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TGF- β 1-Mediated Control of Central Nervous System Inflammation and Autoimmunity through the Inhibitory Receptor CD26¹

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The T cell marker CD26/dipeptidyl peptidase (DP) IV is associated with an effector phenotype and markedly elevated in the human CNS disorder multiple sclerosis. However, little is known about the *in vivo* role of CD26/DP IV in health and disease, and the underlying mechanism of its function in CNS inflammation. To directly address the role of CD26/DP IV *in vivo*, we examined Th1 immune responses and susceptibility to experimental autoimmune encephalomyelitis in CD26^{-/-} mice. We show that gene deletion of CD26 in mice leads to deregulation of Th1 immune responses. Although production of IFN- γ and TNF- α by pathogenic T cells in response to myelin Ag was enhanced in CD26^{-/-} mice, production of the immunosuppressive cytokine TGF- β 1 was diminished *in vivo* and *in vitro*. In contrast to the reduction in TGF- β 1 production, responsiveness to external TGF- β 1 was normal in T cells from CD26^{-/-} mice, excluding alterations in TGF- β 1 sensitivity as a mechanism causing the loss of immune regulation. Natural ligands of CD26/DP IV induced TGF- β 1 production in T cells from wild-type mice. However, natural ligands of CD26/DP IV failed to elicit TGF- β 1 production in T cells from CD26^{-/-} mice. The striking functional deregulation of Th1 immunity was also seen *in vivo*. Thus, clinical experimental autoimmune encephalomyelitis scores were significantly increased in CD26^{-/-} mice immunized with peptide from myelin oligodendrocyte glycoprotein. These results identify CD26/DP IV as a nonredundant inhibitory receptor controlling T cell activation and Th1-mediated autoimmunity, and may have important therapeutic implications for the treatment of autoimmune CNS disease. *The Journal of Immunology*, 2007, 178: 4632–4640.

The T cell marker CD26 is a multifunctional cell surface glycoprotein induced upon T cell activation. The CD26 molecule functions as the cell membrane-associated exopeptidase dipeptidyl peptidase (DP)⁴ IV (EC 3.4.14.5), an en-

zyme capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine residues at the penultimate position (1–3).

Many recent reports have demonstrated that CD26/DP IV serves as an important regulator of immune responses by affecting T cell activation, proliferation, and cytokine production (1–8). Subsequently, multiple roles were predicted for CD26/DP IV in leukocyte homing and inflammation. *In vivo* studies measuring CD26/DP IV expression and activity in autoimmune inflammation demonstrated decreased levels of circulating CD26 in human and experimental arthritis, and implicated CD26 in reducing joint inflammation (9). Pointing to a role for CD26 as a regulator of cell migration into tissues, endogenous CD26 expression on donor stem cells and T cell lymphoma cells negatively regulates engraftment (10) and skin homing (11). Based on these findings, it has been concluded that CD26/DP IV may have a protective role in autoimmune inflammation. In contrast, recent reports found elevated numbers of CD26⁺CD4⁺ T cells in peripheral blood and cerebrospinal fluid from patients with multiple sclerosis (MS), and suggested that CD26/DP IV mediates T cell recruitment to airways in a rat asthma model (12–18). Moreover, *in vitro* and *in vivo* studies conducted with potent synthetic inhibitors identified CD26/DP IV as a target for immunosuppressive therapy (4, 19, 20). Due to their suppression of leukocyte functions, synthetic inhibitors of CD26/DP IV demonstrated profound therapeutic effects in several animal models of human diseases, including rheumatoid arthritis, transplantation, and MS (6–8, 21), but the underlying mechanisms are still elusive.

CD26/DP IV interacts *in vivo* and *in vitro* with a number of ligands that function as enzymatic inhibitors. Although T cells can

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⁴ Abbreviations used in this paper: DP, dipeptidyl peptidase; EAE, experimental autoimmune encephalomyelitis; FoxP3, forkhead-winged helix transcription factor; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; TXA2-R, thromboxane A2 receptor; DS, downstream; US, upstream.

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directly interact with ligands through their surface-expressed CD26/DP IV molecule (19, 22, 23), a secreted form of the DP IV enzyme is able to selectively truncate many soluble mediators of immune functions (1–5, 24). Two structurally and functionally distinct groups of ligands for CD26/DP IV have been identified, as follows: 1) bona fide substrates that are hydrolyzed by DP IV, including the chemokines CXCL12 (stromal cell-derived factor-1), CCL5 (RANTES), or substance P or neuropeptide Y (3, 5, 24), and 2) inhibitory ligands that are not cleaved by DP IV, such as the HIV Tat protein and synthetic inhibitors (4, 19).

Although CD26/DP IV-dependent enzymatic inactivation of the proinflammatory chemokine CXCL12 was found to be associated with protection from human and experimental arthritis (9), additional molecular mechanisms have been identified causing immunosuppression through CD26/CD26 ligand interactions. Notably, many ligands engaging membrane CD26/DP IV at the T cell surface can efficiently induce the regulatory cytokine TGF- β 1 in T cells and inhibit T cell activation and DNA synthesis (8, 19, 23). Thus, we and others have shown that inhibition of CD26/DP IV activity on human or mouse T cells by synthetic or naturally occurring inhibitory ligands results in TGF- β 1-mediated suppression of IL-2 and IFN- γ production, as well as in suppression of T cell proliferation in vitro (4, 8, 19, 23).

Taken together, investigations in inflammation and autoimmunity point to a complex role for CD26/DP IV as an inhibitory leukocyte surface receptor that can serve as a target for ligands blocking enzymatic activity and inhibiting T cell activation. Although previous studies suggest a pivotal role for CD26/DP IV in inflammation (6–8, 21), the precise mechanism by which this ectopeptidase achieves its effects in health and disease remains a critical challenge to elucidate, and the focus of this study was to examine the CD26/DP IV function in vivo.

To directly address the role of CD26/DP IV in Th1 cell immune responses and CNS autoimmunity, we studied the effect of CD26 gene disruption in vivo on the development of experimental autoimmune encephalomyelitis (EAE), using CD26 gene-ablated mice on the C57BL/6 background (25). Specifically, we tested the hypothesis that the CD26 molecule plays a critical regulatory role in autoimmune disease by serving as a target for naturally occurring inhibitory ligands that were previously identified, including ubiquitously expressed proteins such as the thromboxane A2 receptor (TXA2-R) (3, 4, 19, 24). By demonstrating systemic deregulation of Th1 immune responses and exacerbation of EAE in mice lacking CD26/DP IV, we provide evidence for a nonredundant role of this molecule as a negative regulator of T cell functions during inflammatory immune responses in vivo. Our data further show that in CD26^{-/-} mice increased EAE severity is due to a deficit in the regulatory mechanisms that are elicited by natural ligands of CD26/DP IV, thus limiting Th1 immune responses in vivo and in vitro via TGF- β 1 production. These results identify CD26/DP IV as an inhibitory receptor controlling effector T cell functions and Th1 cell-mediated autoimmunity.

