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Targeting Dipeptidyl Peptidase IV (CD26) Suppresses Autoimmune Encephalomyelitis and Up-Regulates TGF-β1 Secretion In Vivo

Andreas Steinbrecher, Dirk Reinhold, Laura Quigley, Ameer Gado, Nancy Tresser, Leonid Izikson, Ilona Born, Jürgen Faust, Klaus Neubert, Roland Martin, Siegfried Anorse, and Stefan Brocke

CD26 or dipeptidyl peptidase IV (DP IV) is expressed on various cell types, including T cells. Although T cells can receive activating signals via CD26, the physiological role of CD26/DP IV is largely unknown. We used the reversible DP IV inhibitor Lys[Z(NO2)]-pyrrolidide (I40) to dissect the role of DP IV in experimental autoimmune encephalomyelitis (EAE) and to explore the therapeutic potential of DP IV inhibition for autoimmunity. I40 administration in vivo decreased and delayed clinical and neuropathological signs of adoptive transfer EAE. I40 blocked DP IV activity in vivo and increased the secretion of the immunosuppressive cytokine TGF-β1 in spinal cord tissue and plasma during acute EAE. In vitro, while suppressing autoreactive T cell proliferation and TNF-α production, I40 consistently up-regulated TGF-β1 secretion. A neutralizing anti-TGF-β1 Ab blocked the inhibitory effect of I40 on T cell proliferation to myelin Ag. DP IV inhibition in vivo was not generally immunosuppressive, neither eliminating encephalitogenic T cells nor inhibiting T cell priming. These data suggest that DP IV inhibition represents a novel and specific therapeutic approach protecting from autoimmune disease by a mechanism that includes an active TGF-β1-mediated antiinflammatory effect at the site of pathology. The Journal of Immunology, 2001, 166: 2041–2048.

O ur current model for the initiation of T cell-mediated inflammatory disease of the CNS includes peripheral Ag-specific T cell activation and Th1 differentiation (1–3). A peripheral T cell activation step appears to be required for autoreactive T cells to enter the CNS via the blood-brain barrier (4). The process of lesion formation is further governed by a complex pattern of cyto- and chemokine expression on restimulation of autoreactive T cells in situ (5, 6). It is widely accepted that Th1 cells, critical for cell-mediated immunity by their production of IL-2, IFN-γ, TNF-α, and lymphotoxin are involved in the immunopathology of organ-specific autoimmune disease (7–9). A role as a serine peptidase catalyzing the cleavage of N-terminal dipeptides from peptides and proteins carrying proline, hydroxyproline, or alanine in the penultimate position (24). Recent evidence suggests that the cell surface dipeptidyl peptidase IV (DP IV, EC 3.4.14.5, CD26) may have a role in T cell activation and homeostasis (19, 20).

DP IV is a highly conserved type II integral membrane protein, constitutively expressed on a wide variety of epithelial, endothelial, and lymphoid cell types (21). It corresponds to the leukocyte differentiation Ag CD26. On CD4+ T cells, CD26/DP IV is tightly regulated, depending on the state of activation (22), and it is found on T cells activated in vivo and memory T cells (23). DP IV acts as a serine peptidase catalyzing the cleavage of N-terminal dipeptides from peptides and proteins carrying proline, hydroxyproline, or alanine in the penultimate position (24). A possible role of CD26/DP IV in T cell-mediated immunity is suggested by: 1) its potent costimulatory activity for T cells activated via the TCR (20); 2) its capacity to interact with extracellular matrix molecules (25, 26); and 3) the suggestion that cleavage by DP IV might regulate the function of numerous immunologically relevant peptides and proteins, including cytokines and chemokines that carry an X-Pro-motif at the N terminus (24). Clinical observations also link CD26/DP IV to autoimmunity. Elevated numbers of CD26+CD4+ T cells were described in peripheral blood and cerebrospinal and synovial fluids from patients with multiple sclerosis (27–29), and clinically active rheumatoid arthritis (30, 31), respectively. Recently, the reversible, competitive DP IV inhibitors, Lys[Z(NO2)]-pyrrolidide (I40) and Lys[Z(NO2)]-thiazolidide (I49) have been extensively analyzed in vitro. They specifically and dose-dependently suppress proliferation and secretion of various cytokines by human and murine T cells (32–34). Interestingly, it is well documented that these inhibitors also induce a 3- to 4-fold

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4 Abbreviations used in this paper: DP IV, dipeptidyl peptidase IV; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; PLP, proteolipid protein; LNC, lymph node cells; Z(NO2), 4-nitrobenzyloxycarbonyl; I40, Lys[Z(NO2)]-pyrrolidide; I49, Lys[Z(NO2)]-thiazolidide; KLH, keyhole limpet hemocyanin.
increase in the secretion of latent TGF-β1 by mitogen-stimulated murine and human T cells (33, 35).

