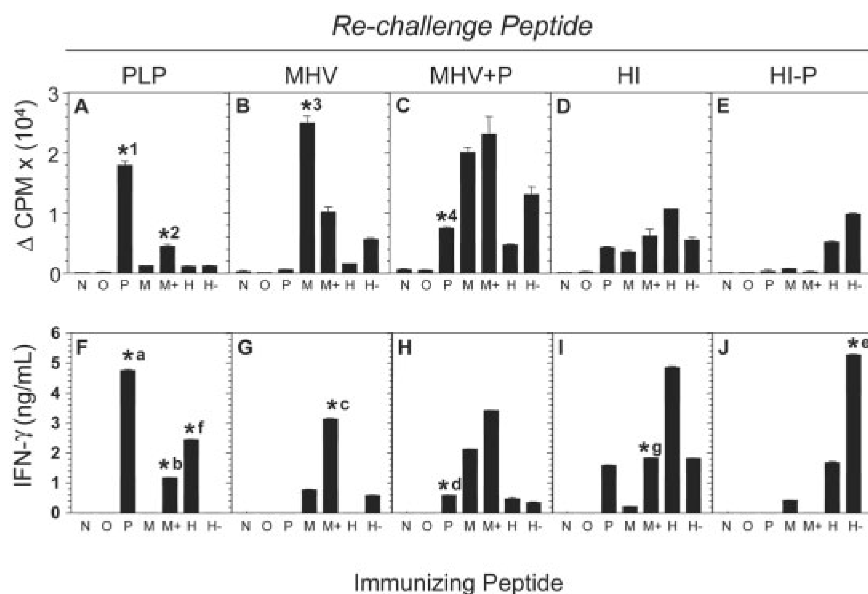

Figure 3. The altered mimic peptide $MHV_{3821-3832}$+P induces PLP-specific T cell responses, but fails to induce EAE. (A–E) Mice were immunized on day 0 with $PLP_{139-151}$ (P), $MHV_{3821-3832}$ (M), $MHV_{3821-3832}$+P (M+), $HI_{574-586}$ (H), $HI_{574-586}$–P (H–) or $OVA_{323-339}$ (O) and observed for signs of clinical EAE. $CD4^+$ T cell proliferative responses were measured in response to $PLP_{139-151}$ (A), $MHV_{3821-3832}$ (B), $MHV_{3821-3832}$+P (C), $HI_{574-586}$ (D) and $HI_{574-586}$–P (E) rechallenge. (F–J) $CD4^+$ T cell IFN-γ responses were measured in response to $PLP_{139-151}$ (F), $MHV_{3821-3832}$ (G), $MHV_{3821-3832}$+P (H), $HI_{574-586}$ (I) and $HI_{574-586}$–P (J) rechallenge. Naive (N) mice demonstrated no responses to rechallenge with any peptide. $^{*1}p<0.05$ compared to naive mice and mice immunized with OVA, MHV, MHV+, HI and HI–, rechallenged with PLP. $^{*2}p<0.05$ compared to MHV- and OVA-immunized mice rechallenged with PLP. $^{*3}p<0.05$ compared to MHV+-immunized mice rechallenged with MHV. $^{*4}p<0.05$ compared to PLP-immunized mice rechallenged with MHV. $^{*a}p<0.05$ compared to naive or OVA-immunized mice rechallenged with PLP. $^{*b}p<0.05$ compared to MHV-immunized mice rechallenged with PLP. $^{*c}p<0.05$ compared to MHV-immunized mice rechallenged with MHV. $^{*d}p<0.05$ compared to PLP-immunized mice rechallenged with MHV. $^{*e}p<0.05$ compared to mice immunized with HI– and rechallenged with HI, MHV, MHV+ and PLP. $^{*f}p<0.05$ compared to mice immunized with HI– and rechallenged with PLP. $^{*g}p<0.05$ compared to MHV-immunized mice rechallenged with HI.

