

Immunopathology and Infectious Diseases

# Therapeutic Effect of Vasoactive Intestinal Peptide on Experimental Autoimmune Encephalomyelitis

## Down-Regulation of Inflammatory and Autoimmune Responses

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**Multiple sclerosis (MS) is a disabling inflammatory, autoimmune demyelinating disease of the central nervous system. Despite intensive investigation, the mechanisms of disease pathogenesis remain unclear, and curative therapies are unavailable for MS. The current study describes a possible new strategy for the treatment of MS, based on the administration of the vasoactive intestinal peptide (VIP), a well-known immunosuppressive neuropeptide. Treatment with VIP significantly reduced incidence and severity of experimental autoimmune encephalomyelitis (EAE), in a MS-related rodent model system. VIP suppressed EAE neuropathology by reducing central nervous system inflammation, including the regulation of a wide spectrum of inflammatory mediators, and by selectively blocking encephalitogenic T-cell reactivity. Importantly, VIP treatment was therapeutically effective in established EAE and prevented the recurrence of the disease. Consequently, VIP represents a novel multistep therapeutic approach for the future treatment of human MS. (Am J Pathol 2006, 168:1179–1188; DOI: 10.2353/ajpath.2006.051081)**

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory, autoimmune demyelinating disease of the central nervous system (CNS). EAE shows pathological and clinical similarities to human multiple sclerosis (MS) and is thus used as a model system to test potential

therapeutic agents.<sup>1</sup> Both EAE and MS are considered archetypal CD4<sup>+</sup> Th1 cell-mediated autoimmune diseases in which reactive Th1 cells, specific to components of the myelin sheath, infiltrate the CNS parenchyma, release proinflammatory cytokines and chemokines, and promote macrophage infiltration and activation.<sup>1</sup> Inflammatory mediators such as cytokines [ie, interleukin (IL)-12, interferon (IFN)- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$ ] and nitric oxide (free radical NO), produced by infiltrating cells and resident microglia, play a critical role in demyelination, oligodendrocyte loss, and degenerative axonal pathology.<sup>2,3</sup> Although available therapies based on immunosuppressive agents inhibit the inflammatory component of MS and either reduce the relapse rate or delay disease onset, they do not suppress progressive clinical disability. This illustrates the need for novel therapeutic approaches to prevent the inflammatory and autoimmune components of the disease and to promote repair and regeneration mechanisms.

The vasoactive intestinal peptide (VIP), a neuropeptide released from the innervation, is also produced by Th2 cells in response to antigen stimulation and under inflammatory/autoimmune conditions.<sup>4</sup> VIP elicits a broad spectrum of biological functions, including immunomodulatory functions, predominantly acting as a potent anti-inflammatory factor.<sup>5</sup> In addition, VIP promotes Th2 differentiation and inhibits Th1 responses, allowing it to emerge as a promising therapeutic factor for the treatment of autoimmune/inflammatory diseases.<sup>6,7</sup> Indeed, VIP is very efficient at ameliorating the pathology of several autoimmune disorders, including rheumatoid arthritis, ulcerative

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colitis, and uveoretinitis.<sup>8–10</sup> In this study, we investigated the potential therapeutic effect of VIP in two murine MS models. We report that treatment with VIP significantly reduces the clinical symptoms and pathology and that its therapeutic effect is associated with the down-regulation of both inflammatory and autoimmune components of the disease.

## Materials and Methods

### Animals and Peptides

Female SJL/J and C57BL/6 mice (8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). PLP<sub>139-151</sub> (HCLGKWLGHDPKF) and MOG<sub>35-55</sub> (MEVGWYRSPFSRVVHLYRNGK) peptides were synthesized using solid phase techniques and high performance liquid chromatography purification by Alpha Diagnostic International (San Antonio, TX). VIP, PACAP38, VIP<sub>10-28</sub>, VIP<sub>1-12</sub>, secretin, and glucagon were purchased from Calbiochem (Laufelfingen, Switzerland). The VPAC1 agonist [K<sup>15</sup>,R<sup>16</sup>,L<sup>27</sup>]VIP<sub>1-7</sub>-GRF<sub>8-27</sub> and the VPAC2 agonist Ro25-1553 have been previously described.<sup>8</sup>

### EAE Induction and Treatment

To induce chronic EAE by active immunization, C57BL/6 mice were immunized subcutaneously with 200  $\mu$ g of MOG<sub>35-55</sub> emulsified in CFA containing 1 mg/ml *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI) on days 0 and 7. Mice also received intraperitoneal injections of 200 ng of pertussis toxin (Sigma, St. Louis, MO) on days 0 and 2. VIP treatment consisted of the intraperitoneal administration of 2 nmol (6.6  $\mu$ g/mouse/day) on days 5, 7, and 9 or on 3 consecutive days after disease onset starting on days 10, 16, or 20.

For the relapsing-remitting (RR)-EAE model, SJL/J mice were immunized subcutaneously with 150  $\mu$ g of PLP<sub>139-151</sub> emulsified in CFA containing 500  $\mu$ g of *M. tuberculosis* H37 RA. The immunized mice were injected intraperitoneally on days 5, 7, and 9 with VIP (0.1 to 10 nmol/day), VIP agonists, or PBS (controls). Alternatively, VIP (2 nmol/day) was administered intraperitoneally starting on days 10 (onset), 16 (acute disease phase), or 20 (relapsing disease phase) for 3 consecutive days. Mice were scored daily for signs of EAE according to the following clinical scoring system: 0, no clinical signs; 0.5, partial loss of tail tonicity; 1, complete loss of tail tonicity; 2, flaccid tail and abnormal gait; 3, hind leg paralysis; 4, hind leg paralysis with hind body paresis; 5, hind and fore leg paralysis; and 6, death.

### Tissue Collection and Cell Isolation

At various time points after immunization, spleen, draining cervical lymph nodes (DLN), brain, and spinal cord were removed. Single-cell suspensions were obtained from spleen or pooled DLNs. Brain mononuclear cells (BMNCs) were isolated by Percoll gradients as described.<sup>11</sup> Antigen-presenting cells (APCs) were pre-

pared by immunomagnetic T-cell depletion of SJL/J spleen cells using microbead-conjugated anti-CD8 and anti-CD4 monoclonal antibodies (mAbs) (Miltenyi Biotech, Bergisch Gladbach, Germany), followed by treatment with 50  $\mu$ g/ml of mitomycin C (Sigma).