In this report, we address the role of CD26/DP IV in murine experimental autoimmune encephalomyelitis (EAE), a well-characterized CD4+ T cell-mediated autoimmune disease leading to CNS inflammation and demyelination in susceptible strains of rodents (1). We demonstrate for the first time that the signs of EAE can be diminished by DP IV inhibition in vivo both in a preventive and therapeutic fashion. CNS inflammation associated with acute EAE can be reduced. Our data suggest that this therapeutic effect may be mediated by up-regulation of the immunosuppressive cytokine TGF-β1 and an inhibition of T cell effector functions in vivo.

Materials and Methods

Mice

Female SJL mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and from the Frederick Cancer Research and Development Center (Frederick, MD). Mice were 7–14 wk of age when experiments were started. All procedures were conducted according to protocols approved by the ACUC of the National Institute of Neurological Disorders and Stroke.

Antigens

Whole myelin basic protein (MBP) was prepared according to the method of Deibler et al. (36) from guinea pig spinal cords (Pel-Freeze Biologicals, Rogers, AR). Peptide 139–151 was prepared from MBP as previously described (37). Mice were examined daily for signs of disease and graded on a scale of increasing severity from 0 to 5 as follows: 0, no signs; 0.5, partial tail weakness; 1, limp tail or slight slowing of righting from supine position; 1.5, limp tail; 2, dragging of hind limb(s) without complete paralysis; 2.5, hind limb paralysis; 3, complete paralysis of at least one hind limb; 3.5, hind limb paralysis and slight weakness of forelimbs; 4, severe forelimb weakness; 4.5, hind limb paralysis and slight weakness of forelimbs; 5, moribund or dead. Treatment effects were assessed using the nonparametric Mann-Whitney rank sum test. All statistical tests were performed with SigmaStat software (Jandel, San Rafael, CA).

Histology

Selected mice were killed with CO2. CNS tissues were fixed in 10% PBS-buffered formalin. Paraffin sections (4 μm) were stained with hematoxylin-eosin or Luxol fast blue (American Histolabs, Gaithersburg, MD). At least two coronal sections from three brain levels and at least two longitudinal and coronal sections from cervical, thoracic, and lumbar sacral levels of the spinal cord were evaluated in a blinded manner by an experienced neuropathologist. Inhibitors and treatment

140 and 149 and the noninhibitory compound Lys(ZNO2)-OH were provided in lyophilized form. For the experiments described, the inhibitors were dissolved in PBS at 10^−3 M and adjusted to neutral pH. In treatment experiments, 1 mg 140 (M = 414.89) was injected from once every other day to three times daily, s.c. or i.p., as indicated below. Mice injected with equal amounts of PBS or Lys(ZNO2)-OH served as controls.

Determination of DP IV activity

The enzymatic activity of DP IV was determined according to the method published by Schönen et al. (38) using 1.6 mM Gly-Pro-4-nitroanilide as substrate for DP IV. The resulting 4-nitroaniline strongly absorbs at 392 nm. The enzymatic activity at 37°C and pH 7.6 is expressed in picomoles. All measurements with substrate and PBS controls were performed in duplicate. To measure DP IV activity in serum, two to three mice per treatment group were bled on the days indicated. Pooling sera were diluted 1:10 before the assay. Brains and spinal cords (caudal from C2) of mice from treatment and control groups (n = 5–6/group) were removed after transcardial perfusion with PBS on the days indicated. Tissues were carefully ground using 1/10THyamine PBS, and a homogenate in a fixed small volume of the detergent was obtained. After 60 min incubation on ice, the homogenate was centrifuged at 100,000 × g and 4°C. The supernatant was diluted 1:10 and immediately used for the assay. The amount of whole protein in the tissue was determined according to the method of Bradford (Bio-Rad Protein Assay Kit II; Bio-Rad, Richmond, CA), with BSA as standard. Sera and CNS tissue from naive mice and from mice transferred with 3 × 10^7 keyhole limpet hemocyanin (KLH)-specific LNC served as controls. The latter were obtained as described above for MBP-specific LNC, using 100 μg KLH for immunization and 10 μg/ml for the 96-h stimulation period in vitro. For the comparison of tissue DP IV activities, a one-way ANOVA was performed. Proliferation assays

MBP- or PLP139–151-specific proliferation of primed LNC or short term T cell lines followed the method previously described (37). All procedures were conducted according to protocols approved by the ACUC of the National Institute of Neurological Disorders and Stroke.