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 $PLP_{139-151}$ (Fig. 3E). IFN-γ secretion was analyzed to determine whether the HI–P peptide was less active than HI in inducing Th1 $PLP_{139-151}$ 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 $PLP_{139-151}$-induced EAE

To further determine the mimic potential of "altered mimic peptide ligands", SJL mice ($n = 8$) were pre-tolerized to MHV+P on day –7/+3 and immunized to $PLP_{139-151}$, to determine whether the altered MHV+P peptide could deliver a cross-reactive tolerogenic signal to $PLP_{139-151}$-specific T cells and inhibit the onset of EAE. Mice tolerized with $PLP_{139-151}$, $HI_{574-586}$ 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 $HI_{574-586}$-tolerized mice, HI–P tolerance failed to protect against $PLP_{139-151}$-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 $PLP_{139-151}$-specific T cells. Mice tolerized to either $PLP_{139-151}$, $HI_{574-586}$ or MHV+P all showed significantly reduced DTH and proliferative responses following rechallenge with $PLP_{139-151}$ 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

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Table 3. Altered mimic peptide tolerance of $PLP_{139-151}$-induced EAE

Tol Ag[a)]	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[b)]	1.0 ± 0.1[b)]	14.5 ± 1.5
HI	3/5	0.8 ± 0.4[b)]	1.3 ± 0.3[b)]	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[b)]	2.0 ± 0.4[b)]	11.4 ± 0.4

a) Separate groups of mice were immunized s.c. on day 0 with 100 μg of $PLP_{139-151}$ and were tolerized to $OVA_{323-339}$ (OVA), $PLP_{139-151}$ (PLP), $HI_{574-586}$ (H), $HI_{574-586}$–P (H–P), $MHV_{3821-3832}$ (MHV), or $MHV_{3821-3832}$+P (MHV+P) at 7 days pre and 3 days post immunization with $PLP_{139-151}$. 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.

b) $p < 0.05$ compared to control OVA-tolerized or non-tolerized mice.

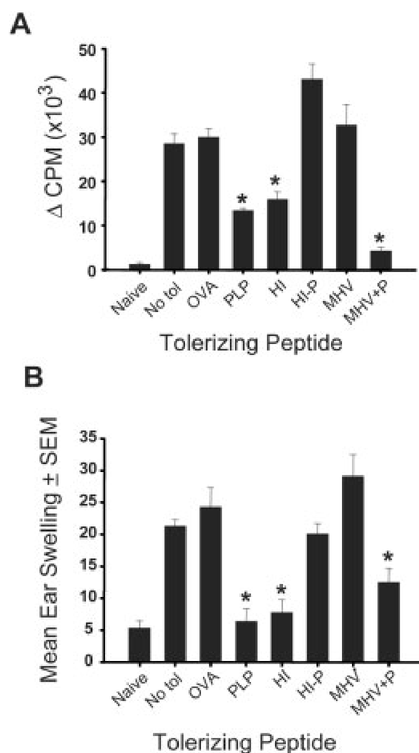

Figure 4. Proline at p9 of the mimic $MHV_{3821-3832}$ or $HI_{574-586}$ peptides is critical for induction of cross-reactivity to $PLP_{139-151}$ as assessed by peripheral tolerance. (A) Separate groups of SJL mice were tolerized to $OVA_{323-339}$ (OVA), $PLP_{139-151}$ (PLP), $HI_{574-586}$ (HI), $HI_{574-586}$–P (HI–P), $MHV_{3821-3832}$ (MHV), or $MHV_{3821-3832}$+P (MHV+P) coupled to syngeneic, ECDI-fixed splenocytes at 7 days pre and 3 days post immunization with $PLP_{139-151}$. $CD4^+$ T cell proliferative responses were measured in response to $PLP_{139-151}$ rechallenge. *Values significantly reduced compared to control levels in $OVA_{323-339}$-tolerized or non-tolerized mice, $p < 0.05$. (B) *In vivo* DTH ear swelling assays on day 14 post priming to $PLP_{139-151}$. Mice tolerized to PLP, HI or MHV+P exhibited reduced DTH responses in contrast to $OVA_{323-339}$- or $MHV_{3821-3832}$-tolerized mice or non-tolerized mice, *$p < 0.05$.

with the native peptide sequences, following rechallenge with $PLP_{139-151}$.

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 $PLP_{139-151}$ mimic peptide (MHV30-BeAn) could induce activation of $PLP_{139-151}$-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 $PLP_{139-151}$ epitope (PLP-BeAn) (Fig. 5A). This was in contrast to mice infected with TMEV expressing a non-PLP mimic OVA peptide. *In vitro* rechallenge with $PLP_{139-151}$ peptide demonstrated $PLP_{139-151}$ 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 $MHV_{3821-3832}$ peptide rechallenge *in vitro* (IFN-γ secretion; Fig. 5C) demonstrated that the MHV 30-mer peptide is a likely natural epitope in SJL mice. Immunohistological analysis of the brain at day 40 post infection demonstrated modest numbers of $CD4^+$ T cells around the hippocampus region of MHV30-BeAn-infected mice (Fig. 5D). In contrast, $CD4^+$ T cells were