### Neuropathology and Immunohistochemistry

For histopathology, perfused transverse sections of the cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cords were stained with Luxol Fast Blue/periodic acid-Schiff to assess demyelination or with hematoxylin and eosin to assess leukocyte infiltration. Semiquantitative analysis of inflammation and demyelination was performed in a blinded manner as described.<sup>12</sup> Immunohistochemistry was performed on adjacent cryosections of brain and spinal cords with Histomouse immunostaining kits (Zymed, San Francisco, CA) as described.<sup>13,14</sup> Positive cells for each marker were counted by automatic video scanning using a Leica Q500 MC, and tissue measurements were performed by using a Scion image analysis system. The number of stained cells per 10<sup>4</sup> square pixels tissue area was calculated. Axonal damage was assessed by Western blot analysis of the abnormally dephosphorylated neurofilament H (NF-H) in whole spinal cord homogenates.<sup>15</sup>

### mRNA Analysis

Total RNA was isolated from spinal cords, brains, and DLN cells, and mRNA expression of a variety of chemokines, chemokine receptors, cytokines, enzymes, and leukocyte markers was quantified using the multiprobe RNase protection assay (RPA) kit from Ambion (Austin, TX), using mCK2b, mCK3b, mCK5c, and mCR5 DNA as templates (BD Pharmingen, San Diego, CA). Sample-to-sample variation in RNA loading was controlled by comparison to the housekeeping gene GAPDH.

### Assessment of T-Cell Autoreactive Response

BMNCs and DLN cells were recovered from the SJL/J mice at the peak of clinical EAE (day 16 after immunization). Cells (10<sup>6</sup> cells/ml) were stimulated in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin) with different concentrations of PLP<sub>139-151</sub> for 48 hours (for cytokine determination) or for 72 hours (for proliferative response). Cell proliferation was evaluated by using a cell proliferation assay (BrdU) from Roche Diagnostics GmbH (Mannheim, Germany). Cytokine and chemokine content in culture supernatants was determined by specific sandwich enzyme-linked immunosorbent assays.<sup>8</sup> The frequency of autoreactive T cells producing IFN- $\gamma$  or IL-4 was determined by the enzyme-linked immunospot (ELISPOT) technique.<sup>6</sup> NO production was determined by measuring oxidized nitrite amounts in culture supernatants by using the Griess reagent.<sup>9</sup> For intracellular analysis of cytokines in stimulated DLN cells, 10<sup>6</sup> cells/ml were collected and stimu-

lated with PMA (1 ng/ml) plus ionomycin (20 ng/ml) for 8 hours in the presence of monensin. Cells were stained with PerCP-anti-CD4 mAbs, fixed, and saponin-permeabilized with Cytofix/Cytoperm, stained with fluorescein isothiocyanate- and phycoerythrin (PE)-conjugated anti-cytokine-specific mAbs (BD Pharmingen), and analyzed using a FACScalibur flow cytometer (BD Pharmingen). To distinguish between monocyte/macrophage and T cell sources, intracellular cytokine analysis was performed exclusively in the PerCP-labeled CD4<sup>+</sup> T-cell population.

### Flow Cytometric Analysis

BMNCs and DLN cells incubated with various mAbs (PE-anti-CD4, PE-anti-F4/80, PE-anti-CD11c, PE-anti-CD8, or PerCP-anti-CD4; 2.5 μg/ml final concentration) were fixed in 1% paraformaldehyde and analyzed on a FACScalibur flow cytometer. We used isotype-matched antibodies as controls and IgG block (Sigma) to avoid nonspecific binding to Fc receptors.

### Proteolipid Protein (PLP)-Specific Antibody Determination in Serum

We used an enzyme-linked immunosorbent assay to determine the specific anti-PLP antibody responses in sera collected at the peak of disease, as previously described.<sup>16</sup> Briefly, Maxisorb plates (Millipore, Bedford, MA) were coated overnight at 4°C with 100 μl of soluble PLP<sub>139-151</sub> peptide (10 μg/ml) in 0.1 mol/L bicarbonate buffer, pH 9.6, followed by blocking and incubation for 2 hours at 37°C with serial dilutions of serum obtained by cardiac puncture from the different treatment groups at day 16 after immunization. Biotinylated anti-IgG, anti-IgG1 or anti-IgG2a antibodies (2.5 μg/ml) (Serotec, Oxford, UK) were added for 1 hour at 37°C. The plates were washed, followed by incubation with streptavidin-horseradish peroxidase and development with the ABTS substrate. A standard curve was constructed for each assay by coating wells with an isotype-specific anti-mouse Ig followed by addition of known concentrations of IgG, IgG1, or IgG2a.

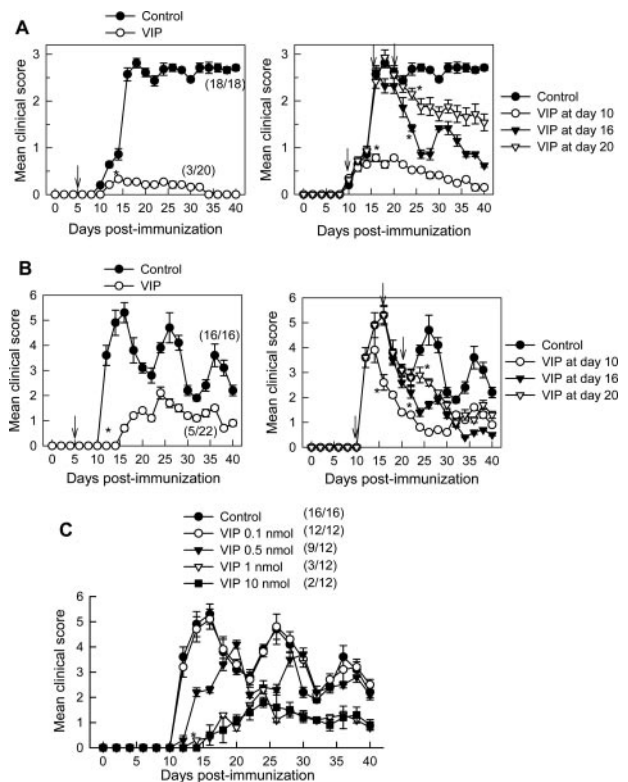
### Statistical Analysis

The Mann-Whitney *U*-test to compare nonparametric data for statistical significance was applied on all clinical results and cell culture experiments.

