Synthetic Peptide Dendrimers Block the Development and Expression of Experimental Allergic Encephalomyelitis

Keith W. Wegmann, Cynthia R. Wagner, Ruth H. Whitham and David J. Hinrichs

*J Immunol* 2008; 181:3301-3309; doi: 10.4049/jimmunol.181.5.3301
http://www.jimmunol.org/content/181/5/3301

**References**
This article cites 60 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/181/5/3301.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Synthetic Peptide Dendrimers Block the Development and Expression of Experimental Allergic Encephalomyelitis

Keith W. Wegmann,*† Cynthia R. Wagner,*†§ Ruth H. Whitham,*† and David J. Hinrichs2*†

Multiple Ag peptides (MAPs) containing eight proteolipid protein (PLP)139–151 peptides arranged around a dendrimeric branched lysine core were used to influence the expression and development of relapsing experimental allergic encephalomyelitis (EAE) in SJL mice. The PLP139–151 MAPs were very efficient agents in preventing the development of clinical disease when administered after immunization with the PLP139–151, monomeric encephalitogenic peptide in CFA. The treatment effect with these MAPs was peptide specific; irrelevant multimeric peptides such as guinea pig myelin basic protein GBPBP72–84 MAP (a dendrimeric octamer composed of the 72–84 peptide) and PLP178–191 MAP (a dendrimeric octamer composed of the PLP178–191 peptide) had no treatment effect on PLP139–151-induced EAE. PLP139–151 MAP treatment initiated after clinical signs of paralysis also altered the subsequent course of EAE; it limited developing signs of paralysis and effectively limited the severity and number of disease relapses in MAP-treated mice over a 60-day observation period. PLP139–151 MAP therapy initiated before disease onset acts to limit the numbers of Th17 and IFN-γ-producing cells that enter into the CNS. However, Foxp3+ cells entered the CNS in numbers equivalent for nontreated and PLP139–151 MAP-treated animals. The net effect of PLP139–151 MAP treatment dramatically increases the ratio of Foxp3+ cells to Th17 and IFN-γ-producing cells in the CNS of PLP139–151 MAP-treated animals. The Journal of Immunology, 2008, 181: 3301–3309.

A nimal models of experimental allergic encephalomyelitis (EAE) have been used to test various immunotherapeutic approaches designed to alter or inhibit the clinical course of this paralytic autoimmune disease. These strategies have included inhibition of costimulation (1–3), infusion of cytokine Abs (4–6), and T cell vaccination (7–9). Additionally, many different studies have used specific encephalitogenic proteins and peptides as part of strategies designed to block the induction or effector function of encephalitogenic T cells (10–12). Two peptide-specific strategies have emerged for the treatment of EAE that involve either 1) an encephalitogenic peptide monomer covalently linked to a MHC class II molecule (referred to as a recombinant T cell ligand, RTL) or 2) linear multimers of the encephalitogenic peptide. Both reagents are made by recombinant technology and have been tested in many systems including the SJL mouse model of relapsing EAE (13–16). Although the mechanism by which these two approaches alter the expression of EAE has yet to be established, it appears that the RTLs may cause a switch to a TH2-type response (15) whereas the linear multimers may induce “high zone tolerance” (13). Both of these reagents have been suggested to alter the course of EAE by interacting directly at the cell surface of either the effector T cell or the APC. RTLs are proposed to act directly with specific TCR (17), whereas the linear multiple peptides may exert their effect by directly cross-linking MHC class II molecules on the surface of APCs (14, 18).

The studies using RTLs and linear peptide multimers demonstrate that varying the structural format of the relevant epitope being presented to the responding immune system can alleviate clinical manifestations of autoimmune disease. Because the results of Ag-specific therapies investigated to date are strongly supportive of this basic principle, we initiated conceptually similar studies with multiple Ag peptides (MAP) synthesized as octamers of encephalitogenic peptides. MAPs are peptide multimers synthesized as dendrimeric octamers (19). They can be tetramers, but typically they are octamers in which during synthesis each of the eight peptides is independently and covalently linked to a branched central lysine matrix (20). When used as peptide immunogens, MAPs have been shown to stimulate humoral immune responses that are superior in comparison to those responses elicited by monomeric peptide or peptide-carrier conjugates (19–22). MAPs also have been found to enhance the sensitivity of immunoassays for peptide-specific Abs and in this capacity have been shown to perform better than tandem repeats of the same peptide (23, 24). When administered in various adjuvant formulations, MAPs have been shown to elicit strong peptide-specific T cell responses, including protective immunity (25–28). Recently, a MAP was found to increase the encephalitogenic response to the 33–55 peptide of myelin oligodendrocyte glycoprotein. In that study, the octameric form of the 33–55 peptide was more encephalitogenic than the monomeric myelin oligodendrocyte glycoprotein peptide when emulsified in adjuvant and injected into C57BL/6 mice (29). Thus, in many systems MAPs elicit an immune response that is more robust than that seen with monomeric forms of the peptide or peptide-carrier conjugates.