Materials and Methods

Mice

Homozygous CD26^{-/-} mice on C57BL/6 genetic background and wild-type C57BL/6 mice were bred in the animal facilities of the Institute of Immunology (25). In all experiments, adult, 8- to 12-wk-old homozygous CD26^{-/-} mice on C57BL/6 genetic background and wild-type C57BL/6 mice were used. Animal breeding and experiments were approved by the local state authorities (IM/G/1/00) and performed according to the institutional guidelines.

Peptides

Myelin oligodendrocyte glycoprotein (MOG) peptide (p)35–55, corresponding to mouse sequence (MEVGWYRSPFSRVVHLYRNGK), 2W-Tat(1–9), and TXA2-R(1–9) was synthesized on a peptide synthesizer by standard 9-fluorenylmethoxycarbonyl chemistry, and purified by HPLC. H-Gly-Pro-Ala-OH and H-Ala-Pro-Gly-OH were purchased from Bachem.

PCR screening of mice

Tissue from tail tips of mice was collected for DNA preparation, followed by a crude DNA preparation using the DNeasy kit (Qiagen), according to manufacturer's instructions. The animals were screened by PCR using a three-primer system (Biotez), as follows: CD26 sense, 5'-GTA GAT GCA TCC AAT GAC CC-3'; CD26 antisense, 5'-ACA TTA GTT GTT AGC GCA AGG CG-3'; and PGK1 prom antisense, 5'-ACT TGT GTA GCG CCA AGT GC-3' (25).

Induction of active EAE and clinical evaluation

Active EAE was induced in 8- to 12-wk-old mice by immunization with MOG p35–55 in CFA (Sigma-Aldrich). A total of 200 μ g of MOG p35–55 peptide and 800 μ g of killed *Mycobacterium tuberculosis* (Difco Laboratories) was emulsified in CFA and injected s.c. by means of four injections over the flanks. In addition, 200 ng of pertussis toxin (List Biological Laboratories) dissolved in 200 μ l of PBS was injected i.p. at the day of immunization and again the day after.

Mice were monitored daily for clinical signs of EAE, and scored according to the following criteria: 0, no signs of disease; 0.5, partial tail weakness; 1, limp tail; 1.5, limp tail and slight slowing of righting; 2, partial hind limb weakness and/or marked slowing of righting; 2.5, dragging of hind limb(s) without complete paralysis; 3, complete paralysis of at least one hind limb; 4, severe forelimb weakness; 5, moribund or dead. Daily clinical scores were calculated and presented as the average (mean) and SEM of all individual disease scores in each group. Statistical comparison of disease severity by clinical score was accomplished by calculating the median clinical score for each mouse from day 8 to day 38 and performing a Wilcoxon analysis (26).

Histology

Selected mice were perfused with 20 ml of cold PBS on day 14 after immunization. Brains and spinal cords were extracted, fixed in 4% (w/v) paraformaldehyde, and embedded in paraffin. Sections were stained with H&E (American Histolabs). Brain, thoracic, and lumbar spinal cord sections were evaluated, and meningeal, parenchymal, and total numbers of inflammatory foci were determined by an examiner blinded to the treatment status of the animal.

Proliferation assays

Lymphocytes and splenocytes were obtained from naive mice or from mice on day 11 or 12 after immunization with MOG p35–55 in CFA by separation of draining lymph nodes or spleen using cell strainers (Falcon). Cells were washed twice in serum-free AIM-V culture medium (Invitrogen Life Technologies) and resuspended in AIM-V culture medium supplemented with 10^{-5} M 2-ME (Merck). For proliferation assays, splenocytes (3×10^5 cells/well) and lymph node cells (2.5×10^5 cells/well) were plated in triplicate cultures in 96-well microtiter culture plates (Falcon). MOG p35–55 was added to final concentrations of 0.5, 5, and 50 μ g/ml PWM to final concentrations of 0.25, 0.5, and 1 μ g/ml, whereas equivalent amount of medium was added to control cultures. In defined experiments, splenocytes (3×10^5 cells/well) and lymph node cells (2.5×10^5 cells/well) were incubated with 50 μ g/ml MOG p35–55 in presence of increasing concentrations of TGF- β 1 (0, 150, 310, 620, 1250 pg/ml; R&D Systems). Culture plates were incubated for 72 h at 37°C. Proliferation was assessed by measuring [3 H]thymidine incorporation added at 0.2 μ Ci/well for the last 16 h. At the end of the incubation period, cells were harvested and radioisotope incorporation was measured as an index of lymphocyte proliferation in a betaplate liquid scintillation counter (Wallac).

Cytokine measurements

For determination of cytokine secretion, lymph node cells or splenocytes were cultured in AIM-V medium supplemented with 10^{-5} M 2-ME. Cells were stimulated with MOG p35–55 (0.5, 5, or 50 μ g/ml). Cell culture supernatants were harvested after 48 h and stored at -70°C until cytokine determination. TNF- α , IFN- γ , IL-2, IL-4, IL-10, and TGF- β 1 concentrations of cell culture supernatants were determined with specific ELISA (R&D Systems), according to the manufacturer's instructions.

In defined experiments, splenocytes of naive mice (3×10^5 cells/well) were incubated in presence and absence of PWM (1 μ g/ml) and of ligand

inhibitor peptides of CD26/DP IV (2W-Tat(1–9), TXA2-R(1–9); 20 μ M) or of substrates of CD26/DP IV (H-Gly-Pro-Ala-OH, H-Ala-Pro-Gly-OH; 100 μ M). In these experiments, cell culture supernatants were harvested after 24 h, and TGF- β 1 concentrations of cell culture supernatants were determined with the specific TGF- β 1 ELISA.

Blood sample collection and TGF- β 1 measurements

Heart blood was collected from CD26 $^{-/-}$ and wild-type mice on day 14 after immunization with MOG p35–55. Citrate at a concentration of 0.105 M was used as an inhibitor of coagulation. Platelet-poor plasma samples were prepared by a three-step centrifugation procedure (700 \times g for 5 min, 1000 \times g for 5 min, and 1300 \times g for 15 min). The plasma samples were stored at -70°C . Plasma concentrations of latent TGF- β 1 were determined with a specific TGF- β 1 ELISA (R&D Systems), according to the manufacturer's instructions.

RNA preparation

Total RNA from splenocytes was prepared using the RNeasy Mini kit (Qiagen). Contaminating DNA was removed by DNase I digestion.