## Results

### Therapeutic Effect of VIP in EAE

We investigated the effect of the administration of the immunomodulatory peptide VIP in two EAE models that mirror different clinical characteristics of MS. Chronic active EAE induced by myelin oligodendrocyte protein (MOG<sub>35-55</sub>) in C57BL/6 mice mimics 20% of clinical MS. VIP treatment during the efferent phase of the disease greatly inhibited the development of EAE and reduced



**Figure 1.** VIP treatment reduces EAE severity and incidence. **A** and **B:** VIP inhibits the progression of actively induced EAE and reduces the severity and relapses of RR-EAE. Active EAE was induced in C57BL/6 mice by immunization with MOG<sub>35-55</sub>, and RR-EAE was induced in SJL/J mice by immunization with PLP<sub>139-151</sub>. Immunized mice were treated intraperitoneally for 3 days with PBS (control) or with VIP (2 nmol/day, **arrows**) starting on day 5 or after the onset of clinical signs (days 10, onset; 16, acute phase; or 20, relapsing phase) as described in Materials and Methods. The data represent the mean clinical score for each group. Numbers in parentheses represent disease incidence of each group (percent mice with disease throughout the entire period). **C:** VIP effect on EAE is dose-dependent. SJL/J mice with RR-EAE were treated with different doses of VIP starting at the onset of clinical signs. The data represent the mean clinical score for each group. Numbers in parentheses represent disease incidence of each group (percent mice with disease throughout the entire period). \**P* < 0.001 versus control from day of onset (*n* = 12 to 22 mice/group).

disease incidence and severity (Figure 1A). Untreated control mice developed moderate (17%; clinical score, 0.5 to 2) to severe (83%; clinical score, 3 to 4) EAE, and recovery from the disease was not observed (Figure 1A). In contrast, in the VIP-treated group, 17 of 20 mice (85%) were entirely asymptomatic whereas the rest (15%) displayed moderate symptoms and completely recovered 20 to 30 days after disease onset (Figure 1A). The complete protection achieved by VIP administration 3 days before disease onset led us to test its effect on active disease. When administered 3 days after disease onset, VIP blocked EAE development, and delaying administration of VIP until disease was well established (5 or 10 days after disease onset) ameliorated the clinical score (Figure 1A).

In the majority of MS patients, clinical disease follows a relapsing-remitting course. Therefore, we tested the effect of VIP on the relapsing-remitting EAE (RR-EAE) model induced by proteolipid protein (PLP<sub>139-151</sub>) in SJL/J mice. Disease severity was substantially reduced in mice receiving VIP treatment during the efferent phase of disease, as reflected by a delay in disease onset, a

**Table 1.** Effect of VIP-Related Peptides in RR-EAE

	Incidence*(%)			Onset (days)	Peak <sup>†</sup>	Relapse (%)	CDI <sup>‡</sup>
	Severe	Mild	None				
Control	19/22 (86)	3/22 (14)	0/22 (0)	11.3 ± 0.8	5.4 ± 0.5	14/22 (64)	56.4 ± 6.6
VIP	0/26 (0)	7/26 (27)	19/26 (73)	21.3 ± 1.3	2.4 ± 0.3	4/26 (15)	21.3 ± 2.7
PACAP	0/12 (0)	4/12 (33)	8/12 (67)	19.4 ± 1.6	2.1 ± 0.4	3/12 (25)	22.4 ± 3.6
VIP <sub>1-12</sub>	10/12 (83)	2/12 (17)	0/12 (0)	12.1 ± 1.1	5.5 ± 0.7	7/12 (58)	54.3 ± 7.1
VIP <sub>10-28</sub>	9/12 (75)	2/12 (17)	1/12 (8)	10.8 ± 1.2	5.4 ± 0.3	7/12 (58)	55.2 ± 3.6
VPAC1	0/12 (0)	3/12 (25)	9/12 (75)	22.6 ± 2.7	2.0 ± 0.3	2/12 (17)	19.7 ± 2.1
VPAC2	3/12 (25)	6/12 (50)	3/12 (25)	15.3 ± 1.6	3.9 ± 0.6	5/12 (42)	39.3 ± 6.1
Secretin	8/10 (80)	2/10 (20)	0/10 (0)	10.7 ± 1.1	5.6 ± 0.7	6/10 (60)	54.3 ± 5.5
Glucagon	7/10 (70)	3/10 (30)	0/10 (0)	11.4 ± 1.2	5.3 ± 0.4	5/10 (50)	53.4 ± 5.6

SJL/J mice with RR-EAE were treated intraperitoneally with PBS (control) or with 2 nmol VIP, PACAP, VIP fragments (VIP<sub>1-12</sub>, VIP<sub>10-28</sub>), VIP receptor agonists (VPAC1, VPAC2), or other peptides of the family (secretin, glucagon) for 3 consecutive days starting at the onset of clinical signs.

\*Disease incidence is graded as severe (clinical score: 4 to 6), mild (clinical score: 0.5 to 3), or none (no clinical signs).

<sup>†</sup>Peak defines the mean of maximal clinical score. Numbers in parentheses represent percentages.

<sup>‡</sup>CDI is the mean of the sum of the daily disease scores.

decrease in the mean clinical score, a decrease in the rate of relapse and a reduction in the cumulative disease index (Figure 1B, Table 1). The therapeutic properties of VIP were also apparent when administered at the onset of clinical symptoms (day 10), at the peak of the disease (day 15, acute phase), or after initial remission (day 20, relapsing phase) (Figure 1B). The VIP effects were dose-dependent, and as previously shown for other autoimmune models,<sup>2-10</sup> 2 nmol was the most efficient therapeutic dose (Figure 1C). In addition, the protective effect of VIP was long lasting, with no clinical symptoms up to 60 days after VIP administration.

To test the specificity of the VIP effect and to identify the VIP receptor involved, we used specific VIP agonists. The VPAC1 agonist mimicked the VIP effects on RR-EAE, whereas the VPAC2 agonist showed only a weak effect (Table 1). VIP specificity was confirmed because neither VIP family peptides (glucagon nor secretin) nor VIP fragments (VIP<sub>1-12</sub> and VIP<sub>10-28</sub>) had any effect (Table 1). In agreement with a recent report,<sup>17</sup> the pituitary adenylate cyclase-activating polypeptide (PACAP), a structurally-related neuropeptide with immunomodulatory effects similar to VIP, reduced EAE progression, severity, and incidence (Table 1). These results indicate that VPAC1 plays a major role in the protective effect of VIP in EAE and that the entire peptide is required for its therapeutic effect.