We initiated the following studies in the SJL mouse model of EAE to determine whether the immunogenic properties of MAPs would also be of value in altering the course of EAE. For these studies we used standard solid phase chemistry to synthesize homogeneous octamers of the encephalitogenic proteolipid protein.
hibiting effect by mechanisms that have not been described either in the target tissue thereby apparently achieving their disease-inhibiting activity by altering Ag-specific cell trafficking and coincidently changing the ratio of regulatory to effector T cells in the target tissue thereby apparently achieving their disease-inhibiting effect by mechanisms that have not been described either forRTLs, linear encephalitogenic multimers, or other Ag-specific strategies designed to limit autoimmune disease.

Materials and Methods

Animals

Female SJL mice (4–6 wk of age) were obtained from The Jackson Laboratory. The animals were housed under specific pathogen-free conditions at the Veterans Affairs Medical Center Animal Care Facility (Portland, OR), according to institutional guidelines. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center (Portland, OR).

Antigenic peptides

The amino acid sequence of the PLP 139–151 peptide used for induction of actively induced EAE was HSLGKWLHGPDKFE, and this peptide monomer was commercially prepared by GenScript (Piscataway, NJ) and supplied at 90% purity. All MAPs were synthesized on a Protein Technologies PS3 peptide synthesizer using standard F-moc chemistry and a double coupling procedure. MAPs were prepared by the sequential addition of amino acid residues to an eight-branched MAP resin (catalog no. 05-24-0151; EMD Chemicals). The MAPs were designed to contain the peptide sequence with added lysine spacers when required to enhance solubility (30). The sequences of the PLP 139–151 MAP with the spacer added is HSLGKWLHDPFKGGK4K2Kβ. The sequence of guinea pig myelin basic protein (GPBP)72–85 octamer peptide is (KKLPQKSSRQDENPVKGG)8-eK4-eK2-eKβ. The PLP 178–191 peptide MAP sequence was (KKNTTWTCQIAFSPPKGGK4K2Kβ. All MAPs were cleaved with a mixture of trifluoroacetic acid (80%), H2O (8%), 1,2-(KKNTTWTCQIAFSPPKGG)8-eK4-eK2-eKβ-tert-butylmethyl ether. The peptide pellets are dissolved in a 1:1 mixture of water to acetonitrile, frozen and lyophilized for long-term storage.

The resulting MAP products were assessed for purity by a commercial service (AAA Laboratories). Because of the nature of the sequence of the MAP octamers the best indication of a successful synthesis is obtained from analysis of complete amino acid composition and then comparing the ratios of amino acid content to the fixed lysine content that constitutes the resin used as the basis of the MAP synthesis. Using the double coupling method for synthesis of the MAP we find that following cleavage from the resin the determined amino acid content was typically at least 90% of theoretical.

Induction of active EAE

EAE was induced in 9–to 12-wk-old female SJL mice by the s.c. injection of 150 μg of PLP 139–151 peptide emulsified in CFA. The injection volume of 0.2 ml was distributed equally between four sites on the flank. The CFA contained 4 mg/ml Mycobacterium tuberculosis strain H37Ra (Difco). EAE was also induced in SJL mice by similar s.c. injection of 200 μg of PLP 178–191 peptide emulsified in CFA.

Scoring of clinical paralysis

Clinical signs of actively induced EAE in SJL mice typically consist of hind limb weakness with limited forelimb involvement. However, animals that develop the most severe clinical signs of disease develop both hind and forelimb paralysis. Degrees of hind limb and forelimb weaknesses were assessed as described and as previously outlined (31). Animals with a flaccid tail were given a clinical severity score of 1. Animals that have difficulty in righting themselves after being flipped onto their backs are given a clinical severity score of 2. Animals with apparent hind limb weakness and that could not right themselves after being flipped onto their backs were given a clinical severity score of 3. Mice that have severe hind limb weakness and could walk upright only with difficulty were given a clinical severity score of 4. Mice that exhibited severe hind limb weakness and who could not walk upright were given a clinical severity score of 5. Animals with hind limb paraplegia that displayed no volitional leg movement were given a clinical severity score of 6. For some experimental groups the average cumulative disease score was determined. The cumulative disease score is the sum of the daily clinical score for an individual mouse over the observation period. The average cumulative disease score is the group’s average daily clinical score summed over the observation period.

Adaptive transfer of EAE

Spleens were removed from PLP 139–151/CFA immune animals 14 days following immunization and single cell suspensions were prepared. The cells were washed twice, and cultured in stimulation medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% sodium pyruvate, 1% glutamine, 1% normal mouse serum, and 2 × 10–5 M 2-ME). The spleen cells were cultured at a concentration of 5 × 106 cells/ml in the presence of 2 μg/ml PLP 139–151 peptide for 72 h at 37°C in 7% CO2. Following Ag activation the spleen cells were washed and viable cells enumerated. Recipient animals were injected i.p. with 3 × 105 viable cells suspended in a volume of 0.2 ml of RPMI 1640.