Results

Short term inhibition of DP IV activity in vivo suppresses adoptive transfer EAE

Adoptive transfer of activated MBP-specific LNC into naive SJL mice induces acute EAE followed by partial recovery and one or more relapses (Fig. 1). To test the effect of synthetic, reversible DP inhibitors by ELISA as described previously (37) a fixed and spinal cords were processed as described above for determination of DP IV activity. Plasma was obtained from deeply anesthetized mice by drawing 0.5 ml cardiac blood into a syringe containing 50 μl citrate as anticoagulant (ACD solution A; Becton Dickinson, Franklin Lakes, NJ). For the ELISA, a mouse monoclonal anti-TGF-β1 Ab (Ab-101-C; R&D Systems, Minneapolis, MN) were added at 10 μg/ml final concentration. The culture medium was based on RPMI-1640 (Life Technologies, Gaithersburg, MD), supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Cytokine measurements

Cytokine secretion was measured by sandwich ELISA in culture supernatants. LNC (4 × 10^5 or 8 × 10^5 per well) were incubated with varying doses of the inhibitors in the presence or absence of Ag in 48- or 24-well plates, respectively, using supplemented AIM-V medium as described above. Cell-free supernatants were aliquoted and stored at −80°C until measurements were performed. Duoset-ELISA kits for IFN-γ, TNF-α, and IL-4 were purchased from Genzyme (Cambridge, MA). For IL-10, InterTest-4X kits were obtained (PharMingen, San Diego, CA). Measurements from at least two dilutions per sample were performed in duplicates according to the manufacturer’s instructions.

TGF-β1 in culture supernatants, tissue homogenates, and plasma was measured by ELISA as described previously (37). A fixed and spinal cords were processed as described above for determination of DP IV activity. Plasma was obtained from deeply anesthetized mice by drawing 0.5 ml cardiac blood into a syringe containing 50 μl citrate as anticoagulant (ACD solution A; Becton Dickinson, Franklin Lakes, NJ). For the ELISA, a mouse monoclonal anti-TGF-β1, β2, β3 Ab (Genzyme) and a chicken anti-TGF-β1 Ab (R&D Systems) were used (40). To release latent TGF-β1, samples were tested before and after transient acidification (40). TGF-β1 concentrations in CNS and plasma were compared by a one-way ANOVA and the Tukey test for multiple pairwise comparisons (41).

Priming studies

Mice were immunized with 400 μg MBP s.c. as described above. On the day of immunization and again 2 days later, the mice were treated i.p. with either 0.5 mg I40 in PBS or an equal volume of PBS. Lymph nodes of treated mice were harvested on day 10, and proliferation from LNC cultures was determined as described above.

Ex vivo studies

In some experiments, spleen cells from recipient mice were serially transferred to test for encephalitogenicity. Single-cell suspensions from spleens harvested at the time points indicated were cultured at 8 × 10^6 per well with 25 μg/ml MBP in 24-well plates as described previously (37). After 4 days, 3 × 10^6 washed cells were injected i.p. into naive mice. Cells from each donor were transferred into two recipients. Proliferation assays and cytokine studies were performed in parallel as described above.


In independent experiments with an observation time of 20 mo, a significant therapeutic effect was seen during the exacerbation.

The difference in median clinical scores between groups was statistically significant between days 8 and 14 and between days 20 and 30. The treatment effect of I40 was not permanent but consistently lasted for \( \sim 2 \) mo as depicted in Fig. 1A. A neuropathological evaluation was performed in mice undergoing neuropathological evaluation on day 14 (three mice per group; see Fig. 2). In this separate experiment, I40 was injected on days 0, 1, 3, 5, 7, 9, 11, and 13, \( p < 0.001 \) for the difference between I40- and PBS-treated mice.