Quantitative RT-PCR

cDNA was generated from 1 μ g of total RNA, and 1/20th of the cDNA mixture was used for quantitative RT-PCR in an iCycler (Bio-Rad). A typical 25 μ l reaction mixture contained 12.5 μ l of HotStarTaq Master Mix (Qiagen), 0.3 μ l of a 1/1000 dilution of SYBR Green I (Molecular Probes), and 0.5 μ mol of the following specific primers: forkhead-winged helix transcription factor (FoxP3)-upstream (US), 5'-TGTCAG TCAACTTCACCAAG-3'; FoxP3-downstream (DS), 5'-AGCTGGTG CATGAAATGTGG-3'; IL-10-US, 5'-GCCTAACATGCTTCGAGA TC-3'; IL-10-DS, 5'-TGATGTCTGGGTCTTGGTTC-3'. An initial denaturation/activation step (15 min, 95°C) was followed by 40 PCR amplification cycles (30 s, 95°C ; 30 s, 58°C ; 45 s, 72°C). The amounts of mRNA were normalized to ribosomal protein large PO (PO-US, 5'-GCACTTTCGCTTCTGGAGGGTGT-3'; PO-DS, 5'-TGACTTGGTT GCTTTGGCGGGATT-3') and analyzed using a one-way ANOVA.

Statistical analysis

Statistical comparison of EAE disease severity was accomplished by performing a Wilcoxon analysis. Statistical analyses of cell proliferation and cytokine production assays were performed using unpaired two-tailed Student's *t* test.

Results

Increased severity of EAE in CD26 $^{-/-}$ mice

To study the role of CD26 *in vivo*, we induced EAE in CD26 $^{-/-}$ and wild-type (CD26 $^{+/+}$) mice. When EAE developed following immunization with MOG p35–55, both wild-type and knockout mice showed paralytic signs of the disease (Fig. 1A). Interestingly, CD26 $^{-/-}$ mice developed significantly higher disease severity than wild-type mice. The mean day of onset of clinical signs in CD26 $^{-/-}$ mice was day 11.0 ± 2.83 , whereas the maximal mean severity score was 3.48 ± 1.13 . In the wild-type group, EAE was consistently less severe, with a later onset of disease (mean onset on day 13.22 ± 3.77 ; $p = 0.0169$) and a lower maximal mean severity score of 2.26 ± 1.01 . Moreover, the difference in the median clinical scores between both groups was statistically significant ($p = 0.048$) as shown by the nonparametric Wilcoxon two-sample test (26).

Histopathologic analysis showed an increased number of meningeal, parenchymal, and total inflammatory foci in CD26 $^{-/-}$ mice compared with wild-type (CD26 $^{+/+}$) mice (Fig. 1B). The differences in the number of foci between the two groups were statistically significant, with probability levels of $p = 0.0003$ (meningeal), 0.0097 (parenchymal), and 0.0023 (total).

Enhanced proliferation and production of Th1 cytokines associated with reduced TGF- β 1 production in MOG-primed T cells from CD26 $^{-/-}$ mice

To address the immunological mechanisms that lead to enhanced disease in CD26 $^{-/-}$ mice, we analyzed the proliferative response

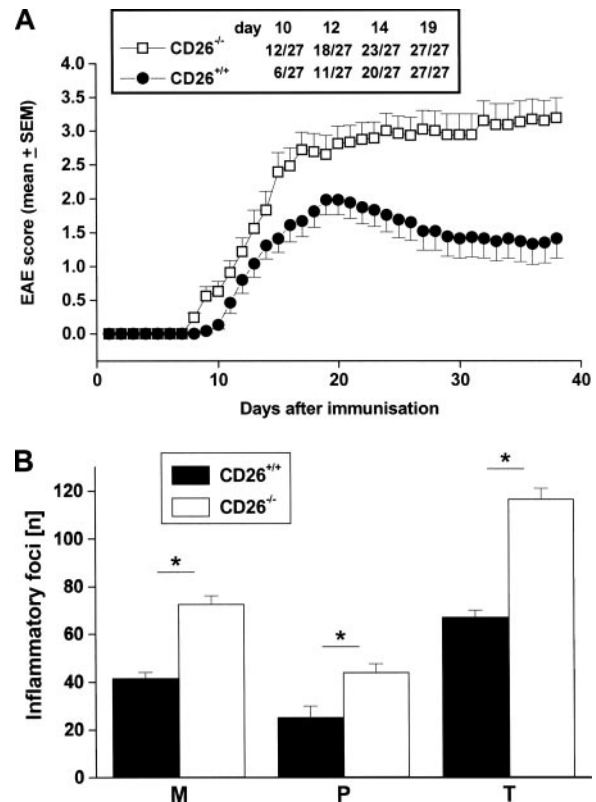
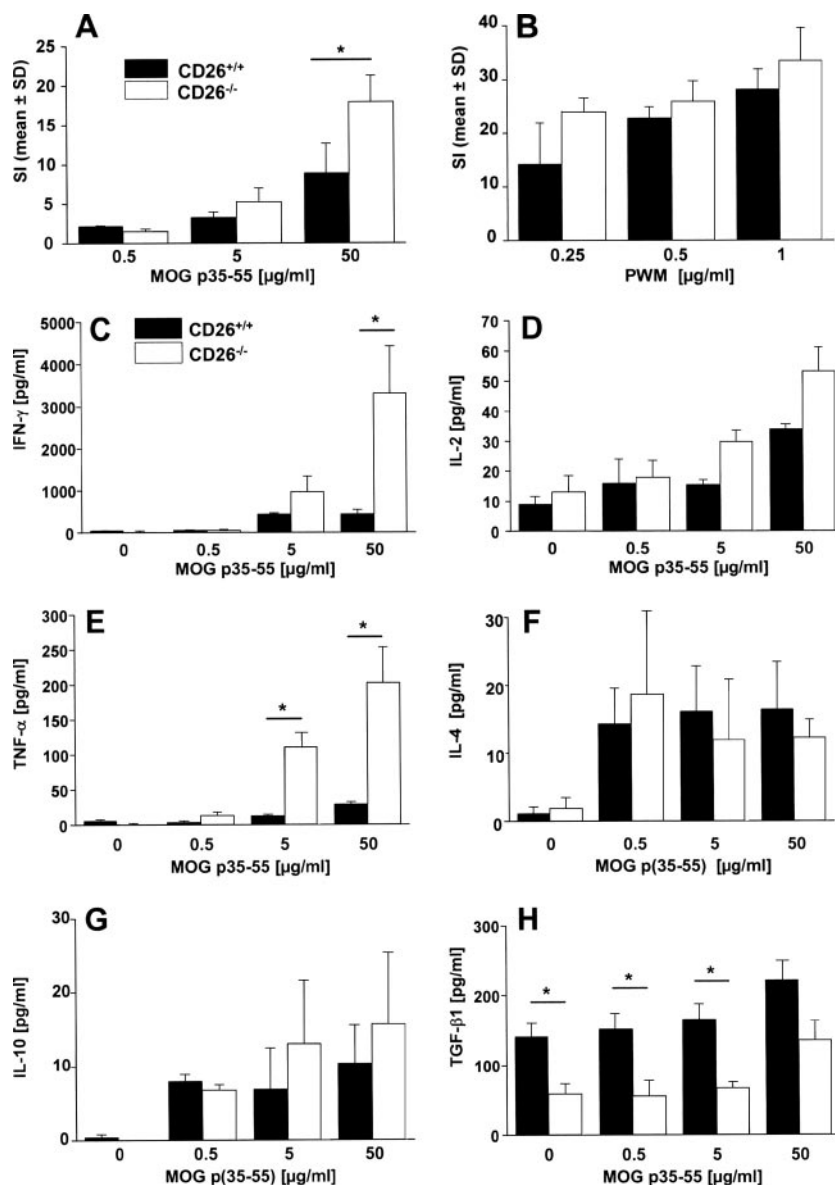