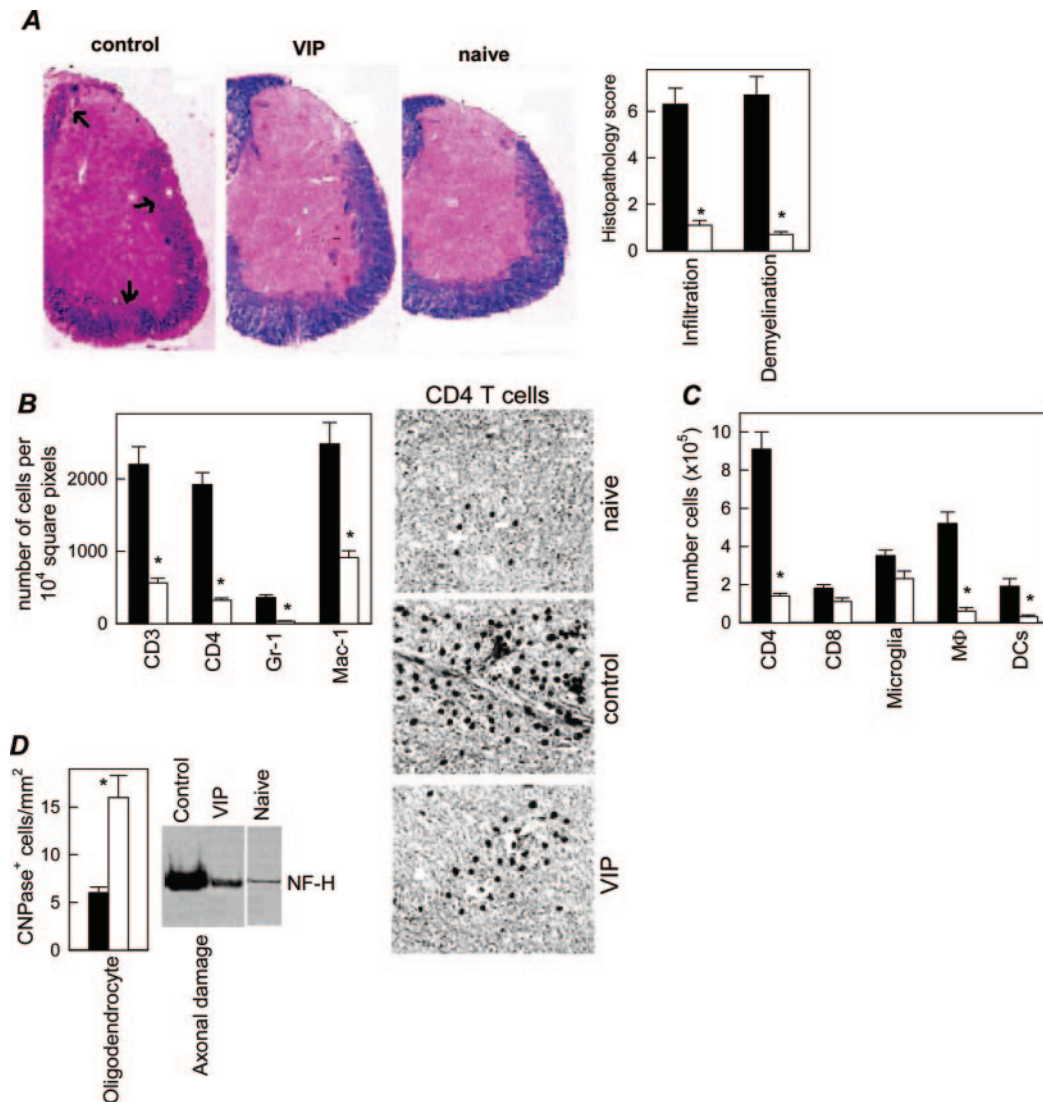
### VIP Treatment Reduces CNS Neuropathology and Inflammation in EAE

We next investigated the mechanisms underlying the amelioration of EAE after VIP treatment. The pathology of MS and EAE features focal areas of inflammatory infiltration and demyelination with oligodendrocyte depletion.<sup>1-3</sup> Histopathological examination of spinal cords confirmed that the beneficial actions of VIP were attributable to a decrease in inflammatory infiltrates and subsequent demyelination (Figure 2A). Immunohistological evaluation of CNS infiltrates in EAE mice revealed that the inflammatory cells close to the perivascular area were mostly CD4<sup>+</sup> T cells and Mac-1<sup>+</sup> macrophages and activated microglia, with few granulocytes and dendritic cells (Figure 2, B and C). VIP administration significantly

decreased the number of infiltrating cells in the CNS ( $4.2 \times 10^5$  compared with  $18 \times 10^5$  cells/brain in control mice), affecting all populations, especially CD4<sup>+</sup> T cells and macrophages (Figure 2, B and C). This suggests that VIP prevents the entry or retention of inflammatory auto-reactive cells into the CNS.

Because myelin and oligodendrocytes are targeted in MS,<sup>2,3</sup> we evaluated the effect of VIP on demyelination, number of oligodendrocytes, and axonal damage. We examined the number of white-matter oligodendrocytes in lumbar dorsal cord columns by immunohistochemistry for the oligodendrocyte marker 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNPase). In mice with EAE, the numbers of oligodendrocytes decreased by an average of 50% in comparison with normal age-matched mice. In contrast, in VIP-treated mice the number of oligodendrocytes remained normal (Figure 2D). Axonal damage, another feature of early MS, was assessed by the abnormal dephosphorylation of neurofilament H (NF-H).<sup>18</sup> Spinal cords of RR-EAE mice exhibited a large increase in dephosphorylated NF-H, whereas VIP treatment resulted in substantially reduced amounts of dephosphorylated NF-H (Figure 2D). Thus, the VIP-induced amelioration of clinical EAE symptoms is associated with substantially reduced demyelination and significant reduction in oligodendrocyte loss and axonal damage.

To examine whether VIP treatment results in the reduction of inflammatory mediators, we analyzed the expression of inflammation-related genes in the spinal cord of EAE mice. VIP administration dramatically reduced the expression of proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IL-18, and IL-12), enzymes (iNOS), chemokines (RANTES, MIP-1, MCP-1, IP-10, and MIP-2) and chemokine receptors (CCR-1, CCR-2, and CCR-5) associated with EAE (Figure 3A). In addition, spinal cords of VIP-treated mice showed increased levels of the anti-inflammatory cytokines IL-10, IL-1Ra, and TGF- $\beta$  (Figure 3A). The decrease in inflammatory mediators could be the result of the reduced number of inflammatory cells in the CNS. However, BMNCs isolated from VIP-treated mice produced lower levels of proinflammatory factors on *in vitro* PLP-stimulation compared to EAE controls (Figure 3B).