**FIGURE 1.** I40 suppresses the clinical severity of EAE in a preventive (A) and therapeutic (B) manner. Naive mice were injected with \( 3 \times 10^7 \) MBP-specific LNC as described in Materials and Methods. A. For EAE-prevention 1 mg I40 in PBS or PBS were injected s.c. on the day of transfer and then every other day until day 10. Boxes show the incidence of EAE in the experimental groups. The difference in median clinical scores between groups was statistically significant between days 8 and 14 and between days 20 and 30 (\( p \leq 0.045 \)). The result is representative of three independent experiments with an observation time of \( >2 \) mo. In two additional experiments, a significant therapeutic effect was seen during the first exacerbation after which CNS tissues were harvested for further analysis. B. For the treatment of early disease, injections were started on day 5, i.e., the day on which mice in both groups developed symptoms. One milligram I40 in PBS or PBS was administered i.p. three times daily until day 13 and then tapered. Significant differences between groups were noted at the peak of disease, on days 8 and 9 (\( p = 0.025 \)), and for the mean maximal scores (\( p = 0.012 \)). In two additional experiments, mice were sacrificed after similar results had been observed during the first exacerbation.

We also investigated whether the treatment with I40 could suppress DP IV activity in serum and in CNS tissue. Although serum DP IV activity increased between days 2 and 10 after transfer in control mice, it did not change significantly from baseline in I40-treated mice (Fig. 3). Spinal cord tissue was examined on day 10 after transfer. In mice with EAE treated with either PBS or Lys[Z(NO\(_2\)\(_2\)]-OH, we observed a 2- to 3-fold increase in DP IV activity compared with naive mice or mice transferred with KLH-specific LNC, indicating that CNS inflammation causes increased DP IV activity in situ (data not shown). I40 treatment was able to partially suppress this increase (Table I).

### Suppression of DP IV activity in vivo

#### Up-regulation of latent TGF-\( \beta \)

DP IV inhibitors have been shown to up-regulate latent TGF-\( \beta \) secretion by polyclonally stimulated murine T cells (33). Many studies have suggested a protective role for endogenously synthesized and therapeutically induced or administered TGF-\( \beta \) in EAE (42–45). We therefore determined the amount of TGF-\( \beta \) secreted into spinal cord tissue of treated mice. Remarkably, in tissue from I40-treated mice, there was a significant increase in the amount of latent TGF-\( \beta \) secreted as compared with naive mice or mice transferred with KLH-specific LNC, indicating that CNS inflammation causes increased DP IV activity in situ (data not shown). I40 treatment was able to partially suppress this increase (Table I).

#### Table I. Effects of I40 treatment during EAE induction in vivo

<table>
<thead>
<tr>
<th></th>
<th>I40</th>
<th>PBS</th>
<th>Lys[Z(NO(_2)(_2)]-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease severity (AUC(^{**}))</td>
<td>7</td>
<td>35.5</td>
<td>27</td>
</tr>
<tr>
<td>DP IV activity in spinal cord(^{**})</td>
<td>0.72 ± 0.05</td>
<td>0.85 ± 0.09</td>
<td>1.03 ± 0.21</td>
</tr>
<tr>
<td>TGF-( \beta )-1 in spinal cord(^{**})</td>
<td>26.0 ± 1.6</td>
<td>18.8 ± 1.8</td>
<td>16.5 ± 1.2</td>
</tr>
<tr>
<td>TGF-( \beta )-1 in plasma(^{**})</td>
<td>13.8 ± 0.7</td>
<td>2.4 ± 0.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{**}\) Groups of six mice were treated with 1 mg I40 or Lys[Z(NO\(_2\)\(_2\)]-OH or PBS s.c. on days 0, 1, 3, 5, 7, and 9. Disease severity is expressed as AUC, i.e., the cumulative daily scores of the mice in each group until day 10 when tissues where harvested.

\(^{**}\) DP IV activity (picokatals/mg protein) from spinal cords (mean ± SE from five to six mice per group) was determined at day 10 as described in Materials and Methods, \( p = 0.006 \) by one-way ANOVA.

\(^{**}\) Latent TGF-\( \beta \)-1 (in nanograms per ml) from cords was determined as described in Materials and Methods. \( p = 0.003 \) for I40 vs Lys[Z(NO\(_2\)\(_2\)]-OH/PBS.

\(^{**}\) No significant difference (\( p = 0.574 \)) between PBS- and Lys[Z(NO\(_2\)\(_2\)]-OH-treated mice.

