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DP IV/CD26, APN/CD13 and related enzymes as regulators of T cell immunity: implications for experimental encephalomyelitis and multiple sclerosis

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1. ABSTRACT

Multiple sclerosis (MS) is the most frequent demyelinating disease of the central nervous system. Peptidases like dipeptidyl peptidase IV (DP IV, CD26) and aminopeptidase N (APN, CD13) play a regulatory role in T cell activation and represent potential targets for the treatment of inflammatory disorders. Synthetic inhibitors of DP IV and/or APN enzymatic activity induce production of the immunosuppressive cytokine TGF-beta1 and subsequently suppress DNA synthesis and Th1 cytokine production of activated human T cells. Compelling evidence has demonstrated that IL-17-producing CD4 cells (Th17) are a major contributor to the pathogenesis of autoimmune inflammation. Here, we report that inhibitors of DP IV-like activity as well as of APN activity inhibit IL-17 production in activated human and mouse T cells. Combining inhibitors of DP IV and APN increases the suppressive effect on T cell specific IL-17 production *in vitro* compared to a single peptidase inhibitor. In the following, we summarize the evidence for the role of both ectoenzymes in T cell activation *in vitro* and *in vivo* and provide a rationale for the use of combined or dual ectopeptidase inhibitors to treat autoimmune diseases like MS.

2. INTRODUCTION

MS is an inflammatory autoimmune disorder and the most frequent demyelinating disease of the central nervous system (CNS). In Northern America and Europe the prevalence of MS is 60 to 100 per 100,000 (1). Epidemiological data indicate that MS susceptibility and outcome are influenced by both genetic and environmental factors (2). MS can affect all functional systems of the CNS. Most frequent symptoms are sensory deficits, weakness of one or several limbs, optic neuritis, cerebellar or brainstem dysfunction, and cognitive impairment. Based on findings in MS patients and animal models, a T cell-mediated immunopathogenesis is widely believed to cause the symptoms and signs of MS. Inflammation and CNS damage in MS patients is thought to be induced by extracerebral activation of autoreactive T cells directed against antigens of the CNS. In the case of failure in the control mechanisms of peripheral tolerance, these T cells are capable of crossing the blood-brain barrier and of entering the brain, where they become re-activated. Subsequently, the release of cytokines and growth factors recruit T cells and other inflammatory cells from the periphery and reinforce the local inflammation. Moreover, cytotoxic mediators cause direct neuronal damage. These

processes lead to local demyelination, damage of oligodendrocytes, axonal degeneration, and neuronal apoptosis (3).

Peptidases like dipeptidyl peptidase IV (DP IV, EC 3.4.14.5) and aminopeptidase N (APN, E.C. 3.4.11.2) are shown to regulate many biological processes and to play a prominent role during T cell activation, immune responses, and autoimmunity (4-8). DP IV, a 110 kD type II transmembrane glycoprotein, is identical with the leukocyte surface antigen CD26. It belongs to the group of post-proline dipeptidyl aminopeptidases, which consists of the four enzymes of the DP IV gene family, DP IV (EC 3.4.14.5), FAP (fibroblast activation protein), DP8 and DP9, and DPII (E.C.3.4.14.2) (6, 7, 9, 10). DP IV is catalyzing the release of N-terminal dipeptides from oligo- and polypeptides preferentially with proline, hydroxyproline and, with less efficiency, alanine in the penultimate position (4-7). These unique substrate specificity of DP IV and related enzymes results in playing a key role in the catabolism of a number of chemo- and cytokines, neuropeptides, immunopeptides, and peptide hormones containing the X-Pro or X-Ala amino terminal sequences, such as CXCL12, substance P, neuropeptide Y, peptide YY, enterostatine, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (GLP-1) (11-13). APN, identical with the myeloid linage antigen CD13, is a 150 kD type II transmembrane metalloprotease. It belongs to the family of zinc-dependent aminopeptidases found in different subcellular organelles, in the cytoplasm and as integral membrane proteins. APN is catalyzing the hydrolysis of neutral amino acids from the N-terminus of oligopeptides. The peptidase stops hydrolysis if proline is in the second position of the N-terminal sequence, thus generating potentially DP IV-susceptible substrates (8). APN is shown to be involved in the degradation of several neuropeptides, angiotensins, cytokines and immunomodulatory peptides. APN also contributes to extracellular matrix degradation and antigen processing (8, 14).