Treatment of actively induced EAE

Stocks of monomer and MAPs were prepared in saline at a concentration of 1.0 mg/ml. Peptide concentrations were adjusted by dilution with sterile saline as required just before treatment. Animals received the indicated amount of peptide (i.p.) in a volume of 0.2 ml. For all studies in which monomer peptide was compared with the MAP we used epitope equivalent amounts of the reagents. For example, the epitope equivalent of 50 μg of the 139–151 monomer is 57.2 μg of the 139–151 octamer because the backbone portion of the octamer contributes ~12.6% to the mass of the octamer.

Proliferation assays

T cell proliferation responses were assayed by plating 5 × 104 spleen cells per well into 96-well flat-bottom tissue culture plates in a volume of 0.2 ml/well of stimulation medium. The cell cultures were incubated for 72 h at 37°C in 7% CO2. The wells were pulsed for the final 18 h with 0.5 μCi per well [3H]thymidine (NEN). The cells were harvested onto glass fiber filters, and [3H]thymidine uptake was measured using a liquid scintillation counter (1205 Betaplate; Wallac). Mean cpm ± SD were calculated for quadruplicate wells.

Isolation of brain mononuclear cells

Mononuclear cells were isolated from the brains and spinal cords of SJL/J mice. For this isolation the mice were anesthetized, perfused with 30 ml of cold saline, and brains and spinal cords removed. A single cell suspension of mononuclear cells was prepared by processing the neural tissue through a 70 μm cell strainer (BD Falcon). The recovered cells were washed in RPMI 1640, resuspended in 8 ml of 40% Percoll (Pharmacia), and then underlaid with 3 ml of 80% Percoll to form a discontinuous gradient in a 15-ml centrifuge tube. The gradient was centrifuged at 500 g for 30 min at room temperature, and the cells at the 40% to 80% interface were harvested. Finally, the cells were washed three times with RPMI 1640 and then stained for FACS analysis or used in ELISPOT assays.

ELISPOT assays

Multi-Screen-HA plates from Millipore were coated overnight at 4°C with 100 μl/well of the capture Abs. Monoclonal Ab clone TC11-1H10.1 at 1.5 μg/ml was used to capture IL-17 and clone AN-18 at 1.25 μg/ml was used to capture IL-17 (1.5 μg/ml) and clone R4-8HA was added for detection of IFN-γ (1.25 μg/ml) (eBioscience). The plates were washed four times with sterile PBS and blocked with RPMI 1640 containing 10% FCS for 1 h. Spleenocytes obtained from mice from the various treatment groups were added to wells in a volume of 0.2 ml of culture medium (RPMI 1640 plus 10% FCS), and cultured with and without PLP 139–151 peptide (2 μg/well). Various concentrations of the CNS mononuclear cells (5 × 104, 2.5 × 104, or 1 × 104) were mixed with feeder cells (5 × 107 naive spleen cells) and added to individual wells in a volume of 0.2 ml of culture medium (RPMI 1640 plus 10% FCS) and cultured with and without PLP 139–151 peptide (2 μg/well). All plates were incubated at 37°C, 7% CO2 for 20 h. The plates were then washed four times with PBS containing 0.05% Tween 20. After washing, detection Abs were added in a volume of 100 μl/well. Monoclonal Ab clone TC11-1H10.1 was added for detection of IL-17 (1.5 μg/ml) and clone R4-8HA was added for detection of IFN-γ (1.25 μg/ml) (eBioscience). After overnight incubation at 4°C, the wells were washed four times with PBS-Tween 20. Streptavidin-alkaline phosphatase (100 μl/well;
FIGURE 1. The encephalitogenicity of MAPs depends on the adjuvant stimulus provided by CFA. SJL mice were immunized with a saline solution of PLP139–151 MAP, or either PLP139–151 monomer or PLP139–151 MAP emulsified in adjuvant (IFA or CFA), and monitored daily for signs of paralysis (n = 6 mice/group). This experiment was repeated once using n = 4 mice/group with similar results. The PLP139–151 MAP was not superior to the peptide monomer in causing EAE when administered in CFA. p > 0.3.

BD Pharmingen) was added to the wells at a concentration of 1 μg/ml and the plates incubated at room temperature for 1 h. The plates were washed four times with PBS-Tween 20. The ELISPOT assay was developed by the addition of 150 μl of 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Kirkegaard & Perry Laboratories). Plates were dried overnight and images of the ELISPOT wells were captured and analyzed with an AID ELISPOT Reader System (Cellular Technology).

Intracellular staining for Foxp3

Cells expressing Foxp3 were detected with a Foxp3 APC staining kit from eBioscience, used according to the manufacturer’s procedure. Cells were surface stained with PerCP-conjugated anti-CD4 Ab (BD Pharmingen), FITC-conjugated anti-CD3 Ab (eBioscience), and PE-conjugated anti-CD25 Ab (eBioscience).

Statistical analysis

Significant differences in maximal disease score, area under the curve or the cumulative disease score between control and treated groups of mice were assessed using the two-tailed Mann-Whitney U test (32). The two-tailed Student t test was used to determine significance differences between experimental groups in the number of IL-17 or IFN-γ secreting cells found in the spleen and CNS. The two-tailed Student t test was also used to analyze the cell trafficking data of CD4+ Foxp3+ cells into the CNS between various treatment groups. All values were calculated using GraphPad Prism Software.