FIGURE 1. CD26 $^{-/-}$ mice show significantly higher clinical severity scores of EAE compared with wild-type mice. **A**, Disease course of EAE (mean score \pm SEM) in wild-type (CD26 $^{+/+}$) and CD26 $^{-/-}$ mice is plotted from day 0 to day 38 (each group $n = 27$ combined from three independent experiments; $p = 0.048$, CD26 $^{+/+}$ vs CD26 $^{-/-}$ mice). *Inset*, Shows incidence of EAE in the experimental groups. **B**, Histopathologic analysis revealed an increased number of meningeal (M), parenchymal (P), and total (T) inflammatory foci in CD26 $^{-/-}$ mice compared with wild-type (CD26 $^{+/+}$) mice. Data represent mean number of inflammatory foci \pm SEM. The differences in the number of foci between the two groups (each $n = 4$) were statistically significant with probability levels of $p = 0.0003$ (M), $p = 0.0097$ (P), and $p = 0.0023$ (T).

(DNA synthesis) to MOG p35–55 in MOG-primed T cells from wild-type and CD26 $^{-/-}$ mice. Briefly, T cells of regional draining lymph nodes from CD26 $^{-/-}$ mice and wild-type mice were isolated on day 11 or 12 following immunization with MOG p35–55. Cells were cultured with MOG p35–55, and proliferation was measured after 72 h. Compared with wild-type mice, MOG p35–55-immunized CD26 $^{-/-}$ mice showed an increased proliferative response of T cells when activated with MOG p35–55 *in vitro* (Fig. 2A). Concerning the proliferation of PWM-stimulated lymph node cells of wild-type CD26 $^{+/+}$ and CD26 $^{-/-}$ mice, no significant differences were found (Fig. 2B).

Th1 cells producing TNF- α , IFN- γ , and IL-2 are implicated in inflammatory pathologies, whereas the immunosuppressive cytokine TGF- β 1 has been shown to limit Th1 responses. To analyze the mechanism underlying increased EAE in CD26 $^{-/-}$ mice, we determined the amounts of Th1 cytokines produced by MOG p35–55-primed T cells from CD26 $^{+/+}$ wild-type and CD26 $^{-/-}$ mice. T cells were isolated from regional draining lymph nodes following immunization with MOG p35–55 and cultured for 48 h in the presence of increasing concentrations of MOG p35–55. Levels of IFN- γ , IL-2, and TNF- α in these cultures were measured by specific ELISA. As shown in Fig. 2, C–E, enhanced MOG p35–55-induced proliferation was associated with increased production of

FIGURE 2. Proliferative response to MOG p35–55 or PWM and cytokine production in MOG-primed T cells from wild-type (CD26^{+/+}) and CD26^{-/-} mice. **A** and **B**, T cells of regional draining lymph nodes from wild-type (CD26^{+/+}) and CD26^{-/-} mice were isolated on day 11 or 12 following immunization with MOG p35–55. Cells were cultured with the indicated concentrations of MOG p35–55 (**A**) or PWM (**B**) for 72 h, and T cell proliferation in each culture was measured using [³H]thymidine incorporation assay. The cell proliferation is shown as stimulation index (SI; mean \pm SD) of four independent experiments (*, $p < 0.05$, CD26^{+/+} vs CD26^{-/-} mice). [³H]Thymidine incorporation in lymph node cells stimulated with 50 μ g/ml MOG p35–55 was determined as 10,332 \pm 4,590 cpm (CD26^{+/+}) and 45,180 \pm 7,530 cpm (CD26^{-/-}), and in PWM-stimulated lymph node cells as 80,482 \pm 30,130 cpm (CD26^{+/+}) and 122,133 \pm 37,608 cpm (CD26^{-/-}). **C–H**, T cells were isolated from regional draining lymph nodes of wild-type (CD26^{+/+}) and CD26^{-/-} mice on day 11 or 12 following immunization with MOG p35–55. MOG-primed lymphocytes were cultured in presence of different concentration of MOG p35–55. Cell culture supernatants were collected 48 h later. Levels of IFN- γ (**C**), IL-2 (**D**), TNF- α (**E**), IL-4 (**F**), IL-10 (**G**), and latent TGF- β 1 (**H**) in the cultures were measured by specific ELISA. Cytokine production is shown as mean \pm SEM of four independent experiments (*, $p < 0.05$).



Th1 cytokines IFN- γ , IL-2, and TNF- α in culture supernatants of MOG p35–55-primed lymph node cells from CD26^{-/-} mice in comparison with wild-type mice. In contrast to increased levels of Th1 cytokines, MOG-specific production of the Th2 cytokines IL-4 and IL-10 did not differ between T cells from CD26^{-/-} or wild-type mice (Fig. 2, *F* and *G*).

Notably, the production of the immunosuppressive cytokine TGF- β 1 was significantly diminished in culture supernatants of both unstimulated and MOG p35–55-stimulated lymph node cells obtained from MOG-primed CD26^{-/-} animals in comparison with cells from CD26^{+/+} wild-type mice (Fig. 2*H*).

CD26^{-/-} mice show diminished TGF- β 1 production in vivo

These findings led us to hypothesize that enhanced activity of autoreactive Th1 cells from CD26^{-/-} mice may be associated with a lack of TGF- β 1 control in vivo. To test this hypothesis, we next measured systemic plasma levels of TGF- β 1 in these mice. In agreement with data from T cell culture experiments, levels of latent TGF- β 1 were significantly reduced in the plasma of CD26^{-/-} mice immunized with MOG p35–55 compared with wild-type control mice (Fig. 3*A*).