Several lines of evidence suggest a role for this group of peptidases within the immune system. DP IV/CD26 is expressed on the surface of resting and activated T cells, activated B cells, and activated NK cells (11, 15). In addition, a soluble form of DP IV occurs in serum (4, 5). DP8 and DP9 are expressed in the cytoplasm of lymphocytes and monocytes (10). The DP IV-like enzyme attractin is expressed on T lymphocytes and monocytes (6, 16, 17). APN/CD13 is strongly expressed on cells of the myelo-monocytic lineage (8, 14). While resting T lymphocytes lack APN/CD13, elevated APN expression is detectable on the surface of activated T cells and on T cells derived from local sites of inflammation (8, 14). APN mRNA expression has also been shown in various T cell populations including unfractionated T cells, $CD4^+$, Th1 and Th2 cells, with highest expression levels detected in $CD4^+CD25^+$ regulatory T cells (8, 14, 18). Activation of human T cells through stimuli such as mitogens, IL-2 or anti-CD3 monoclonal antibodies strongly increase APN mRNA contents, cellular APN enzymatic activity and APN/CD13 surface expression (8, 14). Recent evidence also points to a role of the APN-like peptidase cytosol alanyl-aminopeptidase (cAAP) in the immune response. Expression of cAAP-mRNA was found in $CD4^+$, $CD8^+$, Th1, Th2, and Treg ($CD4^+$ $CD25^+$) T cell subpopulations (18).

3. INHIBITORS OF DP IV, APN AND RELATED ENZYMES MODULATE OF T CELL ACTIVATION *IN VITRO*

Over the last decade, many laboratories have studied the functions of DP IV and/or APN-like enzymes on immune cells using synthetic inhibitors of DP IV and APN enzymatic activity. As a result, a multitude of different synthetic compounds inhibiting DP IV and/or DP IV-like enzymes were tested under different stimulation conditions and on a variety of target cells. The reversible DP IV inhibitors Lys[$Z(NO_2)$]-thiazolidide (I49) and Lys[$Z(NO_2)$]-pyrrolidide (I40) were characterized in most detail by our groups. These synthetic compounds showed strong and dose-dependent inhibitory effects on proliferation of mitogen- and antigen-stimulated human peripheral blood mononuclear cells (PBMC) and T cells, of human T cell clones and of mitogen-stimulated mouse thymocytes and splenocytes (19-23). Moreover, it was shown that these inhibitors suppress the production of IL-2, IL-10, IL-12, and IFN-gamma in pokeweed mitogen (PWM)-stimulated PBMC and purified T cells as well as the production of IL-2, IL-6, and IL-10 in phytohemagglutinin (PHA)-stimulated mouse splenocytes and concanavalin A (Con A)-stimulated mouse thymocytes (20, 21). As demonstrated by competitive RT-PCR, the levels of IL-2 and IFN-gamma mRNA in mitogen-stimulated T cells were decreased after exposure of cells to Lys[$Z(NO_2)$]-thiazolidide (24). The DP IV inhibitors also reduced in a dose-dependent manner IFN-gama, IL-4, and TNF-alpha production of myelin basic protein (MBP)-stimulated T cell clones from patients with MS (25). In contrast to most other cytokines studied, Lys[$Z(NO_2)$]-thiazolidide and Lys[$Z(NO_2)$]-pyrrolidide elicited enhanced mRNA expression and protein secretion of the immunosuppressive cytokine transforming growth factor-beta1 (TGF-beta), a finding that in part explained the inhibitory effects on DNA synthesis and cytokine production in T cells (8, 20, 21, 24).

APN/CD13 inhibitors have also been tested for their immunomodulatory activity on leukocytes functions. The selective APN inhibitors actinonin and probestin caused a dose-dependent suppression of DNA synthesis in mitogen-stimulated human T cells and PBMC (14, 25, 26). Moreover, these inhibitors were shown to suppress IL-1beta and IL-2 production of stimulated human PBMC and T cells (14, 25, 26). Similar to DP IV inhibitors, both actinonin and probestin induced TGF-beta1 production in human PBMC and T cells (14).

