Attractin, a dipeptidyl peptidase IV/CD26-like enzyme, is expressed on human peripheral blood monocytes and potentially influences monocyte function

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Abstract: The ectoenzyme dipeptidyl peptidase IV (DP IV; CD26) was shown to play a crucial role in T cell activation. Several compounds inhibiting DP IV-like activity are currently under investigation for the treatment of Type 2 diabetes, rheumatoid arthritis, colitis ulcerosa, psoriasis, multiple sclerosis, and other diseases. In the present study, we show that human peripheral blood monocytes express a DP IV-like enzyme activity, which could be inhibited completely by the synthetic DP IV inhibitor Lys[Z(NO_2)]-thiazolidide. DP IV immunoreactivity was not detectable on monocytes, and DP IV transcript levels of monocytes were near the detection limit of quantitative polymerase chain reaction. However, monocytes exhibit a strong mRNA expression of the multifunctional DP IV-like ectoenzyme attractin and were highly positive for attractin in flow cytometric analysis. Fluorescence microscopy clearly demonstrated that attractin is located on the cell surface of monocytes. Attractin immunoprecipitates hydrolyzed Gly-Pro-pNA, indicating that monocyte-expressed attractin possesses DP IV-like activity. Inhibitor kinetic studies with purified human plasma attractin revealed that Lys[Z(NO_2)]-thiazolidide not only inhibits DP IV but also attractin (50% inhibition concentration=8.45×10^{-9} M). Studying the influence of this inhibitor on monocyte functions, we observed a clear reduction of cell adhesion to fibronectin-coated culture plates in the presence of Lys[Z(NO_2)]-thiazolidide. Moreover, this inhibitor significantly modulates the production of interleukin-1 (IL-1) receptor antagonist, IL-6, and transforming growth factor-β1 in lipopolysaccharide-stimulated monocyte cultures. In summary, here, we demonstrate for the first time expression of attractin on monocytes and provide first data suggesting that drugs directed to DP IV-like enzyme activity could affect monocyte function via attractin inhibition. *J. Leukoc. Biol.* 80: 621–629; 2006.

Key Words: *DP IV inhibitor · counter-current elutriation · cytokine production · cell adhesion*

INTRODUCTION

In the last years, synthetic inhibitors of dipeptidyl peptidase IV (DP IV; EC 3.4.14.5, CD26) were found to be promising drugs for the treatment of different disorders including autoimmune diseases. A selection of these inhibitors is already in clinical trials for the treatment of Type 2 diabetes and under in vivo investigation in psoriasis, rheumatoid arthritis (RA), colitis, and multiple sclerosis (MS).

DP IV is an ectoenzyme releasing N-terminal dipeptides from peptides with proline in the penultimate position [1]. It exists in a membrane-bound and a secreted form and has access to extracellularly localized substrates. In the immune system, DP IV is expressed on the surface of T cells, B cells, and natural killer cells. Some chemokines, including CC chemokine ligand 5 (regulated on activation, normal T cell expressed and secreted) and CXC chemokine ligand 12 (stromal cell-derived factor-1α), were identified to be hydrolyzed by DP IV [2–4]. During T cell activation, the expression levels and the enzymatic activity of DP IV on T cells clearly increased. It is interesting that inhibition of DP IV activity with synthetic inhibitors such as Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-pyrrolidide, or inhibitory Xaa-Xaa-Pro peptides was shown to suppress proliferation of activated T cells in a way involving the immunosuppressive cytokine transforming growth factor-β1 (TGF-β1; for review, see ref. [5]). However, the physiologic role of the tightly regulated surface expression of DP IV on T cells has not been elucidated in detail as yet.

In recent years, more proteins with DP IV-like activity or DP IV-like structure were discovered and summarized to a protein family designated as "DP IV activity and/or structure homologues" (DASH) [6]. Among them are the ectopeptidases fibroblast-activating protein-α (FAP-α) and attractin (DPPT-L, mouse *Mahogany*, rat *Zitter*), disclosing the fact that DP IV is not a unique ectoenzyme with DP IV-like activity. FAP-α is not expressed on cells of the immune system. Attractin, how-

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ever, was described to be expressed on T cells [7]. Although attractin is not structurally related to DP IV, it is characterized by the facts that its substrate specificity is similar to that of DP IV and that it also exists in soluble, secreted, and transmembrane forms. By addition of attractin to the cell culture medium, it was shown that attractin somehow is involved in interactions between T cells and monocytes [7]. The function of immune cell-expressed attractin, however, remains unsolved until now.