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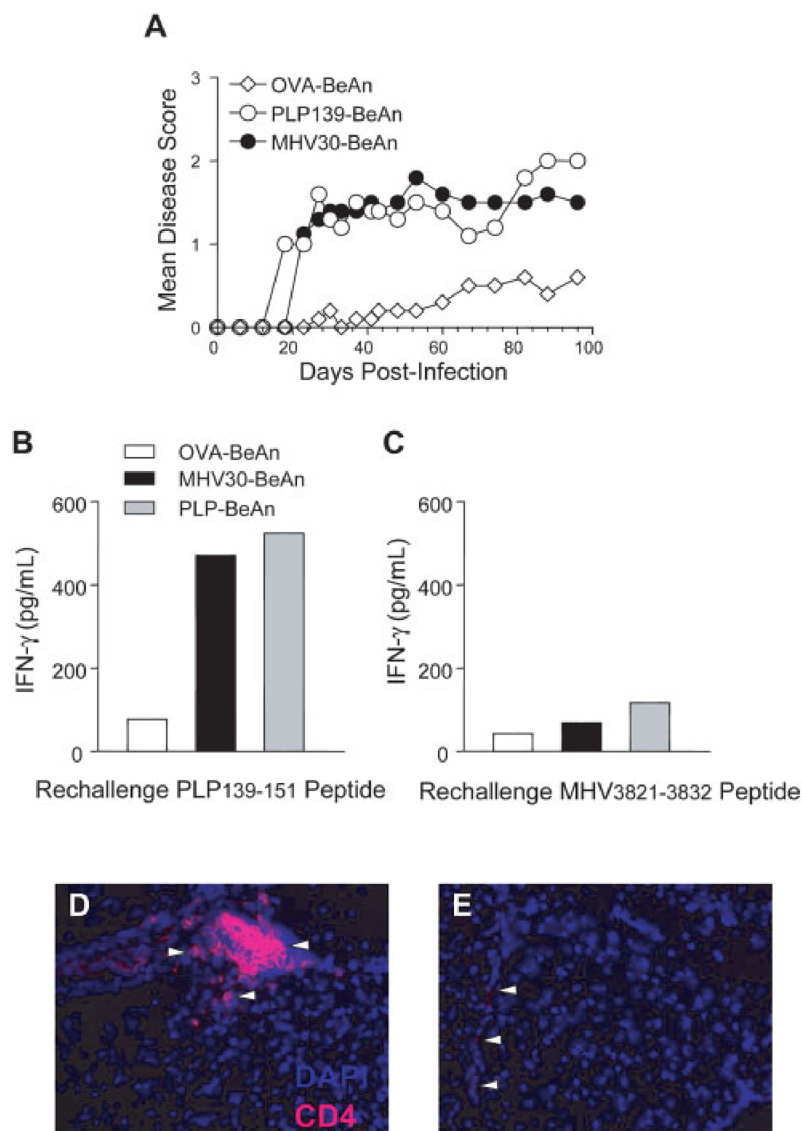

Figure 5. Infection of SJL mice with TMEV engineered to express $MHV_{3813-3842}$ induces CNS autoimmune disease. (A) Separate groups of SJL mice were infected intracerebrally with 9×10^6 PFU PLP139-BeAn (*n* = 5), OVA-BeAn (*n* = 5) or MHV30-BeAn (*n* = 8) and observed for 96 days. MHV30-BeAn-infected mice developed a mild form of flaccid paralytic disease similar in severity and early onset to mice infected with PLP-BeAn. In contrast mice infected with OVA-BeAn exhibited very mild disease with a much delayed onset. Splenic T cells from PLP-BeAn- or MHV30-BeAn-infected mice demonstrated cross-reactive $PLP_{139-151}$ or $MHV_{3821-3832}$ responses at days 20–22 post infection to *in vitro* rechallenge with 50 μM $PLP_{139-151}$ or $MHV_{3821-3832}$ peptide, as measured by IFN-γ secretion (B, C), in contrast to OVA-BeAn-infected mice. Immunohistochemistry of the brain (hippocampus region) at day 40 post infection demonstrated the presence of $CD4^+$ T cells in MHV30-BeAn-infected mice (arrows) compared to very few cells in OVA-BeAn-infected mice (D, E).