\(^{**}\) Latent TGF-\( \beta \)-1 (in nanograms per ml) was measured in plasma obtained from mice undergoing neuropathological evaluation on day 14 (three mice per group; see Fig. 2). In this separate experiment, I40 was injected on days 0, 1, 3, 5, 7, 9, 11, and 13, \( p < 0.001 \) for the difference between I40- and PBS-treated mice.
encephalitogenic T cells themselves might be the source of TGF-β1. Primed LNC were antigenically restimulated in vitro in the presence of I40. Interestingly, during the first 48 h of antigenic stimulation, we observed a modest but consistent increase in the amount of secreted latent TGF-β1 that was detectable after as early as 4 h. (Fig. 4). In most experiments, the maximal effect was achieved by an inhibitor concentration \( \leq 10 \) μM.

Antiinflammatory effects in vitro

To analyze further the potential mechanism(s) of the treatment effect, we examined the effect of DP IV blockade during stimulation of MBP-primed LNC in vitro (Fig. 5). Coincubation of primed LNC and Ag with I40 consistently resulted in a dose-dependent inhibition of proliferation (Fig. 5A) and secretion of the proinflammatory cytokine TNF-α (Fig. 5B). Inhibition of IFN-γ production was seen in two experiments but less consistent in others. Similar results were obtained with a second reversible DP IV inhibitor, I49 (data not shown). IL-10 and IL-4 were usually not secreted in significant amounts by these LNC cultures. In LNC cultures, concentrations of I40 up to 10 μM over 96 h did not cause a significant loss of cell viability (data not shown) as assessed by trypan blue staining.

The antiproliferative effect is mediated by TGF-β1

We next asked whether the inhibitory effect on T cell function might be mediated by TGF-β1. Indeed, a neutralizing anti-TGF-β1 Ab completely blocked the effect of 10 μM I40 on the proliferation of a PLP139–151-specific T cell line, whereas the control Ab had no effect on inhibition by I40 (Fig. 5C). Given that the T cell line was stimulated with peptide presented by irradiated spleen cells, the source of the TGF-β1 neutralized can more likely be attributed to the antigenically stimulated T cells rather than APCs. These results suggest that the effect seen in vivo may be mediated in part by the
FIGURE 4. I40 up-regulates latent TGF-β1 secretion in vitro. A and B, Antigenic restimulation of MBP-specific LNC in the presence of I40 up-regulates the secretion of latent TGF-β1. LNC were obtained 10 days after immunization with MBP (A) and PLP139–151 (B) and cultured with the respective Ag and various concentrations of I40 as described in Materials and Methods. The secretion of latent TGF-β1 in culture supernatants was determined at various time points. Results are shown as means ± SE of duplicate measurements. ø = wells without inhibitor or Ag.

Suppression, not elimination, of encephalitogenic T cells

Our in vivo studies had suggested that I40 treatment during the induction phase does not indefinitely abrogate clinical disease; indeed, after ~2 mo, the clinical signs worsened in the group treated with I40 and reached the severity displayed by the control group (Fig. 1A). Subsequently, as frequently seen in chronic murine EAE, disease scores remained relatively stable, without apparent disease activity. We therefore asked whether worsening of disease several weeks after cessation of treatment was likely to be due to the survival of encephalitogenic T cells after I40 treatment. We addressed this issue by examining the MBP-specific proliferation, cytokine profile, and encephalitogenicity of spleen cells taken after >30 days from animals treated with I40 or PBS either during induction of disease or after disease onset (Table II). In the absence of the inhibitor in vitro, spleen cells from all animals proliferated and secreted proinflammatory cytokines, regardless of whether they were taken from healthy or diseased animals treated with I40 or from PBS-treated control animals with EAE. Furthermore, spleen cells from all mice examined induced typical relapsing EAE after transfer into naive recipients. We conclude that potentially encephalitogenic cells were still present even in animals that had never developed EAE signs. These cells can be reactivated in vitro and produce disease in vivo, suggesting that even after a prolonged period of in vivo treatment with I40 these cells may eventually become susceptible again to antigenic activation in vivo.