No defect in TGF- β 1 sensitivity in CD26^{-/-} mice

Elevated IL-2 production, hyperreactive T cells, and increased autoimmunity have been observed recently in a number of mutant mouse strains. In these studies, it was demonstrated that MRL/Mp or NOD mouse strains and strains deficient in the E3 ubiquitin ligase Cbl-b or the transcription factors NF-ATc2 and NF-ATc3 show reduced effector T cell sensitivity to suppression by TGF- β 1 or CD4⁺CD25⁺ regulatory T cells (27–31). Therefore, we asked the question whether resistance to TGF- β 1 regulation of pathogenic Th1 cells may contribute to the increased autoimmunity seen in CD26^{-/-} mice. To test TGF- β 1 sensitivity, we studied the influence of external TGF- β 1 on MOG p35–55-specific proliferation of primed splenocytes and lymph node cells from CD26^{-/-} mice. As shown in Fig. 3, *B* and *C*, small amounts of TGF- β 1 are capable of suppressing significantly the enhanced proliferative response of MOG p35–55-stimulated splenocytes (Fig. 3*B*) and lymph node cells (Fig. 3*C*) from CD26^{-/-} mice. An amount of 150 pg/ml TGF- β 1 was capable of decreasing the proliferation of CD26^{-/-} T cells up to 40%. These results are comparable to TGF- β 1-mediated antiproliferative effects on wild-type T cells (Fig. 3, *B* and *C*).

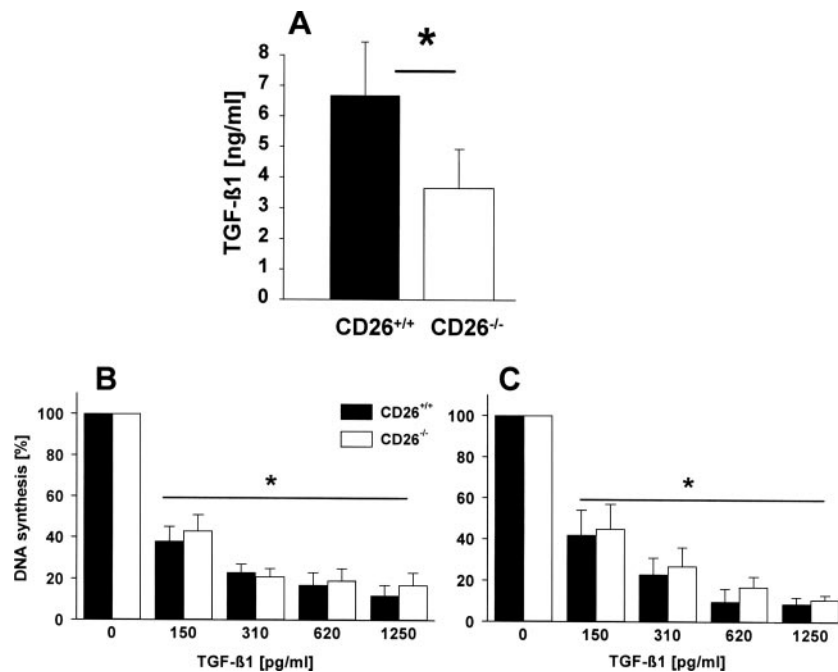


FIGURE 3. TGF- β 1 concentrations in plasma of wild-type (CD26^{+/+}) and homozygous mutant (CD26^{-/-}) mice and influence of TGF- β 1 on the proliferation of MOG-primed splenocytes and lymph node cells from CD26^{-/-} mice. **A**, Heart blood was collected from wild-type and homozygous mutant (CD26^{-/-}) mice ($n = 8$ per group) on day 11 or 12 following immunization with MOG p35–55. Platelet-poor plasma samples were prepared, and latent TGF- β 1 concentrations were measured using a specific TGF- β 1 ELISA system (*, $p < 0.05$, CD26^{+/+} vs CD26^{-/-} mice). Splenocytes (**B**) and cells (**C**) from regional draining lymph nodes of wild-type (CD26^{+/+}) and homozygous mutant (CD26^{-/-}) mice ($n = 3$ in each group) were isolated on day 11 or 12 following immunization with MOG p35–55. Cells were cultured with 50 μ g/ml MOG p35–55 in presence of increasing concentrations of TGF- β 1 for 72 h. T cell proliferation in each culture was measured using [3 H]thymidine incorporation assay. The cell proliferation is shown as percentage of DNA synthesis in control cultures without TGF- β 1 (mean \pm SD) of three independent experiments (*, $p < 0.05$, vs control cultures without TGF- β 1). [3 H]Thymidine incorporation in cultures stimulated with 50 μ g/ml MOG p35–55 alone was measured as 23,460 \pm 9,893 cpm (CD26^{+/+}) and 53,825 \pm 14,023 cpm (CD26^{-/-}) in cultures of lymph node cells, and as 10,859 \pm 5,348 cpm (CD26^{+/+}) and 34,245 \pm 13,269 cpm (CD26^{-/-}) in cultures of splenocytes.

We conclude that T cells from CD26^{-/-} mice are fully sensitive to TGF- β 1-mediated suppression of proliferation. Taken together, our data suggest that in T cells from CD26^{-/-} mice, lack of TGF- β 1 production and not alterations in TGF- β 1 responsiveness may be the primary cause of increased Th1 cell proliferation in response to MOG p35–55 challenge.

Induction of TGF- β in T cells from wild-type mice by ligands of CD26/DP IV

To answer the question whether CD26^{-/-} mice exhibit a deficit of TGF- β 1-mediated immune response shut-off mechanisms, we studied the influence of substrates and ligand inhibitor peptides of CD26/DP IV on TGF- β 1 production in unstimulated and PWM-stimulated splenocytes of naive wild-type (CD26^{+/+}) and homozygous mutant (CD26^{-/-}) mice. Splenocytes of wild-type and CD26^{-/-} mice were isolated and cultured unstimulated and stimulated with PWM (1 μ g/ml) in presence of well-characterized ligand inhibitor peptides of CD26/DP IV (2W-Tat(1–9), TXA2-R(1–9)) and of substrates of CD26/DP IV (H-Gly-Pro-Ala-OH and H-Ala-Pro-Gly-OH) (4, 19). Cell culture supernatants were collected after 24 h, and levels of latent TGF- β 1 were measured using a specific TGF- β 1 ELISA system. As shown in Fig. 4, both ligand inhibitor peptides as well as substrates of CD26/DP IV were capable of inducing the production of the TGF- β 1 in unstimulated and PWM-stimulated splenocytes from CD26^{+/+} wild-type mice.