4. DP IV, APN AND RELATED ENZYMES IN MS

Several years ago, we and others hypothesized that if DP IV, APN and related enzymes modulate functions of activated T cells and regulate immune

responses, inhibiting these peptidases by specific effectors should be beneficial in inflammatory diseases such as MS. Thus, expression and function of DP IV/CD26 and APN/CD13 and the effect of synthetic inhibitors were examined in autoreactive human T cells as well as in several animal models of inflammatory human diseases, such as rheumatoid arthritis, cardiac transplantation, inflammatory bowel disease and experimental autoimmune encephalomyelitis (EAE) (22, 23, 27-32).

Investigating CD26 expression on resting myelin basic protein (MBP)-specific T cell clones from patients with MS, we detected increased CD26 levels by immunostaining and enzymatic assays. In addition, the expression of DP IV/CD26 on resting human autoreactive T cell clones was three- to fourfold higher than on resting peripheral blood T cells from healthy persons (22). Previous studies showed that patients with MS have an increased median percentage of $CD26^+$ cells among $CD4^+$ T cells and $CD8^+$ T cell populations in the peripheral blood (33-40). CD26 expression decreases somewhat in peripheral blood and cerebrospinal fluid after high-dose oral methylprednisolone treatment. Others found correlations between changes in the frequency of CD26-expressing T cells and lesion activity in magnetic resonance imaging of MS patients with relapsing-remitting and chronic progressive disease courses, consistent with the function of CD26 in regulating T cell activation states (41). Jensen *et al.* (33) studied $CD4^+$ T cell activation in patients with clinically isolated syndromes suggesting an initial attack of MS. They demonstrated that the percentage of blood $CD26^+$ $CD4^+$ T cells was increased in these patients, and correlated with disease activity measured by magnetic resonance imaging and with clinical disease severity. In contrast, the percentage of $CD25^+$ $CD4^+$ T cells, a phenotype of regulatory T cells in the cerebrospinal fluid correlated negatively with the concentration of MBP and the presence of IgG oligoclonal bands. Finally, gene expression profiling in MS patients and healthy controls identified higher average expression of DP IV/CD26 in peripheral blood mononuclear cells of MS patients (42, 43). Collectively, these data indicate that increased CD26 expression and enzymatic activity on human $CD4^+$ T cells correlates with disease activity in MS. The elevation of CD26 may arise from an increased frequency of activated T cells in MS patients and could thus reflect the presence of pathogenic myelin-reactive T cells. Of note, also the expression of APN/CD13 was found to be increased on PBMC in patients with MS (44-46). In recent studies, Ziaber *et al.* presented evidence for APN playing a role as an immunological marker of various clinical forms of MS. Mechanistic studies suggested that CD13 antigen expression may be an important functional marker for transendothelial migration properties of PBMC (45, 46).

We recently addressed the role of DP IV/CD26 in murine EAE, a well characterized $CD4^+$ T-cell mediated autoimmune disease, characterized by CNS inflammation and demyelination (23, 47). We demonstrated that the clinical signs of EAE are suppressed by Lys[$Z(NO_2)$]-pyrrolidide, an inhibitor of DP IV enzymatic activity, both in a preventive and therapeutic fashion. CNS inflammation associated with acute EAE was reduced. Moreover, we found an up-regulation of latent TGF-betaproduction *in vivo* both in spinal cord tissues and in plasma from DP IV inhibitor-treated mice as compared to mice treated with PBS or control substances. These data suggest that the therapeutic effect of inhibitors of DP IV enzymatic activity in EAE is mediated by upregulation of the immunosuppressive cytokine TGF-beta *in situ* and the inhibition of T cell effector functions (23). Based on results of these studies, DP IV enzymatic activity has attracted major interest as a potential target for anti-inflammatory therapy in MS. A significant challenge for future studies will be to determine whether the anti-inflammatory effects of synthetic inhibitors of DP IV enzymatic activity are the result of selective inhibition of DP IV or involve the interaction with other newly discovered DP IV-like enzymes, for example DP8, DP9 or DP II.