T cell priming occurs during DP IV inhibition in vivo

We finally asked whether DP IV inhibition in vivo interferes with Ag-specific priming responses. Mice were treated with I40 or PBS on the day of immunization with MBP in CFA and again 48 h later. LNC from both groups of mice proliferated equally well to MBP on secondary stimulation suggesting similar priming efficacy (Fig. 6). These data suggest that during and after treatment with I40, a disease perpetuation and/or exacerbation may be possible via epitope spreading which can be considered as an endogenous immunization by released myelin Ags (46)

Discussion

The present study in the prototypical T cell-mediated autoimmune disease, EAE, demonstrates a critical role for CD26/DP IV in the modulation of effector functions of CD4+ T lymphocytes. Signs of EAE were partially suppressed by administration of the specific CD26/DP IV inhibitor I40 in vivo, in a preventive as well as in a therapeutic manner. The reduction of clinical signs was associated with reduced CNS inflammation. This result raises two major questions. 1) Is this a specific and nontoxic effect, mediated by

Table II. Encephalitogenic T cells are not eliminated by I40 treatment in vivo

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Scorea</th>
<th>Δ cpm</th>
<th>TNF-α  (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>EAE in Recipients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Incidence</td>
</tr>
<tr>
<td>1</td>
<td>I40</td>
<td>0</td>
<td>111,376</td>
<td>518</td>
<td>11,500</td>
<td>2/2</td>
</tr>
<tr>
<td>2</td>
<td>I40</td>
<td>0</td>
<td>82,050</td>
<td>517</td>
<td>11,425</td>
<td>2/2</td>
</tr>
<tr>
<td>3</td>
<td>I40</td>
<td>3</td>
<td>104,684</td>
<td>643</td>
<td>11,871</td>
<td>2/2</td>
</tr>
<tr>
<td>4</td>
<td>I40</td>
<td>4</td>
<td>36,471</td>
<td>236</td>
<td>6,817</td>
<td>2/2</td>
</tr>
<tr>
<td>5</td>
<td>Controls</td>
<td>4</td>
<td>43,895</td>
<td>368</td>
<td>10,671</td>
<td>1/2</td>
</tr>
<tr>
<td>6</td>
<td>Controls</td>
<td>3</td>
<td>69,877</td>
<td>795</td>
<td>9,880</td>
<td>2/2</td>
</tr>
<tr>
<td>7</td>
<td>I40</td>
<td>0</td>
<td>42,535</td>
<td>424</td>
<td>6,103</td>
<td>2/2</td>
</tr>
<tr>
<td>8</td>
<td>I40</td>
<td>0</td>
<td>64,879</td>
<td>550</td>
<td>5,780</td>
<td>2/2</td>
</tr>
<tr>
<td>9</td>
<td>Controls</td>
<td>2</td>
<td>141,534</td>
<td>695</td>
<td>9,031</td>
<td>2/2</td>
</tr>
<tr>
<td>10</td>
<td>Controls</td>
<td>4</td>
<td>51,751</td>
<td>955</td>
<td>11,633</td>
<td>2/2</td>
</tr>
</tbody>
</table>

a Spleens were obtained from 10 recipient mice used for in vivo treatment experiments. In parallel cultures for spleen cells from individual mice, the cells were stimulated with 25 μg/ml MBP. After 96 h, 3 × 10^6 spleen cells were transferred i.p. into each of two naive mice per donor spleen. Supernatants were derived from the same cultures and analyzed by ELISA (SD of duplicate measurements usually <5%). Proliferation assays over 96 h were performed in parallel with results given after subtraction of background counts (SD of triplicates usually <10%). Mice 1–6 were sacrificed on day 38 from an experiment with daily I40 treatment from days 0 to 21 (twice daily from days 7 to 19). For mice 7–10, treatment was started on the first day of clinical disease and continued until day 19 as shown in Fig. 1B. Spleens were taken on day 31.
b Maximal clinical score of the individual donor mice.

* Mean day of onset and mean maximal score (MMS) from two recipient mice.
targeting DP IV activity in vivo? 2) What mechanism/s may account for it?

Is the treatment effect DP IV specific? Several lines of evidence suggest that DP IV is the specific target of I40 in vitro as well as in vivo during the treatment of EAE. 1) We treated mice with Lys[Z(NO2)]-OH, a truncated form of I40 that lacks the pyrroliidine moiety, rendering it noninhibitory in vitro. This substance did not have a significant effect on either the early clinical course of EAE or the DP IV activity and latent TGF-β1 secretion in spinal cord (Table I). 2) We observed an increase in serum DP IV activity in control mice during the first 10 days after the transfer of encephalitogenic T cells that did not occur in I40-treated mice (Fig. 3). Our conclusion is further supported by a recent study in a rat transplantation model (47). Treatment with the irreversible DP IV inhibitor prodipine prevented the increase in serum DP IV normally seen during the first days after cardiac allotransplantation. Both allograft rejection and the concomitant increase of DP IV activity in transplant tissue were delayed (47). In another study, arthritis signs in rats could be suppressed by several biochemically distinct DP IV inhibitors, including I49, indicating that the effect was very likely to be due to the specific inhibitory effect on DP IV (48). In vitro, most studies found a correlation between the level of DP IV activity and the effect of DP IV inhibitors on cellular activation (reviewed in Ref. 20).