CD26/DP IV ligands fail to induce TGF- β 1 production in cells from CD26^{-/-} mice in vitro

The finding that ligands of CD26/DPIV induce TGF- β 1 production by splenocytes led us to hypothesize that in CD26^{-/-} mice,

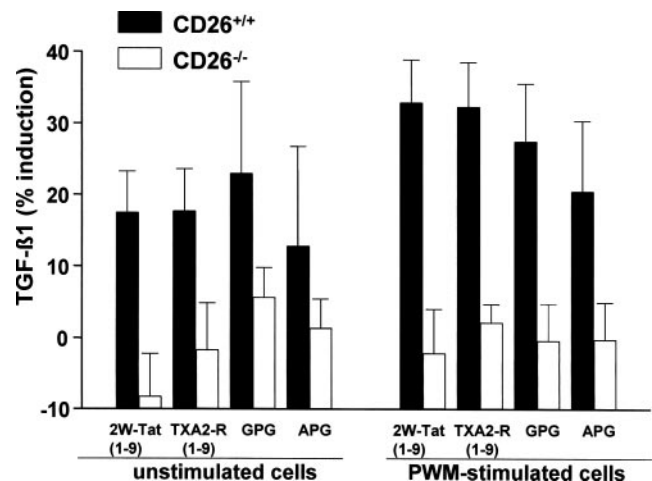


FIGURE 4. Influence of CD26/DP IV ligands on TGF- β 1 production in unstimulated and PWM-stimulated splenocytes of naive wild-type (CD26^{+/+}) and CD26^{-/-} mice. Splenocytes of wild-type (CD26^{+/+}) and of CD26^{-/-} mice were cultured in presence and absence of PWM (1 μ g/ml) and of ligand inhibitor peptides of CD26/DP IV (2W-Tat(1–9), TXA2-R(1–9), 20 μ M) or of substrates of CD26/DP IV (H-Gly-Pro-Ala-OH, GPA; H-Ala-Pro-Gly-OH, APG; 100 μ M). Cell culture supernatants were collected 24 h later. Levels of latent TGF- β 1 in the cultures were measured using a specific ELISA system. TGF- β 1 production is shown as percentage of TGF- β 1 induction (mean \pm SEM) of four independent experiments. The differences between TGF- β 1 production of splenocytes from wild-type and CD26^{-/-} mice were significant ($p < 0.05$) in each case. TGF- β 1 concentrations of unstimulated cultures are 173 \pm 62 pg/ml (CD26^{+/+}) and 183 \pm 42 pg/ml (CD26^{-/-}), and of PWM-stimulated cultures 164 \pm 60 pg/ml (CD26^{+/+}) and 204 \pm 50 pg/ml (CD26^{-/-}), respectively.

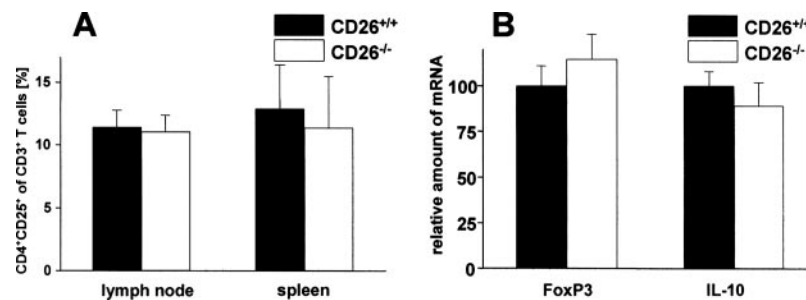


FIGURE 5. CD4⁺CD25⁺ regulatory T cell subsets and activity in wild-type (CD26^{+/+}) and CD26^{-/-} mice with EAE. *A*, Percentage of CD4⁺CD25⁺ of total CD3⁺ T cells in lymph nodes and spleen from wild-type and CD26^{-/-} mice on day 14 following immunization with MOG p35–55 ($n = 4$ per group). *B*, FoxP3 and IL-10 transcript levels quantified by real-time RT-PCR in splenocytes of wild-type (CD26^{+/+}) and CD26^{-/-} mice on day 14 following immunization with MOG p35–55 in CFA ($n = 4$ per group). mRNA expression is shown as relative amount of FoxP3 or IL-10 mRNA (percentage of CD26^{+/+} mice normalized to mRNA of the housekeeping gene ribosomal protein large PO).

the observed reduction in TGF- β 1 production in vitro and in vivo may be caused by the lack of ligand response in CD26^{-/-} mice. Indeed, as results shown in Fig. 4 demonstrate, we were unable to detect an increase in TGF- β 1 production by splenocytes from CD26^{-/-} mice in response to CD26/DP IV ligands.

No numerical change of CD4⁺CD25⁺ T regulatory cells in CD26^{-/-} mice

A possible source of TGF- β 1 could be myelin-specific Th1 cells (Fig. 2*E*) (8). Alternatively, a regulatory T cell subset might provide TGF- β 1 involved in immune regulation through CD26/DP IV. Work in recent years identified naturally occurring and IL-10-producing regulatory T cells as an important mechanism limiting autoimmune inflammation. IL-10 and TGF- β 1 produced by CD4⁺CD25⁺ regulatory T cells are involved in protection from a variety of autoimmune diseases, including EAE (32–35). For these reasons, we analyzed levels of the CD4⁺CD25⁺ regulatory T cell population in spleen and lymph nodes of CD26^{-/-} mice and wild-type mice. Moreover, we measured expression of IL-10 and FoxP3, which is functionally associated with naturally occurring CD4⁺CD25⁺ regulatory T cells. As seen in Fig. 5*A*, the CD4⁺CD25⁺ regulatory T cell subset represents ~10% of the peripheral T cell compartment in both wild-type and CD26^{-/-} mice. Thus, no decrease of naturally occurring CD4⁺CD25⁺ regulatory T cells was seen in lymph nodes or spleen of CD26^{-/-} animals. In addition, there was no evidence of a diminished activity of CD4⁺CD25⁺ regulatory T cells when FoxP3 or IL-10 transcripts of CD26^{-/-} mice were quantified in splenocytes by real-time RT-PCR and expression levels compared with wild-type control mice (Fig. 5*B*). Taken together, these data show no numerical change in CD4⁺CD25⁺ regulatory T cells and argue against a functional perturbation of this cell population.

Discussion

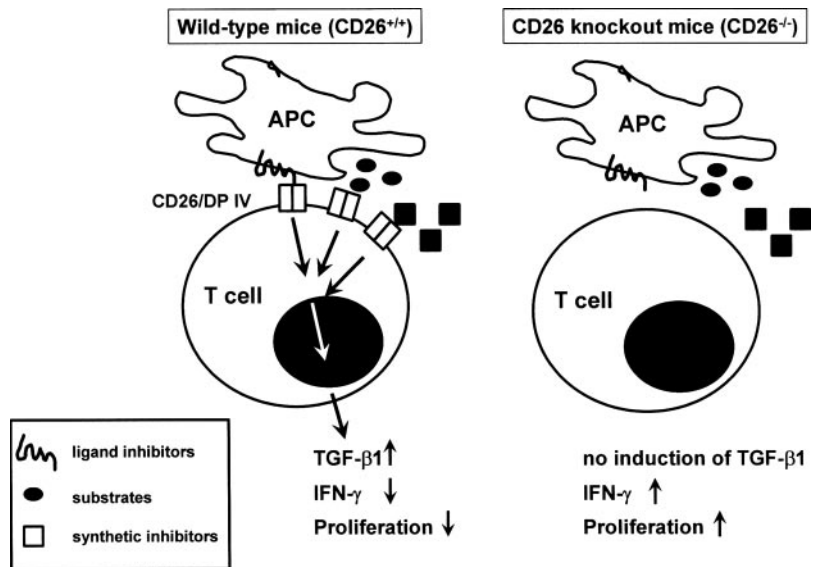
An association between CD26/DP IV and immune cell functions has long been recognized. Inasmuch as previous studies showed protection from EAE by engaging the CD26/DP IV molecule with synthetic inhibitors of its enzymatic DP activity, the question whether this ectopeptidase achieves its effects in EAE as an enzymatic effector molecule or inhibitory cell surface receptor remained a critical challenge to address. In this study, we compared susceptibility to EAE induction between CD26^{-/-} mice backcrossed onto the C57BL/6 background and wild-type control mice. Whereas mice deficient in CD26/DP IV developed more severe EAE, T cell proliferation and IL-2 production in response to the CNS autoantigen MOG p35–55 were significantly increased in lymph node cells from CD26-deficient mice immunized with the