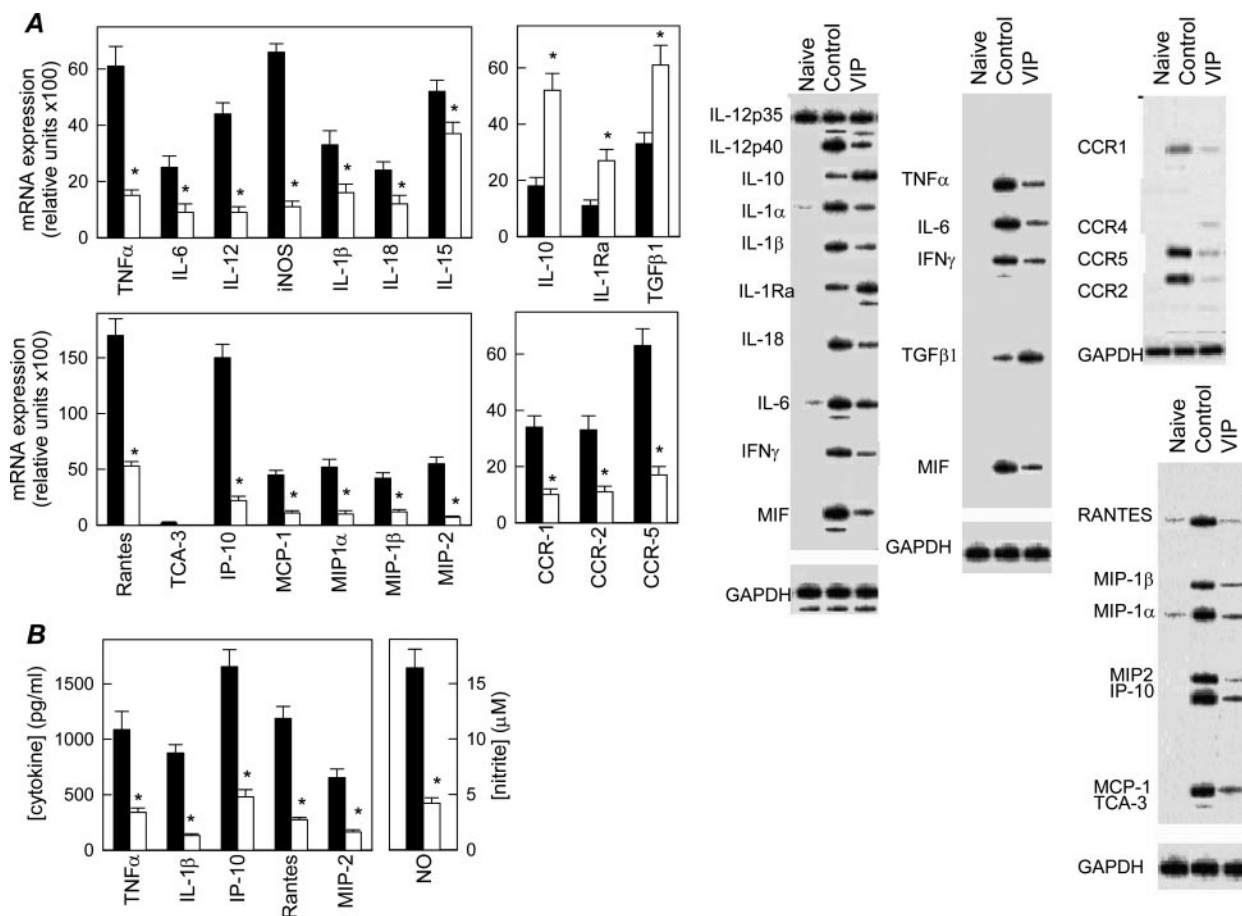


**Figure 2.** VIP reduces the histopathology in the CNS of mice with EAE. SJL/J mice were induced with RR-EAE and treated with PBS (control, ■) or with VIP at the onset of disease (□) as in Figure 1. Naïve animals without any treatment were used as negative controls. **A–C:** VIP treatment decreases demyelination, oligodendrocyte cell death, and inflammatory infiltration in the CNS. **A:** Transverse sections of several regions of the spinal cord ( $n = 6$ ) randomly selected at the peak of clinical disease were stained with Luxol Fast Blue/periodic acid-Schiff (for demyelination) or with H&E (for inflammatory infiltration). A representative histological section from a control EAE mouse shows areas of demyelination (arrows) that correspond to areas of leukocytic infiltration (not shown). A comparable region of spinal cord from a VIP-treated mouse shows no histopathological signs, similar to naïve animals. Mean pathological scores of demyelination and lymphocyte infiltration were determined as described in Materials and Methods. **B:** Immunohistological evaluation of CNS infiltrates. Numbers of CD3<sup>+</sup>, CD4<sup>+</sup>, Gr-1<sup>+</sup>, and Mac-1<sup>+</sup> cells in infiltrates per 10<sup>4</sup> square pixels from spinal cord sections were determined ( $n = 6$ ). Immunohistological samples for CD4<sup>+</sup> T cells are shown. **C:** Infiltrating mononuclear cells were isolated from brain at the peak of disease, and the numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, microglia (F4/80<sup>+</sup>CD45<sup>low</sup>), macrophages (Mφ, F4/80<sup>+</sup>CD45<sup>high</sup>), and dendritic cells (DCs, CD11c<sup>+</sup>) were determined by flow cytometry ( $n = 5$ ). Numbers of infiltrating cells in naïve sham animals were lower than 10<sup>4</sup> cells, less microglia that were  $2.4 \times 10^5$  cells. **D:** The numbers of white matter oligodendrocytes in the lumbar dorsal cord were determined by immunohistochemistry for CNPase. Axonal damage was determined by Western blot analysis for abnormally dephosphorylated neurofilament H (NF-H) in whole spinal cord homogenates. Naïve animals showed  $17.2 \pm 2.3$  CNPase<sup>+</sup> cells/mm<sup>2</sup>. \* $P < 0.001$  versus control.