5. COMBINED INHIBITION OF DP IV AND APN ENZYMATIC ACTIVITY ON STIMULATED T CELLS *IN VITRO*

Both *in vitro* and *in vivo* studies have shown that inhibitors of DP IV and APN enzymatic activity act in concert suppressing proliferation and effector functions of proinflammatory T cells (48, 49). Since inhibition of DP IV enzymatic activity was shown to protect from autoimmune disease and inhibitors of DP IV and APN enzymatic activity acted in a concerted manner to suppress proliferation and effector functions of T cells, we hypothesized that the combined application of DP IV and APN inhibitors could exhibit increased effects in regulating T cell activation and may yield increased therapeutic benefits in autoimmune diseases like EAE. In a recent study, we evaluated the combined action of DP IV and APN inhibitors on T cell functions (31). Human PBMC and purified T cells were stimulated with PHA or PWM in the presence and absence of different concentrations of the inhibitor of DP IV enzymatic activity Lys[$Z(NO_2)$]-pyrrolidide and of the inhibitor of the APN enzymatic activity actinonin. We showed that both substances act synergistically to fully abrogate PHA- or PWM-induced DNA synthesis of PBMC and T cells. The simultaneous inhibition of DP IV and APN enzymatic activity led to more pronounced reduction of proliferation of PBMC and T cells than the one observed in response to each of the inhibitors alone (31). The same additive effect on proliferation was seen also in other modes of stimulation and in other cell models. Figure 1 summarizes the effects of Lys[$Z(NO_2)$]-thiazolidide (I49), Lys[$Z(NO_2)$]-pyrrolidide (I40), actinonin and the combination of these substances on the DNA synthesis of PWM-stimulated splenocytes of wild-type C57BL/6 mice. Of note, synergistic effects of combined inhibition of DP IV and APN activity were observed in these cells.

As previously reported, Lys[$Z(NO_2)$]-pyrrolidide and Lys[$Z(NO_2)$]-thiazolidide as well as actinonin were capable of inducing TGF-beta in activated PBMC and T cells. Interestingly, the combined inhibition of DP IV and APN enzymatic activity increased the production and release of TGF-beta in an additive (Lys[$Z(NO_2)$]-

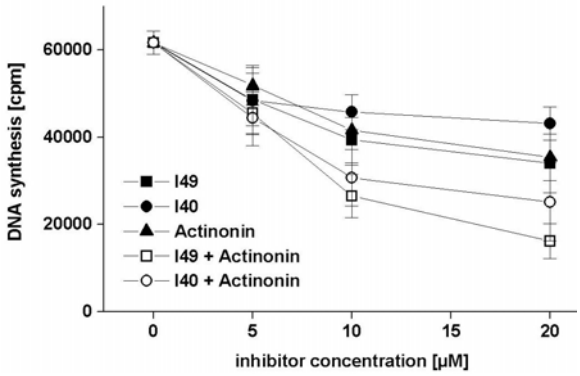

Figure 1. Influence of inhibitors of DP IV and/or APN enzymatic activity on DNA synthesis of PWM-stimulated splenocytes of C57BL/6 mice. Splenocytes were obtained from naive mice by separation of the spleen using cell strainers. Cells were stimulated in 96-well microtiter culture plates (10^5 cells/well) with PWM (1 µg/ml) in the presence of different concentrations of the inhibitors of DP IV activity, Lys[Z(NO_2)]-thiazolidide (I49) and Lys[Z(NO_2)]-pyrrolidide (I40), or the APN inhibitor actinonin alone and in combination. Cells were cultured for 72 hours and DNA synthesis determined by standard ^{3}H-thymidine uptake. [^{3}H-TdR] incorporation is shown as mean ± SD of four independent experiments.

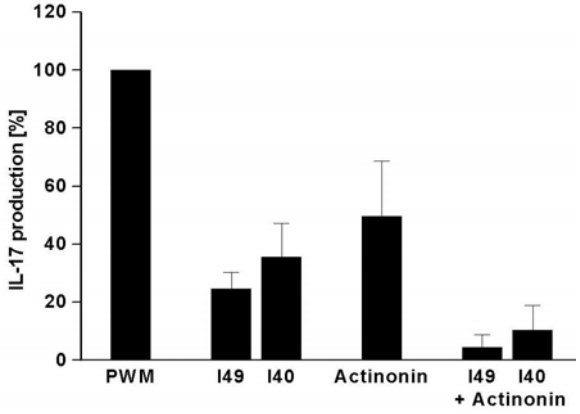

Figure 2. Influence of inhibitors of DP IV and/or APN enzymatic activity on IL-17 production of PWM-stimulated human T cells. T cells were enriched by nylon wool adherence from PBMC of healthy donors isolated by Ficoll-Paque gradient centrifugation. Cells were stimulated with PWM (2 µg/ml) in the presence of the inhibitors of DP IV activity Lys[Z(NO_2)]-thiazolidide (I49) or Lys[Z(NO_2)]-pyrrolidide (I40) and the inhibitor of APN activity actinonin alone and in combination (all 20 µM). Cell culture supernatants were harvested after 72 hr and IL-17 concentrations were determined with a specific enzyme-linked immunosorbent assay (human IL-17-ELISA) according to the manufacturer´s instructions. IL-17 production is shown as mean ± SD of three independent experiments.