MOG peptide. These data are in line with the previous observation that the severity of Ag-induced arthritis is increased in CD26-deficient mice (9). Based on the disease phenotype revealed in these mice, we conclude that CD26/DP IV acts as a negative regulator molecule in autoimmunity.

The activation of CD4⁺ autoreactive T cells and their differentiation into a Th1 phenotype are thought to be crucial events during the initial steps in the initiation of EAE and probably MS, and these cells may also be important players in the long-term evolution of these disorders (36–38). In the EAE model, the injection of myelin Ags into susceptible animals leads to a Th1 CD4⁺ T cell-mediated autoimmune disease that shares similarities with MS and can be adoptively transferred by encephalitogenic CD4⁺ T cells into a naive animal. Because EAE scores and T cell proliferation were increased in CD26^{-/-} mice, we hypothesized that ablation of the CD26/DP IV gene in vivo results in the reduction of regulation of Th1 cell immune responses, causing enhanced autoimmune disease in the EAE model. Indeed, production of IFN- γ and TNF- α was found to be elevated in cultures of draining lymph node cells from mice immunized with MOG p35–55 in a dose-dependent manner. As these cytokines are associated with the encephalitogenic potency of myelin Ag-specific T cells, we conclude that enhanced Th1 cell reactivity in response to MOG is part of the underlying mechanism causing enhanced disease scores in CD26^{-/-} mice. In contrast, the production of the Th2 cytokines IL-4 and IL-10 was found to be normal in cultures of draining lymph node cells from CD26^{-/-} mice immunized with MOG p35–55 in comparison with wild-type mice.

Previous reports had shown that CD26/DP IV inhibitors suppressed EAE, and that this suppression was associated with the up-regulation of TGF- β in vivo and in vitro (8). It is now well established that TGF- β 1 is a cytokine with powerful immunoregulatory effects in EAE (39). Anti-TGF- β 1 Ab treatment in vivo aggravates EAE severity, whereas TGF- β 1 treatment can prevent EAE and suppress disease (39, 40). Miller et al. (41) showed that the protective effect of oral administration of myelin Ag is abrogated by anti-TGF- β 1. Oral low dose feeding of myelin Ags induced a specific regulatory and protective population of Th3 cells secreting TGF- β 1, IL-10, and IL-4 (42, 43). Notably, treatment of myelin Ag-specific T cells in vitro with TGF- β 1 suppresses their proliferation, secretion of TNF- α and IFN- γ , and capacity to induce EAE. A recent report demonstrates that both CD4⁺CD25⁺ and CD4⁺TGF- β latency-associated peptide⁺ regulatory T cells mediate recovery from EAE in SJL mice in which TGF- β plays an important role (44). These regulatory T cells inhibited proliferation of encephalitogenic T cells to autoantigen or anti-CD3 Ab stimulation. Depletion of CD4⁺CD25⁺ T cells during the recovery

FIGURE 6. Working model of the hypothetical function of CD26/DP IV on immune cells.



phase exacerbated disease, resulted in the expansion of Ag-specific T cells, and enhanced IFN- γ production in a TGF- β -dependent fashion. In addition, TGF- β was shown to be involved in the recovery from EAE because the percentage of CD4⁺ cells expressing TGF- β latency-associated peptide on the cell surface increased significantly in blood and spleen of EAE-recovered mice as compared with the naive mice and in vivo neutralization of TGF- β abolished recovery from disease (44).

Because EAE scores were significantly increased in CD26^{-/-} mice, and in vitro, T cell proliferation as well as the production of Th1 cytokines were enhanced when T cells from CD26^{-/-} mice were stimulated with MOG p35–55, we hypothesized that CD26^{-/-} mice produce less TGF- β both in vivo and in response to Ag stimulation in vitro.

Indeed, we found that increased EAE severity seen in CD26^{-/-} mice was associated with a significant reduction in TGF- β 1 production in vivo and in vitro in response to MOG p35–55. Consistent with our hypothesis, reconstitution of TGF- β 1 at concentrations seen in wild-type cultures profoundly reduced proliferation of MOG p35–55-specific T cell from CD26^{-/-} mice, indicating that CD26-negative T cells are fully susceptible to TGF- β 1 control at a level comparable to cells from wild-type mice. We conclude that lack of T cell responsiveness to TGF- β 1 (27–31) does not cause the enhanced autoimmunity seen in CD26^{-/-} mice. Thus, our results suggest that enhanced susceptibility to EAE induction and deregulated Th1 immunity in CD26^{-/-} mice are associated with a defect in TGF- β 1 production in response to challenge with autoantigen, and that this lack of intrinsic TGF- β 1 release may cause exacerbation of EAE.

Why might CD26/DP IV be involved in TGF- β 1-mediated protection from EAE? Previously, we and others reported results from therapeutic studies with potent synthetic CD26/DP IV inhibitors that were initiated in immune disorders (6–8, 21). The rationale to target CD26/DP IV in diseases with an autoimmune pathogenesis is based on results from previous studies showing elevated numbers of CD26⁺ CD4⁺ T cells present in peripheral blood and cerebrospinal fluid from patients with MS (13–18). In addition, myelin-specific CD4⁺ T cell clones from patients with MS express high levels of surface CD26/DP IV (45). Thus, CD26/DP IV appears to be a T cell activation marker that is elevated in MS. Recently, we addressed the role of CD26/DP IV in mouse EAE, an animal model for MS (36–38), and demonstrated that clinical and histopatho-

logical signs of the disease can be suppressed by CD26/DP IV inhibition in vivo (8). We used the reversible DP IV inhibitor Lys[Z(NO₂)]-pyrrolidide to dissect the role of DP IV in EAE and to explore the therapeutic potential of DP IV inhibition for autoimmunity. Lys[Z(NO₂)]-pyrrolidide administration in vivo decreased and delayed clinical and neuropathological signs of adoptive transfer EAE. We showed that Lys[Z(NO₂)]-pyrrolidide blocked DP IV activity in vivo and increased the secretion of the immunosuppressive cytokine TGF- β 1 in spinal cord tissue and plasma during acute EAE. In vitro, while suppressing autoreactive T cell proliferation and TNF- α production, Lys[Z(NO₂)]-pyrrolidide consistently up-regulated TGF- β 1 secretion. A neutralizing anti-TGF- β 1 Ab blocked the inhibitory effect of Lys[Z(NO₂)]-pyrrolidide on T cell proliferation to myelin Ag. CD26/DP IV inhibition in vivo was not generally immunosuppressive, neither eliminating encephalitogenic T cells nor inhibiting T cell priming (8). Suppression of Th1 memory cell effector functions, most likely through TGF- β 1 production, was thus identified as a mechanism by which CD26/DP IV inhibition attenuates EAE.