Results

Peptides in a MAP configuration have been demonstrated to be strongly immunogenic, yet their encephalitogenicity, like that of monomeric encephalitogenic peptides, remains dependent on the adjuvant stimulus provided by CFA. For example, PLP139–151 MAP emulsified in CFA consistently induced EAE in SJL mice, whereas PLP139–151 MAP in IFA or in a saline solution did not induce EAE (Fig. 1). At a lower dose (i.e., 30 μg) of encephalitogen the PLP139–151 MAP induced modest signs of clinical disease. At that lower dose neither the monomer peptide nor the PLP139–151 MAP caused the development of relapsing EAE. Although the PLP139–151 MAP was encephalitogenic when administered within the CFA emulsion it was not significantly superior as an encephalitogen when compared with the response elicited by the 139–151 peptide monomer.

To determine whether, when presented in a nonencephalitogenic form, a MAP would alter the development of actively induced EAE, we administered soluble PLP139–151 MAP to SJL mice subsequent to immunization with the encephalitogenic PLP peptide 139–151 monomer emulsified in CFA. Fig. 2A shows that treatment of SJL mice with PLP139–151 MAP inhibited EAE development when administered on days 2, 6, and 10 following immunization with the PLP139–151 peptide monomer in CFA. Treatment with the 139–151 monomer had no influence on the initial course of disease development, as has been reported by others (13). Fig. 2B shows the course of disease development over an extended observation period, demonstrating that in addition to modifying the initial signs of clinical disease, treatment on postimmunization days 2, 6, and 10 with the various doses of PLP139–151 MAP continued to inhibit clinical disease over an observation period of 90 days. MAP treatment was peptide specific, as treatment with PLP178–191 MAP had no significant influence on the development of 139–151 PLP peptide/CFA-induced disease.

To determine the effectiveness of MAP treatment on EAE when the treatment is initiated at later time points, that is, at time points subsequent to initial T cell Ag recognition and resulting activation,
we conducted experiments in which PLP\textsubscript{\textasciitilde139–151} MAP was administered on days 10–12 following immunization. It has been reported that eight days following immunization with 139–151 PLP monomer peptide emulsified in CFA, in the spleens, but not yet in the CNS, of SJL mice, peptide-specific T cells can be detected by Ag-specific proliferation and cytokine production (33). When we assessed mice 10 days following immunization we found that some mice had an infiltration of mononuclear cells in their CNS and that cells within that population produced IL-17 and IFN-γ in response to stimulation with 139–151 PLP peptide. No animals had any evidence of clinical disease at day 10 and some mice had no mononuclear infiltration of their CNS at that time point (data not shown). When treatment was started at that later time point (i.e., 10 days after encephalitogenic challenge), PLP\textsubscript{\textasciitilde139–151} MAP treatment reduced the subsequent development of EAE clinical signs (Fig. 3). Epitope equivalent injections with the 139–151 peptide monomer did not impact the development of clinical disease. Thus MAP treatment also is effective in reducing development of clinical disease when administered after T cell priming but before disease-inducing-T cell target-tissue infiltration has occurred.

Subsequent to demonstrating that MAP treatment can effectively alter the progression to clinical disease, we evaluated the influence of MAP treatment when initiated following the onset of clinical disease. In these experiments groups of mice were immunized with the PLP\textsubscript{\textasciitilde139–151} encephalitogenic peptide in CFA, and then after paralytic disease was evident these mice were treated with either PLP\textsubscript{\textasciitilde139–151} MAP, a MAP containing the 72–85 peptide sequence of GPBP (GPBP\textsubscript{72–85} MAP), as an Ag specificity control, or the PLP peptide (139–151 monomer). The treated animals received MAP therapy for 2 days at a dose of 100 μg of MAP per day. Fig. 4 shows that mice with clinical signs of EAE treated with the PLP\textsubscript{\textasciitilde139–151} monomer, or the GPBP\textsubscript{72–85} MAP developed additional episodes of clinical disease as is characteristic of not-treated relapsing disease progression in this mouse strain (34). Mice with clinical signs of EAE treated with PLP\textsubscript{\textasciitilde139–151} MAP continued to recover from the initial episode of disease at the time of treatment, but in contrast to the mice in the control treatment groups, the PLP\textsubscript{\textasciitilde139–151} MAP-treated mice developed a reduced number of subsequent episodes of disease that also were less severe. The duration and number of relapses were greatly reduced only in the PLP\textsubscript{\textasciitilde139–151} MAP treatment group (only 2 of 11 animals relapsed to have two additional paralytic events) compared with PLP 139–151 peptide monomer or the GPBP\textsubscript{72–85} MAP treatment groups where all animals relapsed to have multiple paralytic events. In these experiments epitope-specific MAP therapy of clinically ill mice prevented progression to more severe clinical disease and subsequent relapses were rare and of modest clinical impact, demonstrating that peptide-specific MAP treatment not only can prevent development of initial disease but also can limit episodes of primary and recurring disease.