pyrrolidide/actinonin) or superadditive (Lys[Z(NO_2)]-thiazolidide/actinonin) manner (31). Compelling evidence has recently demonstrated that IL-17-producing CD4 cells (Th17) are a major contributor to the pathogenesis of autoimmune inflammation (48-51). IL-17 is shown to play an important role in the development of EAE (52, 53). Consistent with these observations, the therapeutic efficacy of IL-17 neutralization and IL-17 vaccination has been demonstrated by different groups (54-56). Based on these studies, we investigated the effect of inhibitors of DP IV and/or APN enzymatic activity on IL-17 production of PWM-stimulated human T cells and of PWM-stimulated splenocytes from C57BL/6 mice. To test the ability of these inhibitors on IL-17 production, human T cells were stimulated with PWM in the presence of the inhibitors of DP IV activity Lys[Z(NO_2)]-thiazolidide (I49) or Lys[Z(NO_2)]-pyrrolidide (I40) and the inhibitor of APN activity actinonin alone and in combination. Cell culture supernatants were harvested after 72 hr and concentrations of IL-17 were determined with a specific human IL-17-ELISA. As shown in Figure 2, we found that inhibitors of DP IV-like activity as well as of APN activity inhibit IL-17 production in human mitogen-stimulated T cells. Combining inhibitors of DP IV and APN activity increases the suppressive effect on T cell IL-17 production *in vitro* in comparison to a single peptidase inhibitor. A similar effect of these inhibitors could be shown on IL-17 production of PWM-stimulated splenocytes of C57BL/6 mice (Figure 3).

The synergistic or additive effects of combined inhibition of DP IV and APN can be explained in part by different non-redundant signaling pathways demonstrated for these enzymes (8, 11, 14, 15). An alternative explanation for the observed inhibitory effects, particularly on T cells, could be based on the finding that DP IV and APN inhibitors are preferentially affecting different T cell populations. This view is supported by recent studies that implicated APN inhibitors in supporting the phenotype and function of $CD4^+CD25^+$ regulatory T cells (57-59). This unique cell population may act to powerfully suppress and control inflammatory processes by direct cell-to-cell action on effector T cells or via release of anti-inflammatory cytokines like TGF-beta or IL-10 (60, 61). Thus, induction of regulatory T cells may be a mechanism by which ectopeptidase inhibitors suppress inflammation (60).

6. TREATMENT OF EAE WITH INHIBITORS OF BOTH DP IV AND APN ENZYMATIC ACTIVITY

Based on the summarized *in vitro* observations, inhibition of both DP IV/CD26 and APN/CD13 activity *in vivo* may provide a new approach to modulate T cell functions and tissue-specific autoimmunity in the CNS. These results should have important implications for the treatment of human diseases with a putative autoimmune pathogenesis. Major research efforts are directed at the investigation of DP IV, APN and related enzymes as potentially powerful and safe pharmacological targets for new classes of selective and combined inhibitors. Recently, we studied the combined application of DP IV and APN inhibitors in EAE. SJL/J mice with chronic EAE were treated with Lys[Z(NO_2)]-pyrrolidide or actinonin alone or with both inhibitors in combination (31). Treatment of animals was initiated around the peak of the first episode of