### VIP Decreases the Th1 Cell Autoreactive Response in EAE

EAE is a Th1-type cell-mediated autoimmune disease. High levels of Th1-type cytokines (eg, IFN- $\gamma$ ) are detected in the CNS in both MS and EAE, and conversely, neutralizing Th1 cytokine antibodies generally ameliorate disease progression in the murine model.<sup>19</sup> In addition, Th2-type cytokines are predominantly present in murine brain during recovery, and treatment based on Th2-type cytokines suppresses the disease, suggesting a switch from Th1- to Th2-type responses during remission.<sup>19,20</sup>

VIP could prevent or ameliorate EAE by reducing encephalitogenic T-cell responses and/or migration to the CNS. We first determined the proliferation and cytokine profile of peripheral T cells from VIP-treated EAE mice. T lymphocytes derived from the cervical DLN of EAE mice showed marked PLP antigen-dependent proliferation, whereas T cells from VIP-treated mice proliferated much less (Figure 4A). This suggests that VIP administration during EAE progression partially inhibits autoreactive T-cell clonal expansion. To assess the effector Th phenotype, we analyzed the cytokine profile. EAE results in the generation of PLP-specific effector T cells producing high



**Figure 3.** VIP reduces the inflammation in the CNS of mice with EAE. SJL/J mice were induced with RR-EAE and treated with PBS (control, ■) or with VIP at the onset of disease (□) as in Figure 1. **A:** VIP treatment decreases the expression of inflammatory mediators in the CNS. Total RNA was purified from spinal cords harvested at the peak of clinical disease and the expression of inflammatory cytokines, chemokines, chemokine receptors, and iNOS was determined by RPA. Data represent the mean  $\pm$  SD of five mice per group. Representative RPA blots for each set of genes are shown. Samples from naive animals were used as control for basal gene expression. **B:** BMNCs were pooled from five mice per group at the peak of disease and stimulated *in vitro* with PLP<sub>139-151</sub> and splenocytes (as antigen-presenting cells, APCs). Cytokine and nitric oxide (NO) levels in supernatants were determined. Cells obtained from naive animals or from EAE mice cultured with an irrelevant Ag (OVA, 10  $\mu$ g/ml) or with medium alone did not produce any cytokines: <30 pg IP-10/ml, <20 pg TNF- $\alpha$ /ml, <15 pg IL-1 $\beta$ /ml, <50 pg RANTES/ml, <0.1 ng MIP-2/ml. Data represent the mean  $\pm$  SD of five mice per group. \**P* < 0.001 versus control.

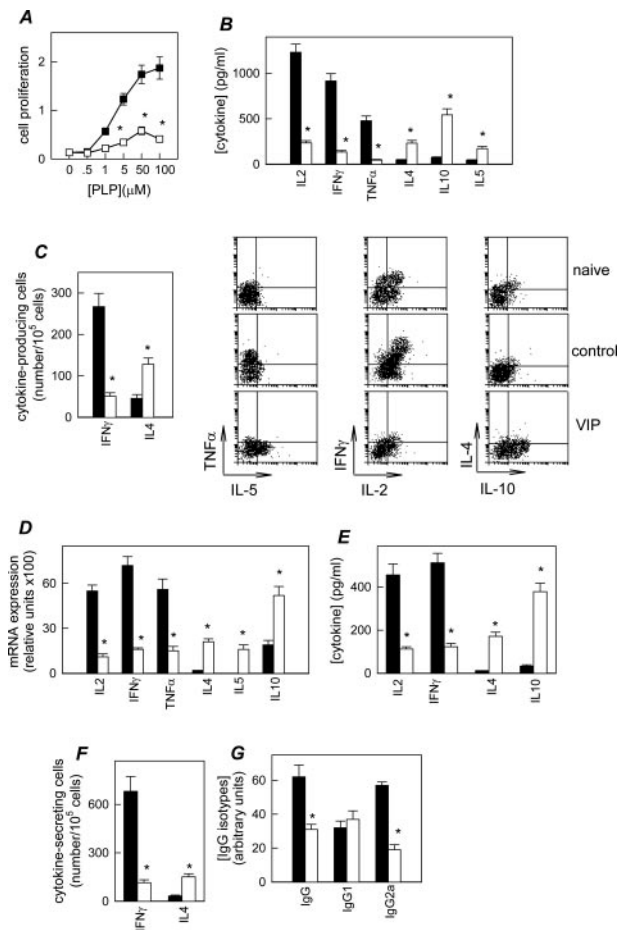
levels of Th1-type cytokines (IFN- $\gamma$ , IL-2, and TNF- $\alpha$ ) and low levels of Th2-type cytokines (IL-4, IL-5, and IL-10) (Figure 4B). In contrast, T cells from VIP-treated EAE mice produced lower levels of Th1 and higher levels of Th2 cytokines (Figure 4B), indicating a shift in the Th1/Th2 balance. ELISPOT assays confirmed these results (Figure 4C, left panel). Given that some of the cytokines assayed could be produced by monocytes/macrophages, B cells, or CD8<sup>+</sup> T cells, in addition to CD4<sup>+</sup> T cells, we determined the intracellular expression of these cytokines by flow cytometry in sorted DLN CD4<sup>+</sup> T cells after stimulation. CD4<sup>+</sup> T cells from VIP-treated EAE mice showed significantly lower numbers of IL-2/TNF- $\alpha$ /IFN- $\gamma$ -producing Th1 cells and increased numbers of IL-10/IL-4/IL-5-producing cells. To assess whether VIP has a similar effect on the CNS cytokine pattern, we determined the expression levels of Th1 and Th2 cytokines in spinal cord and brain. VIP-treated EAE mice showed a decreased expression of Th1 cytokines and increased levels of Th2 cytokines (Figure 4D). Because this might be due to a reduced CNS T-cell infiltration, we evaluated the cytokine profile in cultures of isolated BMNCs stimulated with PLP.

In contrast to control EAE mice, mononuclear cells isolated from the VIP-treated mice produced low levels of Th1 cytokines and high amounts of Th2 cytokines (Figure 4, E and F).

High levels of circulating antibodies directed against myelin antigens invariably accompany the development of MS, and their production is a major factor in determining susceptibility to the disease.<sup>1-3</sup> VIP administration resulted in reduced serum levels of PLP-specific IgG, particularly autoreactive IgG2a antibodies, generally reflective of Th1 activity (Figure 4G). These data provide further evidence that VIP administration during EAE reduces the Th1 autoreactive response and promotes Th2 responses both in the CNS and the periphery.

### Discussion

The initial stages of EAE involve multiple steps that can be divided into two main phases: early events associated with initiation and establishment of autoimmunity and later events associated with the evolving immune and