It should be noted that when administered after clinical disease was evident, in some mice monomer peptide or PLP\textsubscript{\textasciitilde139–151} MAP treatment elicited signs of anaphylaxis. Occasionally the anaphylaxis was fatal. The development of anaphylaxis in SJL mice injected with soluble peptide after immunization with the 139–151 peptide emulsified in CFA has been reported (35, 36). The development of Abs following immunization with some peptides in CFA puts animals at risk for anaphylaxis and in the SJL mouse this tendency may be increased due to the higher levels of mast cells in this mouse strain (37). Although anaphylaxis did not occur in animals treated early following immunization, the development of this potential problem prevented us from analyzing the influence of the PLP\textsubscript{\textasciitilde139–151} MAP reagent at later postrecovery time points in this mouse model of relapsing EAE.

Because MAP treatment prevented clinical signs of EAE we examined by standard histology the consequence of PLP\textsubscript{\textasciitilde139–151} MAP therapy on the cellular infiltrate and degree of demyelination seen in the brains and spinal cords of SJL mice immunized with 139–151 PLP peptide monomer in CFA. Microscopic analysis of these CNS tissues from mice treated with PLP\textsubscript{\textasciitilde139–151} MAP on days 2, 6, and 10 as well as animals treated on days 10–12 showed few inflammatory foci and no evident demyelination (data not
shown). This is in keeping with the lack of disease in these PLP<sub>139–151</sub> MAP-treated mice.

To assess the impact of PLP<sub>139–151</sub> MAP treatment on the properties of immune cell populations, lymphocytes infiltrating CNS tissue were compared with those in the spleens of mice in control and treatment groups. For these experiments mice were immunized with PLP<sub>139–151</sub> peptide in CFA, treated with PLP<sub>139–151</sub> MAP on days 10–12 following encephalitogenic immunization, and then spleen cells and CNS cells were isolated on day fourteen following immunization (a time point where all control animals exhibited clinical disease). The isolated cells were then assessed by ELISPOT analysis for the peptide-specific stimulation of IL-17 or IFN-γ production. Fig. 5, A and B, show a significant increase in the relative numbers of both IL-17 and IFN-γ-producing cells in the spleens of mice in the PLP<sub>139–151</sub> MAP treatment groups compared with those in the no treatment group. Cells isolated from the CNS showed a concomitant significant decrease in Ag-specific cytokine-secreting cells in the PLP<sub>139–151</sub> MAP treatment groups vs the control group (Fig. 5, C and D). For example, the total number of IL-17-secreting cells isolated from the CNS of untreated mice ranged from 35 to 50,000, whereas in the PLP<sub>139–151</sub> MAP-treated mice the number of CNS IL-17-producing cells ranged from a high of 9000 to fewer than 1000. This decrease was seen only in CNS cells isolated from mice treated with the PLP<sub>139–151</sub> MAP reagent and not from mice treated with the PLP<sub>139–151</sub> monomer peptide or the PLP<sub>178–191</sub> MAP reagent (data not shown).

Animals immunized with 139–151 PLP peptide in CFA and treated with PLP<sub>139–151</sub> MAP before the onset of EAE do not develop clinical signs of EAE yet they possess a large number of encephalitogen-specific cells within the spleen that produce IL-17 and IFN-γ in response to specific peptide stimulation in vitro. To determine additional properties of splenocytes from the PLP<sub>139–151</sub> MAP-treated mice we measured their proliferative response to peptide in vitro and subsequently assessed their encephalitogenic potential by adoptive transfer. Fig. 6 shows that when spleens cells from PLP<sub>139–151</sub> MAP-treated mice were stimulated in vitro with monomeric peptide that they retained an undiminished proliferative response to Ag. Those spleen cells were obtained from mice 14 days following encephalitogenic immunization, a sample time point at which all no-treatment mice have clinical signs of EAE and all PLP<sub>139–151</sub> MAP-treated mice had been and were disease free. In addition to their undiminished proliferative potential in response to peptide-specific stimulation, the spleen cells from the PLP<sub>139–151</sub> MAP treatment groups also had the capacity to transfer clinical EAE. For this assessment spleen cells were stimulated in bulk culture with specific peptide for 3 days and then transferred into naive recipients. As can be seen in Fig. 7, recipients of spleen cells from PLP<sub>139–151</sub> MAP-treated mice developed disease ~5 days following cell transfer and all recipients developed clinical signs of disease. Thus, PLP<sub>139–151</sub> MAP-treated disease-free mice have peptide-reactive cells that have the potential to develop into encephalitogenic effector cells.