As a result of their blocking effects on T cell activation and proliferation, inhibitors of DP IV-like activities were assumed to be useful for immunosuppressive therapy. Korom et al. [8, 9] observed abrogation of acute allograft rejection in a rat cardiac transplantation model. Inhibition of DP IV activity also turned out to be a promising approach for the therapy of T cell and macrophage-mediated autoimmune diseases. It was demonstrated that substances inhibiting DP IV-like enzymes, such as Ala-boro-Pro, Lys[Z(NO_2)-thiazolidide, Ala-Pro-nitrobenzoylhydroxylamine, TMC-2A, and TSL-225, suppress collagen- and alkyldiamine-induced arthritis, representing animal models for RA [10, 11].

In MS, a devastating autoimmune disease of the central nervous system triggered at least in part by autoreactive myelin antigen-specific T cells [12, 13], we observed that myelin-specific T cell clones of patients with MS express increased DP IV activity as compared with T cells of healthy controls [14]. Furthermore, we could show for the first time that targeting DP IV with synthetic inhibitors suppresses experimental autoimmune encephalomyelitis (EAE) in mice. In this MS animal model, injections of Lys[Z(NO_2)]-pyrrolidide reduced disease severity in a preventive as well as in a therapeutic manner, indicating that DP IV inhibition represents a novel therapeutic approach for the treatment of this autoimmune disorder [14, 15].

Beside T cells, monocytes were reported to express a "DP IV-like enzyme activity" on the cell surface, which has not been characterized until now [16]. Thus, inhibitors of DP IV could also target monocytes. For RA, the causative role of macrophages in the immunopathogenesis is evident. In MS, mononuclear phagocytes are shown to be involved in the destruction of the axonal myelin sheath [17]. So, substances suppressing monocyte function could be beneficial for patients suffering from these diseases.

Here, we show that attractin, a DP IV-like ectoenzyme, is expressed on the surface of human peripheral blood monocytes. Moreover, the DP IV inhibitor Lys[Z(NO_2)]-thiazolidide is capable of modulating adhesion and cytokine production of these cells.

MATERIALS AND METHODS

Preparation of human peripheral blood mononuclear cells (PBMC) and monocytes

PBMC were prepared from heparinised blood of healthy volunteers as described by Reinhold et al. [18]. Monocytes were enriched by counter-current centrifugal elutriation (Beckman Coulter, Unterschleissheim, Germany) [19, 20] and resuspended in AIM-V serum-free culture medium (Gibco-BRL, Invitrogen, Karlsruhe, Germany). Flow cytometric analysis indicated that 90 ± 5% of these cells were positive for CD14 and CD45.

Analysis by flow cytometry

Flow cytometric analysis was carried out according to standard procedures. Anti-CD26-phycoerythrin (Clone L272), anti-CD14-fluorescein isothiocyanate (FITC; Clone MΦP9), anti-CD45-FITC (Clone 2D1), anti-attractin (Clone 14), and isotype control antibodies were purchased from Becton Dickinson (Heidelberg, Germany). Immunoglobulin G2b (IgG2b)-FITC (Clone DAK-G09) was supplied by Dako (Hamburg, Germany) and FITC-conjugated donkey anti-mouse F(ab)2 fragment, by Dianova (Hamburg, Germany). Briefly, cells (5×10^5) were incubated with monoclonal antibodies at saturating concentrations in cell wash (Becton Dickinson) for 30 min at 4°C. Controls were performed with conjugated, irrelevant antibodies of the corresponding isotype or unconjugated antibodies in conjunction with the secondary antibody. Cells were washed and analyzed using a FACSCalibur (Becton Dickinson). Cells were fixed with 1% paraformaldehyde and stored at 4°C if the analysis could not be performed immediately.

Preparation of membranes from peripheral blood monocytes

Freshly prepared peripheral blood monocytes were homogenized on ice by ultrasonic in 5 mM Tris HCl, pH 7.4, with 300 mM sucrose (homogenization buffer). Nuclei and unbroken cells were removed by 10 min centrifugation with 1000 *g* at 4°C, resuspended with 1/10 vol homogenization buffer, and centrifuged again. The supernatants of both centrifugations were pooled and centrifuged at 16,000 *g* for 30 min at 4°C to separate soluble and particulate fraction. The pellet was resuspended in hypo-osmotic homogenization buffer without sucrose to break open membrane vesicles. After 30 min incubation on ice, the membranes were sedimented by centrifugation with 16,000 *g* for 45 min at 4°C. Peripheral blood monocyte membranes were solubilized in phosphate-buffered saline (PBS), pH 7.4, with 0.5% Igepal CA-630 (Sigma-Aldrich, Schnelldorf, Germany).