**Figure 4.** VIP decreases the Th1 cell autoreactive response in EAE. **A–C:** VIP treatment decreases Th1-mediated response in the periphery. SJL/J mice suffering from RR-EAE were treated with PBS (control, ■) or with VIP at the onset of disease (□). DLN cells isolated at the peak of clinical disease were stimulated with different concentrations of PLP<sub>139-151</sub>, and the cell proliferative response (**A**) and the cytokine levels in supernatants (**B**) were determined. The number of PLP-specific T cells producing IFN-γ or IL-4 was determined by ELISPOT (**C, left**) and the expression of intracellular cytokines was determined by flow cytometry in gated CD4<sup>+</sup> cells (**C, right**). DLN cells from naïve animals or from EAE mice cultured with an irrelevant Ag (OVA, 10 μg/ml) or with medium alone did not proliferate ( $A_{450} < 0.110$ ) or produce any cytokines: <0.1 ng IFN-γ/ml, <30 pg IL-4/ml, <20 pg TNF-α/ml, <0.1 ng IL-10/ml, <0.1 ng/ml IL-2, and <50 pg IL-5/ml. Data represent the mean ± SD of eight mice per group. **D–F:** VIP regulates the Th1/Th2 balance in the CNS. Spinal cord and brain were isolated from the different experimental groups ( $n = 8$ ) at the peak of clinical disease. **D:** Total RNA from spinal cords was isolated and the levels of gene expression for different Th1/Th2 cytokines were determined by RPA. BMNCs were stimulated with PLP<sub>139-151</sub> and spleen APCs. **E:** Cytokine levels in supernatants were determined by enzyme-linked immunosorbent assay. Cells cultured with medium alone did not induce any cytokines. **F:** Number of PLP-specific T cells producing IFN-γ or IL-4 was determined by ELISPOT. Data are the mean ± SD of eight mice per group. **G:** VIP treatment regulates PLP-specific IgG levels. Sera were collected and the levels of PLP-specific IgG, IgG1, and IgG2a were determined by enzyme-linked immunosorbent assay. Serum obtained from naïve animals showed undetectable levels of PLP-specific IgG. Data are represented as the mean ± SD using arbitrary units, as analyzed in three separate experiments (eight mice per group per experiment). \* $P < 0.001$  versus control.

inflammatory responses. The crucial process underlying disease initiation is the induction of autoimmunity to myelin sheath components; later events involve a destructive inflammatory process. Progression of the autoimmune response involves the development of reactive Th1 cells with encephalitogenic potential, their entry into the

CNS, and future recruitment of inflammatory cells through multiple mediators.<sup>1–3</sup> Certain therapeutic approaches address the autoimmune component of EAE and MS, complementing existing anti-inflammatory therapies.<sup>19–24</sup> In this study we show that the neuropeptide VIP provides a highly effective therapy for EAE. The therapeutic effect of VIP is associated with a striking reduction of the two deleterious components of the disease, ie, the autoimmune and inflammatory response. VIP treatment decreased the presence of encephalitogenic Th1 cells in the periphery and the CNS. In addition, VIP strongly reduced the inflammatory response during EAE progression by down-regulating the production of several inflammatory mediators, such as TNF-α, IL-6, IL-1β, IL-18, IL-12, NO, and various chemokines and their receptors in both spinal cord and brain parenchyma. At the same time, VIP induced the production of the anti-inflammatory cytokines IL-10, IL-1Ra, and TGF-β, which ameliorate the disease. As a consequence, VIP reduced the appearance of inflammatory infiltrates in the CNS and the subsequent demyelination and axonal damage typical of EAE.

The capacity of VIP to regulate a wide spectrum of inflammatory mediators might offer a therapeutic advantage over neutralizing antibodies and receptor antagonists directed against a single mediator. Chemokines are responsible for the CNS infiltration and activation of various leukocyte populations that contribute to MS neuropathology. The inhibitory effect of VIP on chemokine production by macrophages and microglia could partially explain the absence of infiltrates in the parenchyma,<sup>5</sup> being especially relevant for chemokines as MIP-2 (chemotactic for neutrophils), IP-10 (for Th1 cells), and RANTES/MIP-1α (for macrophages and T cells), all involved in MS pathogenesis.<sup>2,20,21</sup> In addition, Grimm and colleagues<sup>25</sup> have recently reported that VIP exerts part of its anti-inflammatory effect *in vivo* by *trans*-deactivating chemokine receptors, a mechanism that could also be participating in the effect of VIP on EAE. Furthermore, the few infiltrating cells found in VIP-treated mice were not able to produce Th1-type cytokines, suggesting that VIP directly suppresses the inflammatory/autoreactive cells. Several previous studies identified VIP as a potent deactivating factor for macrophages and resident microglia and as a suppressor of the Th1 response.<sup>4–7</sup> However, it is a mistake to consider VIP as a cause of general immunosuppression. In general, the administration of VIP *in vivo* inhibits any antigen-specific, active Th1 response converting it to Th2, an effect mediated by several non-excluding mechanisms involving both an effect of VIP at the level of Th1/Th2 generation, either directly or through effects on APCs, and/or at the level of the already generated effectors, by preferentially promoting Th2 proliferation, survival, or accumulation.<sup>4,5</sup> However, this VIP-mediated Th1/Th2 class switch is more pronounced for autoantigens under several inflammatory conditions (present study)<sup>8,10</sup> and depends on the concentration of antigen used. Indeed, VIP inhibited the classical antigen-driven Th1 response and generated antigen-specific memory Th2 cells when a high dose of antigen was administered to TCR-transgenic mice.<sup>26</sup> However, with

low/tolerant amounts of antigen, VIP did not induce any Th2 response but generated antigen-specific regulatory T cells (Treg) and dendritic cells with capacity to induce Tr1-like Treg.<sup>27,28</sup>

Therefore, the potential induction of Treg by VIP adds a new player to this scenario because several studies have indicated that a subtype of T cells, namely Treg, confer significant protection against EAE by promoting protective Th2 responses and decreasing the CNS homing of autoreactive cells.<sup>29–33</sup> In this sense, the present study shows that VIP treatment also inhibits events in the inflammatory phase of EAE after the activation/differentiation of antigen-specific effector Th1 cells. In addition, whereas T-cell proliferation to the autoantigen PLP is almost abolished in VIP-treated animals, Th2 cytokines produced by these low-proliferating cells are significantly increased. Therefore, there exists the possibility that VIP induces Treg with suppressive activity during the progression of the disease. Indeed, we have recently demonstrated that the administration of VIP to EAE mice induces the expansion of IL-10/TGF- $\beta$ -producing CD4<sup>+</sup>CD25<sup>+</sup> Foxp3-expressing Treg cells in the periphery and the CNS.<sup>34</sup> In addition to expanding the CD4<sup>+</sup>CD25<sup>+</sup> population, VIP also induced more efficient Treg, in terms of both cytokine secretion and suppressive activity. The VIP-induced CD4<sup>+</sup> Treg cells produce high levels of IL-10 and TGF- $\beta$ 1.<sup>34</sup> Also, on a per cell basis, the VIP-induced CD4<sup>+</sup> Treg cells are very strong suppressors of responder autoreactive T-cell proliferation, particularly at low regulatory T cell/autoreactive T cell ratios.<sup>34</sup> The mechanisms involved in the generation/activation of Treg by VIP during EAE are not fully understood. Whether VIP acts directly on T cells inducing the generation or expansion of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells remains to be established. However, we have found that they can be peripherally generated from the CD4<sup>+</sup>CD25<sup>-</sup> compartment, because VIP treatment prevented EAE progression in CD25-depleted mice and restored the number of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells.<sup>34</sup> In addition, VIP is able to convert *in vitro* activated antigen-primed CD4<sup>+</sup>CD25<sup>-</sup> cells to very efficient CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>CTLA4<sup>+</sup>.<sup>34</sup> Additionally, VIP indirectly induced Tr1-like regulatory T cells through the generation of tolerogenic dendritic cells,<sup>28</sup> which are able to inhibit encephalitogenic T-cell activation and ameliorate EAE progression.<sup>35</sup> Alternatively, the VIP-induced presence of Treg in the DLN and CNS of EAE mice could simply be a consequence of a recruitment of Treg to these sites through the effect on the production of certain APC/T-cell-related chemokines. However, whereas VIP treatment of EAE mice increased the expression in the CNS of CCR-4 (Figure 3A), a chemokine receptor specifically expressed in Treg cells,<sup>36</sup> reflecting an increased presence of Treg, it failed to increase the expression of the ligands for CCR-4 (not shown), arguing against this possibility. VIP-induced Treg generation during EAE correlates with our data showing that VIP treatment increases the production of IL-10 and TGF- $\beta$ 1, two of the major mediators of regulatory T cells, in CNS and the periphery. Interestingly, VIP induced long-term Treg on EAE mice, because VIP-treated mice that