It has been established that the development and expression of EAE is controlled by regulatory T cells (T<sub>reg</sub>) and that these cells,
identified by the presence of the transcription factor Foxp3 (38, 39), are present at the target tissue as well as the secondary lymphoid organs (40–42). Although it is evident that Treg have an impact on the development of encephalitogenic effector cells, Treg may have only a modest impact on the disease-inducing capacity of existing encephalitogenic effector T cells (40). We determined the percentage of Foxp3+CD4+ mononuclear cells in the CNS of no-treatment as well as PLP 139–151 MAP-treated (days 10–12 treated) mice on day 14 following immunization with the 139–151 monomer in CFA. All no-treatment animals had clinical scores of at least three and all PLP139–151 MAP-treated mice were disease free. As shown in Fig. 8, the most apparent effect of PLP139–151 MAP treatment was the change in the ratio of Foxp3+CD4+ T cells to IL-17 or IFN-γ cytokine-secreting cells. PLP139–151 MAP treatment did not significantly influence the number of Foxp3+CD4+ T cells found in the CNS of treated compared with nontreated controls, but because PLP139–151 MAP treatment significantly reduces the appearance of cytokine-secreting cells in the CNS, the ratio of Foxp3+CD4+ T cells to IL-17 or IFN-γ secreting CD4+ T cells increases significantly. For those studies the cells were stimulated in vitro with PLP139–151 peptide for ELISPOT detection of cytokine production. When we measured IL-17 production by cells from the CNS without any in vitro peptide stimulation of the cells, the percentage of CNS cells producing IL-17 was much lower for cells isolated from the PLP139–151 MAP treatment group compared with as many as 4% of the cells from the CNS of animals with EAE (data not shown). These cells were collected on day 14 following immunization with the 139–151 PLP peptide, a time point at which clinical disease was evident in all mice of the no-treatment control group. Thus MAP treatment reduces not only the absolute percentage of cytokine-secreting cells in the CNS relative to nontreatment controls, but also appears to impact the activation state of the cells that appear within the target tissue.

**Discussion**

Ag-specific regulation of an autoimmune response offers a means to alter disease expression without compromising the varying demands on the immune system to respond to other antigenic stimuli.
Our study has found that MAPs, nonlinear peptide dendrimers that are readily synthesized as homogeneous octamers of an encephalitogenic peptide with each peptide tethered to a central lysine core, are highly effective reagents in altering the course of disease in the SJL murine model of relapsing EAE. The PLP<sub>139–151</sub> peptide is the dominant encephalitogen in this mouse strain due in part to the large number of T cells specific to this peptide that escape thymic deletion (43). Thus for studies using the SJL mouse strain, blocking the development of clinical disease following activation of T cells to this PLP peptide target has been the objective of many studies. In addition, not all neuroantigens elicit a humoral response that leads to peptide-induced anaphylaxis (36, 45), an allergic reaction that can result in death. It is possible that each of these Ag-specific approaches involves the incorporation of the relevant encephalitogenic peptide, but they differ in their peptide delivery format (13, 15, 53, 54). These approaches primarily use recombinant methods to produce the soluble reagents while an additional method uses peptide-conjugated Ags that develop as a consequence of the peptide therapy (44). This concern is especially true for peptide-based treatment in SJL mice; others have reported the development of treatment-elicted anaphylaxis in SJL mice immunized with the 139–151 PLP peptide in CFA (35, 36). We have not established if repeated injections of the PLP<sub>139–151</sub> MAP into naïve mice causes the production of Abs that will elicit anaphylaxis. However, we have noted that by day 14 following immunization with the 139–151 PLP peptide in CFA, at least some of the immunized SJL mice have developed a sufficient humoral peptide-specific response to place them at risk for the development of anaphylaxis when exposed to soluble peptide including the PLP<sub>139–151</sub> MAP used in this study. Because of this development and increased potential for anaphylaxis, we did not expand these studies to include later time points following immunization, especially at the time of relapse that typically occurs between days 20 and 30 following immunization. It is important to note that the development of Abs causing anaphylaxis resulted from the immunization with PLP peptide in CFA that is required to trigger the cellular autoimmune disease. In spontaneous autoimmune disease, Abs reactive to specific MAPs used for treatment may not develop as part of the peptide-based disease therapy. In addition, not all neuroantigens elicit a humoral response that leads to peptide-induced anaphylaxis (36, 45), an observation that may correlate with the thymic expression of the peptide in question.

Although the mechanism of action for this MAP-based therapy has yet to be determined, it is evident that at least some of the basis for treatment success is associated with changes in trafficking that occurs following MAP administration. In both treatment groups where PLP<sub>139–151</sub> MAP treatment was initiated before the onset of clinical signs of disease, PLP<sub>139–151</sub> MAP specific therapy was associated with an increase in the number of Ag-specific cells found in the spleen and at the same time a reduction in the number of Ag-specific cells found in the CNS (Fig. 5, A and B). Although no or very limited disease developed in these PLP<sub>139–151</sub> MAP-treated mice, the encephalitogen-specific cells in their spleens retained the capacity to respond to specific peptide as measured in vitro by proliferation, cytokine production, and, following in vitro stimulation, with the capacity to transfer clinical disease (Fig. 7). Thus MAP therapy does not initially render cells anergic or cause their deletion as is seen with some therapies (46), but apparently alters their trafficking to the CNS. Altered cell trafficking of encephalitogen-specific T cells was recently reported to be seen in an adoptive transfer model of rat EAE where, using intravital microscopy, the authors showed that soluble Ag causes an immediate effect on peptide-specific cells resulting in cell clustering and retention within the spleen (47).