These findings from in vitro and in vivo studies show that targeting CD26/DP IV enzymatic activity with synthetic inhibitors attenuated autoimmune disease by an acute TGF- β 1-mediated immunosuppression of CD26-expressing T cells (4, 8, 19, 23), but did not address the intrinsic role of CD26/DP IV during inflammatory immune responses in vivo. The ability to block autoimmunity by inhibition of the external enzymatic activity of DP IV initially led to the hypothesis that CD26/DP IV may act as an effector molecule in immune responses. However, this possibility is unlikely in view of recent reports indicating that CD26/DP IV negatively regulates leukocyte homing and experimental rheumatoid arthritis (9, 10).

The present study fills the gap of mechanistic studies by defining the role of CD26 in CNS autoimmunity using CD26^{-/-} mice. Our data clearly demonstrate that expression of CD26/DP IV is not necessary for autoimmune inflammation to occur. In contrast, we identify CD26/DP IV as a nonredundant negative regulator of T cell functions. These data are consistent with a recent report demonstrating that deficiency of DP IV/CD26 in mice results in changes of T cell cytokine secretion, including an increase of IFN- γ , without further addressing the mechanism (46).

Because many naturally occurring CD26/DP IV ligands act as partial enzymatic inhibitors, we hypothesized that membrane-bound CD26/DP IV acts as a constitutive and nonredundant inhibitory receptor of effector T cell activation and autoimmune responses by interacting with CD26/DP IV ligands. It was shown that in wild-type mice, proteins and peptides that are part of the constitutive biological repertoire bind to CD26/DP IV and function as endogenous DP IV inhibitors (3, 4, 19). Several such ligands of CD26/DP IV, including naturally occurring inhibitors, have been identified by us and others (3–5, 19, 24). Surprisingly, we found that these natural ligands of CD26/DP IV act in an immunosuppressive fashion, much like synthetic inhibitors of CD26/DP IV. Thus, we report in this study that inhibitors of CD26/DP IV include ligand peptides such as 2W-Tat(1–9) or TXA2-R(1–9) and CD26/DP IV substrates such as H-Gly-Pro-Ala-OH or H-Ala-Pro-Gly-OH. Consistent with our underlying hypothesis, we found that these ligands act only on T cells from wild-type, but not CD26^{−/−} mice by induction of TGF-β1. We conclude that these naturally occurring ligands may control inflammation via continuous TGF-β1-mediated suppression of Th1 immunity, whereas CD26^{−/−} mice lack this regulatory loop (Fig. 6).

Although our data did not indicate significant changes in numbers of CD4⁺CD25⁺ regulatory T cells in CD26^{−/−} mice, the role of this cell population in the control of autoimmunity through the CD26/DP IV receptor remains to be further investigated.

Negative regulators of T cell activation and autoimmune responses have previously been identified (47), including CTLA-4, an inhibitory TCR that competes with CD28 for CD80/CD86 ligands and thus increases the threshold for T cell activation or delivers inhibitory biochemical signals that actively block signals from the TCR and/or CD28 (48, 49). Interestingly, CTLA-4^{−/−} T cells are capable of proliferating spontaneous in vitro and secreting increased amounts of cytokines, including IFN-γ upon TCR engagement compared with normal mice. Moreover, CTLA-4^{−/−} mice develop spontaneous autoimmunity (48, 49).

Although the precise signals delivered by CTLA-4 and their level of action remain uncertain, CTLA-4 signaling down-regulates cytokine production and regulates cell proliferation (50–52). In contrast to CD26/DP IV signaling, TGF-β1 induction has not been clearly observed in response to CTLA-4 engagement. Besides distinct signaling pathways and inhibitory mechanisms, one of the major differences between negative regulation of T cell responses through CTLA-4 and CD26/DP IV lies in the multitude of potential inhibitory ligands available for interaction with the latter, including TXA2-R(1–9) (3, 4, 19, 24). Notably, TXA2-R, a G protein-coupled receptor through which the arachidonic acid metabolite thromboxane A2 exerts its effects, was identified as a CD26/DP IV ligand (19). Thus, TXA2-R(1–9), the N-terminal nonapeptide of TXA2-R, exerts significant inhibitory effects on CD26/DP IV activity and on DNA synthesis as well as IL-2 production of Ag-stimulated PBMC. Moreover, TXA2-R(1–9) induces production of TGF-β1 (19). These data identified the N-terminal part of TXA2-R as an endogenous inhibitory ligand of CD26/DP IV that modulates T cell activation via CD26/DP IV inhibition. In line with data presented in this study, it was shown that Ag-specific immune responses are enhanced in vivo in TXA2-R-deficient mice, which also develop marked lymphadenopathy with age (20). Thus, it was established that the thromboxane-TXA2-R axis negatively regulates T cell activity. In light of our results, lack of an ubiquitous inhibitory ligand for CD26/DP IV could enhance the immune dysregulation observed in TXA2-R^{−/−} mice.

Because of its potentially important role in leukocyte migration and inflammation, CD26 is a molecule of great interest to clinical

science and medicine. In this study, we observed a deregulation of the autoimmune process in EAE, the prototypic model of an autoimmune-mediated CNS disorder. In summary, our results demonstrate a nonredundant role of the CD26/DP IV molecule in regulating Th1 autoimmunity, presumably through release of TGF-β1 via naturally occurring CD26/DP IV ligands that have been previously identified (3, 4, 19). Thus, up-regulation of CD26/DP IV during effector T cell activation may render pathogenic T cells more susceptible to autocrine suppression by TGF-β1 (Fig. 6). Externally applied synthetic inhibitors of CD26/DP IV use this pathway in a therapeutic fashion (8). Taken together, our results support the notion of a critical regulatory role for the CD26/DP IV molecule within the immune system and provide a mechanism for targeting CD26/DP IV with a variety of natural and/or synthetic inhibitory ligands as a promising strategy for immunotherapy.

Disclosures

The authors have no financial conflict of interest.

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