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, $HI_{574-586}$, but not priming with the core $HI_{574-586}$ epitope in CFA, could induce a non-classical form of CNS inflammatory autoimmune disease, by the cross-activation of $PLP_{139-151}$-specific Th1 cells [12, 13]. $MHV_{3821-3832}$ is another potential $PLP_{139-151}$ mimic peptide with 23% homology to the major encephalitogenic peptide in SJL mice, $PLP_{139-151}$ [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. $MHV_{3821-3832}$ is located in the ORF1a gene which codes for the replicative polyprotein PL1-PRO (Papain-like proteinases), a multifunctional protein involved in the transcription of negative-stranded RNA, leader RNA, subgenomic mRNA and progeny virion RNA, as well as

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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 $HI_{574–586}$ can induce expansion of $PLP_{139–151}$-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 $\pm$ 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 $PLP_{139–151}$-induced recall responses, particularly IFN-γ production. Nevertheless, these data demonstrated the potential for cross-activation of $PLP_{139–151}$ T cells by the MHV peptide. In $PLP_{139–151}$-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, $MHV_{3821–3832}$ peptide-coupled splenocyte tolerance of $PLP_{139–151}$-induced EAE was ineffective at inhibiting disease and T cell recall responses, in contrast to $PLP_{139–151}$- or $HI_{574–586}$-induced tolerance.

Previously, it was shown that the critical anchor residues in the I-A^s 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-A^s-associated autoimmune disease with regard to presentation of self peptides [16]. To take this observation further and to attempt to understand the differences in $PLP_{139–151}$-specific T cell cross-activating potential between the $HI_{574–586}$ and $MHV_{3821–3832}$ mimic peptides, we sought to determine the influence of the p9 MHC class II proline anchor residue in molecular mimicry between the "self" $PLP_{139–151}$ 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 $HI_{574–586}$ mimic peptide.

Both $PLP_{139–151}$ and $HI_{574–586}$ have a proline residue (P, aliphatic) at p9 in the MHC class II ligand motif, whereas $MHV_{3821–3832}$ contains asparagine (N, amidic). Interestingly, substitution of the secondary MHC class II contact residue at p9 (N$\rightarrow$P) conferred an ability on the MHV peptide to induce stronger cross-reactive $PLP_{139–151}$-specific T cell responses, as measured by T cell proliferation or IFN-γ ELISA assays. In contrast, substitution of P$\rightarrow$N at p9 of the HI mimic peptide (HI–P) abrogated the ability of that peptide to induce cross-activation of $PLP_{139–151}$-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 $HI_{574–586}$-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 $PLP_{139–151}$ response (*i.e.*, $PLP_{139–151}$-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 $PLP_{139–151}$-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 $PLP_{139–151}$-specific T cells by the MHV peptide. Interestingly, Th1 recall responses, particularly

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IFN-γ, to the self $PLP_{139–151}$ 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 $PLP_{139–151}$-specific $CD4^+$ T cells by self peptide endogenously released as a consequence of chronic myelin damage presented locally in the CNS by infiltrating dendritic cells [22], and the increased affinity of $PLP_{139–151}$ (IC_{50} 87 nM) for $I-A^s$ compared to $MHV_{3821–3832}$ (IC_{50} 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 ($I-A^s$) anchor residue at p9 plays a critical role in the ability of $PLP_{139–151}$ 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 (TTMLSLATAKVIAKWLAVNV-LYFTDVPQIK – 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 $MgCl_2$,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 $PLP_{139–151}$ distributed over three sites on the lateral and dorsal hind flanks. For $HI_{574–586}$ or $OVA_{323–339}$ 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 following peptides were purchased from Peptides International (Louisville, KY): $PLP_{130–159}$ 30-mer (QAHSLER VCHCLGKWLGHPDKFVGITYALT), $PLP_{139–151}$ (HSLGKWLGH PDKF), $HI_{574–586}$ (EQLVKWLGLPAPI), HI-P (EQLVKWLGLNA PI), $MHV_{3821–3832}$ (KVIAKWLAVNVL), $MHV_{3813–3842}$ 30-mer (TMLSLATAKVIAKWLAVNVLYFTDVPQIKL), MHV+P (KVIAK WLAVPVL), and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR). 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, $PLP_{139–151}$ 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

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determined by ELISA (Endogen Minikits, Woburn, MA) or by Luminex® 100 System (Upstate, VA, USA).

Induction of peripheral tolerance

Tolerance was induced by i.v. injection of 5×10^7 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 (FcγIII/IIR, 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 the Mann-Whitney non-parametric ranking test. Analysis of cytokine secretion, DTH and proliferation responses from multiple groups was performed by one-way ANOVA.

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