Although the PLP<sub>139–151</sub> MAP treatments diminish trafficking of peptide-specific encephalitogenic cells into the CNS, we did not find a coincident reduction in the number of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells found in the CNS. It has been reported that both endogenous as well as Ag-specific T<sub>reg</sub> influence the development and recovery course of EAE, that these cells are active early in disease in the responding T cells of the lymphoid organs as well as in the target tissue, and that they are required for clinical recovery (48–51). Although it is likely that fully differentiated encephalitogenic T cells are less prone to the influence of T<sub>reg</sub> cells (40), T<sub>reg</sub> cells do influence the development of encephalitogenic T cells within secondary lymphoid organs and may play a role at the dendritic cell interface within the CNS thereby preventing the full development of IL-17-producing T cells in the target tissue (52). In our study, Foxp3<sup>+</sup> CD4<sup>+</sup> T cells were found in the spleen as well as in the CNS following PLP<sub>139–151</sub> MAP treatment, and their numbers and overall distribution did not appear to be influenced by PLP<sub>139–151</sub> MAP treatment. Whether these cells contributed to the inhibited development of encephalitogen-specific T cells to encephalitogenic effector cells is not addressed in this study. However the fact that PLP<sub>139–151</sub> MAP treatment does not diminish the number of Foxp3<sup>+</sup> CD4<sup>+</sup> T cells found in the CNS results in a significantly increased ratio of T<sub>reg</sub> to encephalitogenic cells and their precursors in the CNS, a ratio that may also contribute to the limited development of disease that is seen in PLP<sub>139–151</sub> MAP-treated mice.

In addition to our use of MAPs to alter the course of EAE, a number of Ag-specific strategies have been reported to inhibit successfully the development of EAE. These approaches have in common the incorporation of the relevant encephalitogenic peptide, but they differ in their peptide delivery format (13, 15, 53, 54). These approaches primarily use recombinant methods to produce the soluble reagents while an additional method uses peptide-conjugated cells to alter the course of disease. Specifically, the one method uses ethylencarbodimide to facilitate the covalent association of the peptide with nucleated target cells (55). Each of these methods is successful in modifying the expression of clinical disease in the SJL mouse model albeit each report offers different explanations for success. It is possible that each of these Ag-specific approaches involves a common mechanism that contributes to their influence on the course of clinical disease. Specifically, following systemic administration each of the reagents may be captured within the spleen with subsequent MHC class II presentation of the encephalitogenic peptide component that is common to all of these reagents. Such a presentation would provide a non-CNS target for encephalitogenic T cells or their precursors. Ag-specific retention in the spleen in the
absence of additional stimuli would further diminish the encepha-
ilitogenic potential of the retained cells and perhaps result in the
development of anergy as has been suggested for the method using
ethylenecarbodiimide-facilitated conjugation of peptide (10).

The cellular events within the spleen are yet to be determined for
each of these methods but an alteration in trafficking and local
modification of the Ag-specific T cell population cannot be ruled
out from the published studies. For example, the IgG-peptide chi-
merya reagent is likely to be processed by the spleen because it is
most effective when delivered in an aggregated form. This aggre-
gated form induces production of IL-10, a cytokine that would
reduce the development and expression of encephalitogenic effec-
tor cells (54). It has also been reported that the peptide-conjugated
cells, due to the treatment with ethylenecarbodiimide for conjuga-
tion, become apoptotic and their influence on disease development
most likely occurs due to cross-presentation (55). The observation
that these conjugated cells are most effective when administered
i.v. (56) suggests that initial processing within the spleen sets up a
condition that would target effector cells and their precursors to the
spleen in the absence of additional inflammatory stimuli with an
overall result of limiting the development or trafficking of patho-
genic effector cells to the CNS. Furthermore the peptide-conju-
gated cells also effectively alter the course of disease when the
peptide is conjugated to allogeneic cells (55), an observation con-
sistent with the need for cross-presentation and the involvement of
the spleen due to the i.v. administration of the peptide-conjugated
cells. It has also been found that the RTL reagents do not need to
contain a syngeneic MHC class II molecule to prevent the de-
velopment of clinical EAE induced by encephalitogenic peptide (A.
Vandenbark, unpublished observation), an observation also sugges-
ting that additional targets to divert encephalitogenic cells would
be generated in an Ag-presenting, but not target tissue, such as
the spleen. Recent trafficking studies with the RTL reagent
found that RTL treatment significantly reduced Ag-specific T cell
infiltration into the brain. Whether these cells were the found in
the spleen or other sites was not addressed in this study (57).