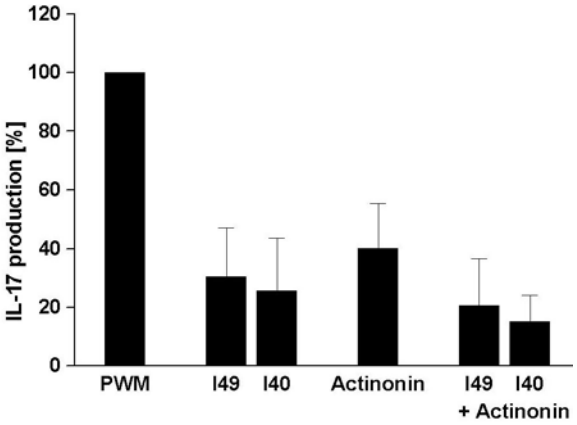

Figure 3. Influence of inhibitors of DP IV and/or APN enzymatic activity on IL-17 production of PWM-stimulated splenocytes of C57BL/6 mice. Splenocytes were cultured in serumfree AIM-V medium supplemented with 10^{-5} M 2-mercaptoethanol. Cells were stimulated with PWM (2 µg/ml) in the presence of the inhibitors of DP IV activity Lys[Z(NO_2)]-thiazolidide (I49) or Lys[Z(NO_2)]-pyrrolidide (I40) and the inhibitor of APN activity actinonin alone and in combination (all 20 µM). Cell culture supernatants were harvested after 72 hr and concentrations of murine IL-17 were determined with a specific enzyme-linked immunosorbent assay (murine IL-17-ELISA) according to the manufacturer´s instructions. IL-17 production is shown as mean ± SD of four independent experiments.

acute clinical disease - between days 16 and day 46. We showed that the simultaneous application of inhibitors of DP IV and APN activity led to a more profound reduction of the clinical severity of the EAE compared to the use DP IV or APN inhibitors alone. The combined treatment even with low doses of each inhibitor (0.1 mg every 3 days) significantly reduced the clinical severity of chronic EAE (31). Further highlighting the efficacy of dual peptidase inhibition in autoimmunity, a comparable therapeutic effect of these inhibitors as well as the newly developed dual DP IV/APN inhibitor IP12.C6 was demonstrated in a mouse model of colitis (32). In summary, current data raise the possibility that simultaneous inhibition of DP IV and APN enzymatic activity is advantageous over targeting a single ectopeptidase, and support the development of inhibitors with dual specificities for both ectopeptidases. Due to the lack of reported side effects and their unique mode of action by local upregulation of TGF-beta at the site of inflammatory lesion formation, we envision development and clinical testing of dual inhibitors of DP IV and APN activity for a wide range of human disorders, including MS, associated with Th17 cell-mediated organ-specific inflammation.

7. CONCLUSION AND PERSPECTIVE

DP IV/CD26, APN/CD13 and related enzymes have generated interest as immunopharmacological targets based on the following findings: (1) these peptidases are highly upregulated in activated T cells (8, 10, 11,14, 15); (2) a number of different inhibitors of DP IV and APN enzymatic activity were shown to reduce T cell activation and functions *in vitro* (11,14, 15, 19-24); (3) single synthetic DP IV inhibitors were successfully used as treatment in several animal models of human diseases, including rheumatoid arthritis, multiple sclerosis and transplantation (23, 27-30); (4) combined application of DP IV and APN inhibitors suppress proliferation of stimulated human PBMC and T cells in a synergistic or additive manner *in vitro* (31); (5) APN/CD13 inhibitors activate $CD4^+CD25^+$ regulatory T cells harnessing them for the treatment of autoimmune diseases (32, 58, 59); and (6) combined treatment of SJL/J mice with chronic EAE with DP IV and APN inhibitors significantly reduce the clinical severity of disease (31). Because of the limited specificity of inhibitors of DP IV and APN enzymatic activity it should considered, however, that also other members of the two peptidase families, e. g. DP8 and DP9 or cAAP, could be involved actively in these processes. Collectively, *in vitro* and *in vivo* data demonstrate that dual inhibition of DP IV/CD26 and APN/CD13 activity provides a new approach to modulate T cell activation and T cell-mediated autoimmunity in the CNS. These results may have important implications for the treatment of human diseases with a putative autoimmune pathogenesis.

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Abbreviations:APN: Aminopeptidase N, CNS: central nervous system, DP: dipeptidyl peptidase, EAE: experimental autoimmune encephalomyelitis, FAP: fibroblast activation protein, IFN: Interferon, IL: interleukin, I40: Lys[Z(NO_2)]-pyrrolidide, I49: Lys[Z(NO_2)]-thiazolidide, MBP: myelin basic protein, MS: Multiple Sclerosis, PBMC: peripheral blood mononuclear cells, PHA: phytohemagglutinin, PWM: pokeweed mitogen, TGF-beta1: transforming growth factor beta 1, TNF-alpha: Tumor Necrosis Factor-alpha

Key words: Dipeptidyl peptidase IV, CD26, Aminopeptidase N, CD13, T Cell Activation, Experimental Autoimmune Encephalomyelitis, IL-17 Production, Multiple Sclerosis, Peptidase Inhibitors, Review

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