The inhibition seen in vitro is not a toxic effect toward T cells. After 96 h of coculture with I40, an increased percentage of trypan blue-staining T cells was only observed at a concentration of 50 μM or higher. The inhibitory effects on proliferation and cytokine production, however, were already seen at lower concentrations (IC50 ~10 μM). The up-regulation of TGF-β1 was even maximal at concentrations of ≤10 μM. Moreover, the antiproliferative effect of 10 μM I40 on an autoreactive T cell line was completely blocked by a neutralizing anti-TGF-β1 Ab. Various techniques to determine cell viability or apoptosis induction, respectively, have previously shown that these inhibitors do not adversely affect cell viability in inhibitory doses up to 10 μM (19). Three further observations exclude depletion of lymphocytes in vivo as a major mechanism of peripheral tolerance under effective treatment conditions: 1) T cells may be primed during I40-treatment (Fig. 6); 2) autoreactive T cells capable of transferring EAE after stimulation in vitro were recovered from mice that were previously injected with encephalitogenic T cells and treated with I40 (Table II); 3) animals developed EAE weeks after treatment with I40 had been stopped (Fig. 1A).

What is the mechanism of autoimmune disease suppression? We suggest that the protective effect of DP IV inhibition is caused by a modulation of T cell effector function. In a biochemical assay, we found DP IV activity on the cell surface of all autoreactive T cell clones examined, on resting as well as on activated T cells. The DP IV inhibitors I40 and I49 had strong antiproliferative effects in vitro on the T cell clones examined, on both an encephalitogenic Th1 clone and a nonencephalitogenic Th2 clone (data not shown). I40 and I49 also suppressed the proliferation of LNC and, importantly, their secretion of TNF-α and, to a lesser extent, IFN-γ. These data confirm and extend earlier reports showing that DNA synthesis and the secretion of IL-2, IL-6, and IL-10 from mouse spleen cells and thymocytes (33) are suppressed by DP IV inhibitors. Likewise, in human T cells proliferation and the secretion of various cytokines including TNF-α and IFN-γ were suppressed (34, 35). It is widely accepted that EAE can be mediated by Th1 CD4+ T cells typically secreting IFN-γ, TNF-α, and lymphotoxin. Various therapeutic approaches that target Th1 cytokines in vivo have been found to be effective in EAE (reviewed in Refs. 7–9 and

FIGURE 5. I40 suppresses the proliferation and secretion of proinflammatory cytokines in cultures of MBP-specific LNC. MBP-specific LNC were obtained and cultured as described in Materials and Methods over 96 h, with 25 μg/ml MBP and various concentrations of I40. Wells without inhibitor and without MBP (ø) served as controls. A. The proliferation of LNC is shown as the mean ± SD of quadruplicate cultures. The results are representative of four independent experiments. B. Secretion of TNF-α as determined by ELISA. The results (means of duplicates) are representative of four independent experiments. C. The inhibitory effect on T cell proliferation is neutralized by anti-TGF-β1. A PLP139–151-specific T cell line was antigenically stimulated and incubated with various concentrations of I40 and/or 10 μg/ml of either anti-TGF-β1 or control Ab as described in Materials and Methods. The proliferation of quadruplicate cultures (SD ≤20%) after 72 h is shown as percent of the counts in the respective control conditions which are normalized to 100%.