Because peptide-specific encephalitogenic T cells must reach the
CNS to initiate disease, any additional display of the MHC class
II presented peptide in non-target tissue would present a spe-
cific target but one without a clinical impact (i.e., disease symp-
toms would not be apparent). Each of these protocols, including
ours implementing MAPs, uses peptide-containing molecules that
because of their increased molecular size may not be cleared as
rapidly as the monomeric peptide, thereby resulting in a longer in
vivo half-life and potentially enhanced processing by MHC class II
positive cells. Such processing would occur in the absence of ad-
tional costimulatory signals thus providing a platform for Ag
recognition by Ag-specific T cells but without their subsequent
development into encephalitogenic effector cells. We are currently
evaluating the long-term fate of the T cells that are initially en-
riched in the spleen of PLP139–151 MAP-treated mice to determine
whether this treatment also eventually leads to anergy or deletion
of these cells.

The SJL mouse model of EAE is well defined in terms of en-
cephalitogenic peptides and disease relapses that develop after
immunization with a single encephalitogenic peptide. It is likely
that the additional episodes of clinical disease are due to sensiti-
ization to additional encephalitogenic peptides released during
the initial target-tissue attack (58, 59). Thus it was surprising that
mice treated with PLP139–151 MAP after clinical evidence of dis-
ease rarely developed additional episodes of disease. Because in
some experiments, PLP139–151 MAP treatment was delayed until
disease was evident, sensitization to the other potentially encepha-
loitogenic peptides should not have been prevented. It should be
noted that the peptide-specific RTL treatment of EAE in SJL mice
also inhibits the development of relapsing EAE when targeted to
the PLP139–151 peptide (15). We currently are investigating
whether MAP-delivered peptide-specific therapy can be used to
alter the course of relapsing disease that purportedly develops fol-
lowing sensitization to additional, endogenously released peptides
(52, 58, 60).

It is evident from this study that synthetic peptide multimers
effectively inhibit the development of clinical EAE and that this
inhibition is peptide specific. Although conceptually similar to pre-
vious reports of peptide-specific inhibition of EAE using various recombinant proteins, our studies differ from these previous re-
ports by using readily synthesized peptide reagents. Our studies to
date suggest that the effectiveness of MAP therapy relates to a
decrease in the accumulation of Ag-specific encephalitogenic T
cells at the target organ and a significant retention of peptide-
specific T cells within the spleen. Whether long-term effects of
MAP therapy induce additional regulatory events has yet to be
investigated.

Acknowledgments

We thank Archie Bouwer for discussion and comments during the execu-
tion of this study. We also thank Arthur Vandenbark for discussion of
unpublished findings using RTLs in the mouse model of EAE.

Disclosures

The authors have no financial conflict of interest.

References

1. Perrin, P. J., D. Scott, L. Quigley, P. S. Albert, O. Feder, G. S. Gray, R. Abe,
chronic relapsing experimental allergic encephalomyelitis. J. Immunol. 154:
1481–1490.

2. Howard, L. M., A. J. Miga, C. L. Vanderlugt, M. C. Dal Canto, J. D. Laman,
vention by anti-CD40L (CD154) antibody in an animal model of multiple sclero-

3. Chimits, T., N. Najafian, K. A. Abdallah, V. Dong, H. Yaptia, M. H. Sayegh, and
S. J. Khoury. 2001. CD82-independent induction of experimental autoimmune

4. Broh, H. P. M., M. van Meurs, E. Blezer, A. Schantz, D. Peritt, G. Treacy,
autoimmune encephalomyelitis in common marmosets using an anti-IL-12p40

5. Constantinouc, C. S., M. Wysocka, B. Hilliard, E. S. Ventura, E. Lavi,
G. Trinchieri, and A. Rostami. 1998. Antibodies against IL-12 prevent superan-
tigen-induced and spontaneous relapses of experimental autoimmune encepha-

M. Kamoun, and A. Rostami. 2003. Role of IL-12 receptor [beta]1 in regulation of
T-cell response by APC in experimental autoimmune encephalomyelitis. J. Im-
munol. 171: 4485–4492.


8. Bewsher, H. G., and D. J. Hinrichs. 1996. T-cell vaccination prevents EAE ef-
ector cell development but does not inhibit priming of MBP responsive cells.

cination against experimental autoimmune encephalomyelitis: evaluation of vac-

and S. D. Miller. 1990. Inhibition of murine relapsing experimental autoimmune
encephalomyelitis by immune tolerance to proteolipid protein and its encepha-

R. Gold, and J. L. Strominger. 2001. Vaccination, prevention, and

cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by
a synthetic peptide or proteins requiring endocytic processing. J. Exp. Med.
185: 1043–1054.

13. Falk, K., O. Rotzsche, L. Santambrogio, M. E. Dorf, C. Brosnan, and
J. L. Strominger. 2000. Induction and suppression of an autoimmune disease by
oligomerized T cell epitopes: enhanced in vivo potency of encephalitogenic pep-

14. Steiner, N., Neureiter, M., K. Falk, O. Rotzsche, A. Weishaupt, C. Schneider,
K. V. Toyka, R. Gold, and J. L. Strominger. 2001. Vaccination, prevention, and


The Journal of Immunology

3309

Downloaded from http://www.jimmunol.org/ by guest on June 3, 2017