recovered from EAE showed a higher percentage of DLN CD4<sup>+</sup>CD25<sup>+</sup> T cells (14.2% versus 3.4%) than untreated mice that recovered from EAE at the end of the disease (day 45). These increased Treg numbers in the VIP-treated mice were paralleled by a maintained shift from Th1 to Th2 response (25 of 84 versus 145 of 14 IFN- $\gamma$ /IL-4-producing cells in DLN of VIP-treated EAE mice versus recovered control EAE mice). In addition, we reported that unfractionated CD4<sup>+</sup> cells isolated from VIP-treated EAE mice showed a significant therapeutic effect on experiments of adoptive EAE transfer and that this effect was almost abrogated when they were depleted of CD4<sup>+</sup>CD25<sup>+</sup> cells.<sup>34</sup> However, although within the CD4<sup>+</sup> T cells induced by VIP, the CD4<sup>+</sup>CD25<sup>+</sup> population conveys the major suppressive capacity, the CD4<sup>+</sup>CD25<sup>-</sup> population, presumably containing Th2 cells, also showed a partial therapeutic effect on EAE.<sup>34</sup> Furthermore, *in vivo* blockade of the Treg mediators CTLA-4, TGF- $\beta$ 1, and IL-10, but not of the Th2-type cytokine IL-4, significantly reversed the therapeutic effect of VIP on EAE (unpublished data), supporting a major role of Treg versus Th2 cells in the VIP action. Therefore, the generation of Treg by VIP could also explain the selective inhibition of Th1 immune responses once T cells have completed differentiation into Th1 effector cells as evidenced by the therapeutic effect of delayed administration of VIP in established EAE. Importantly, we have recently found that Th1 effectors are more susceptible than Th2 cells to the suppression by VIP-induced Treg.<sup>27</sup> However, because VIP has been found to directly affect Th1/Th2 balance through multiple mechanisms,<sup>4–7</sup> a Treg-independent effect of VIP in the differentiation of Th1 cells cannot be excluded in EAE. In this sense, VIP inhibited the *in vitro* production of inflammatory mediators by BMNCs isolated from EAE mice in a time course that excludes the involvement of newly generated Treg.

Recently, attention has focused on repair and regeneration mechanisms as targets for therapy in MS, especially in the secondary progressive phase of the disease. Interestingly, the delayed administration of VIP not only prevented the progression of the disease but induced significant recovery, suggesting a role of VIP in repair and/or neuroregeneration. In fact, in our study VIP inhibited oligodendrocyte cell loss and axonal damage, and we observed that VIP stimulates T cells to produce the brain-derived neurotrophic factor (unpublished data), which has been reported to induce axonal outgrowth, remyelination, regeneration, and rescue of degenerating neurons.<sup>37</sup> Similarly, VIP has been reported to induce astrocytes to produce other neurotrophic factors.<sup>38,39</sup> This may indicate that VIP is not solely an anti-inflammatory agent but that it also contributes to neuroprotection and neuroregeneration.

Our study is also in agreement with a recent report of Kato and colleagues<sup>17</sup> describing that the VIP-related peptide PACAP prevents EAE progression by using the model of MOG-induced EAE in C57BL/6 mice. They also describe that the effect of PACAP is mediated by down-regulating both inflammatory and Th1-type autoreactive responses, attributing a major role to APCs, mainly macrophages and resident microglia, and in which down-

regulation of the co-stimulatory molecule CD86 is clearly involved. Therefore this mechanism could also be involved in the therapeutic effect of VIP on EAE, because VIP has been found to inhibit the expression of co-stimulatory molecules, such as CD40, CD80, and CD86, by activated dendritic cells, macrophages, and microglia.<sup>4,5</sup>

The immunological effects of VIP are exerted through interaction with a family of G protein-coupled receptors, namely VPAC1, VPAC2, and PAC1, which mainly stimulate the adenylate cyclase pathway.<sup>5</sup> Similar to other inflammatory models,<sup>8–10</sup> our results indicate that VIP mediates its effect on EAE through VPAC1, which is expressed by polymorphonuclear cells, macrophages, microglia, and T and B cells, supporting the potential involvement of these cell types in the curative effects of VIP. The fact that VPAC1 is the major mediator not only represents an advantage for the design of more specific and stable therapeutic drugs in the future but also supports a major involvement of Treg versus Th2 cells in the action of VIP on EAE because VPAC2 has been related to VIP-induced Th2 response and VPAC1 to VIP-induced Treg.<sup>4–6,28</sup>

Our results serve as the basis for proposing a novel MS treatment strategy that is targeted to the inhibition of the different neuropathological components of the disease. It is important to note that VIP has been tested in human patients,<sup>40</sup> and it is well tolerated in doses similar to those that are able to prevent EAE. In our study, the animals did not exhibit side effects, probably because a short period of treatment with the peptide is enough to get a significant disease remission without recurrence, and plasma VIP levels only slightly increased after VIP treatment (35 pg/ml versus 64 pg/ml). The ability of VIP, on delayed administration, to ameliorate the ongoing disease also fulfills an essential prerequisite for a therapeutic agent. These observations provide a powerful rationale for the assessment of the efficacy of VIP as a novel therapeutic approach to the treatment of MS.

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