FIGURE 6. I40 treatment in vivo does not suppress the Ag-specific priming response. Six mice per group were treated with either 0.5 mg I40 or PBS i.p. on days 0 and 2 with regard to immunization with MBP as described in Materials and Methods. LNC from lymph nodes harvested on day 10 were incubated for 96 h with varying concentrations of MBP as described above. LNC proliferation is shown as the mean ± SD of quadruplicate cultures. The results are representative of two independent experiments.
TGF-β is a cytokine with powerful immunoregulatory effects (reviewed in Ref. 51). A protective role for TGF-β1 in EAE has been clearly established. The endogenous TGF-β1 production is up-regulated in the CNS and presumably plays a down-modulatory role during the recovery phase of acute EAE (43, 44). Although anti-TGF-β1 Ab treatment in vivo aggravates EAE severity (43, 45, 52–54), TGF-β1 treatment can prevent EAE and suppress disease already established (42, 55). Weiner et al. (45) showed that the protective effect of oral administration of myelin Ags is abrogated by anti-TGF-β1. Oral low dose feeding of myelin Ags induced a specific regulatory and protective population of Th3 cells initiating the infiltrate and by T cells that are attracted and activated during the later stages of lesion formation in a bystander fashion. Not surprisingly, macrophages and microglia, in addition to a majority of T cells, appear to produce TGF-β1 in acute EAE lesions (50). Whether these or other cell types are susceptible to regulation of DP IV remains to be investigated.

TGF-β1 is a cytokine with powerful immunoregulatory effects (reviewed in Ref. 51). A protective role for TGF-β1 in EAE has been clearly established. The endogenous TGF-β1 production is up-regulated in the CNS and presumably plays a down-modulatory role during the recovery phase of acute EAE (43, 44). Although anti-TGF-β1 Ab treatment in vivo aggravates EAE severity (43, 45, 52–54), TGF-β1 treatment can prevent EAE and suppress disease already established (42, 55). Weiner et al. (45) showed that the protective effect of oral administration of myelin Ags is abrogated by anti-TGF-β1. Oral low dose feeding of myelin Ags induced a specific regulatory and protective population of Th3 cells secreting TGF-β1, IL-10, and IL-4 (13, 15). Treatment of myelin Ag-specific T cells in vitro with TGF-β1 suppresses their proliferation, secretion of TNF-α and IFN-γ, and capacity to induce EAE (55). Finally, TGF-β1 has anti-inflammatory effects in situ by suppressing the production of NO, and TNF-α by microglia and of cytokine-induced MHC class II, TNF-α, and ICAM-1 expression by rodent astrocytes (56). A recent study showed that adoptive transfer of activated MBP-specific Th1 clones transduced to secrete latent TGF-β1 delayed and ameliorated EAE signs in mice immunized with PLP (57). Taken together, up-regulation of latent TGF-β1 production by DP IV inhibition may be an important mechanism of autoimmune disease suppression. It may represent TGF-β1-mediated bystander suppression that also appears to be one of the protective mechanisms of oral tolerance (14) and of transduced self-reactive T cells as shown by Chen et al. (57).

The partial inhibition of DP IV activity in vivo and the moderate up-regulation of TGF-β1 may explain why we were not able to completely abrogate EAE by increasing the amount of I40 administered, especially in a therapeutic setting. However, the transduced T cells producing considerably higher amounts of latent TGF-β1 in vitro than our I40-treated LNC did not completely abrogate EAE either (57). Indeed, results from others and our own preliminary data indicate a complex role of DP IV inhibition and TGF-β1-mediated disease modulation, respectively, in acute vs chronic EAE. Different treatment effects have been demonstrated depending on the exact time the animals were exposed to TGF-β1 (52). Furthermore, it appears that TGF-β1-mediated treatment effects are temporally and spatially confined to the inflammatory infiltrate in the CNS, explaining the lack of general immunosuppression during treatment with I40. In particular, DP IV inhibition in vivo did not eliminate encephalitogenic T cells (Table II). In addition, despite the antiinflammatory effects of I40 on LNC in vitro (Fig. 5), incidence and severity of EAE were similar when equal numbers of DP IV inhibitor- and PBS-pretreated lymph node cells were injected into naive mice (data not shown). Finally, DP IV inhibition during antigenic priming did not suppress secondary Ag-specific T cell proliferation (Fig. 6). Because T cell priming is not affected by DP IV inhibition, epitope spreading may still occur which has been involved in the clinical progression of EAE induced with PLP139–151-specific T cells (46, 58). Currently, we are investigating systematically the effects of long term treatment with DP IV inhibitors on chronic disease.

In conclusion, our findings show that the DP IV activity associated with CD26 plays an important role in the activation of autoreactive T cells. Inhibition of DP IV activity in vivo provides a new approach to down-modulate tissue-specific autoimmunity. These results could have important implications with regard to the treatment of human diseases thought to be mediated by T cell-mediated autoimmune mechanisms.

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References