Immunoprecipitation of attractin from cell lysates

Peripheral blood monocytes were lysed on ice in 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, containing 1% Triton X-100 and 0.1% Igepal CA-630 (Sigma-Aldrich). After 20 min incubation on ice with frequent mixing, unlysed cell fragments were removed by centrifugation with 16,000 *g* for 30 min. Lysates (20×10^6 cells/ml lysis buffer) were incubated with 5 μg/ml rabbit preserum (control) or 5 μg/ml affinity-purified rabbit anti-attractin antiserum, kindly provided by Jonathan Duke-Cohan [7], and protein A-sepharose overnight at 4°C. Immunoprecipitates were washed three times with 1 ml ice-cold lysis buffer and resuspended in 200 μl lysis buffer.

Determination of DP IV-like enzyme activities

DP IV-like activity was measured using Gly-Pro-*p*NA as substrate, according to a slightly modified method described previously [21]. Lys[Z(NO_2)]-thiazolidide was synthesized by coupling of Boc-Lys[Z(NO_2)]-OH to thiazolidine [22]. Briefly, separated peripheral blood monocytes were seeded in 48-well plates for 1 h to allow adhesion (2.5×10^5 cells in 100 μl AIM-V/well). Nonadherent cells were removed, and adherent cells were preincubated with 50 μM Lys[Z(NO_2)]-thiazolidide or AIM-V medium for 10 min at 37°C. The enzymatic reaction was started by addition of 10 μl Gly-Pro-*p*NA to a final concentration of 1.5 mM. The zero values were stopped immediately after substrate addition with 9 vol 1 M sodium acetate, pH 4.4 (stop buffer). The samples were incubated at 37°C. After an appropriate incubation time, the substrate hydrolysis was stopped with 9 vol stop buffer. DP IV-like activities of PBMC were measured in a similar way, omitting the adhesion step. Samples of the membrane preparation and the immunoprecipitation were measured analogically. For determination of spontaneous substrate decomposition, a buffer control was treated in the same manner. The samples were centrifuged, and extinctions of the supernatants were measured at 392 nm. Enzymatic activity was calculated with the extinction coefficient $\varepsilon_{392\ nm} = 11.5 \times 10^6\ mmol^{-1}\ cm^{-1}$ in pkat/10^6 cells.

Determination of cell viability

To examine cell viability, a lactate dehydrogenase (LDH) cytotoxicity detection assay was used (Roche Diagnostics, Mannheim, Germany).

Fluorescence microscopy of attractin-stained monocytes

For microscopic analysis, freshly separated monocytes were stained in the same manner as for flow cytometry. Stained monocytes were seeded on poly-L-lysine-coated coverslips, fixed with 1% paraformaldehyde and 0.025% glutaraldehyde in PBS, and visualized using a fluorescence microscope (DMRE 7, Leica Microsystems, Heidelberg, Germany), equipped with a 100×/NA1.4 objective and a charged-coupled device camera, Spot RT (digital instruments).

Preparation of total RNA from peripheral blood monocytes and PBMC

Total RNA was isolated from separated monocytes and PBMC using nucleospin RNA II kit (Machery-Nagel, Düren, Germany). For RNA isolation, cells were lysed with lysis buffer provided in the kit and stored at −80°C. Frozen samples were thawed slowly and treated according to the Nucleospin RNA II purification protocol. RNA was freed from DNA by treatment with DNase I. RNA preparations were checked on agarose gels, and the RNA content was quantified spectrophotometrically using a GeneQuant (GE Healthcare, Freiburg, Germany). For reverse transcription (RT) to cDNA, 1 μg RNA was incubated in a final volume of 20 μl with 20 units avian myloblastosis virus RT (Promega, Mannheim, Germany) in the supplied buffer, 0.5 mM deoxynucleoside triphosphates, and 10 mM random hexanucleotides (Boehringer, Mannheim, Germany) in the presence of 50 units placenta RNase inhibitor (Ambion, Austin, TX) for 1 h at 37°C. The enzymatic reaction was stopped by a 10-min incubation at 65°C.

Relative RNA quantification by real-time polymerse chain reaction (PCR)

The RNA transcript levels were measured by real-time PCR using β-actin for internal standard and determined according to the relative standard curve method to assess the different PCR efficiencies of the primer pairs. For DP IV, the human-specific primers E3 GATGCTACAGCTGACAGTCGC and E7 TGGTGACCATGTGACCCACTG; for attractin, the primers TGGCTCATTGAAGGACAGCC and CAGTTGTCTGTACAGTGAGG; and for β-actin, the primers CATGCCATCCTGCGTCTGGACC and ACATGGTGGTGCCGCCAGACAG were used. For the generation of the standards, the PCR products were cloned into PCR topoisomerase II vector (Gibco-BRL, Invitrogen). The vectors were propagated in Top F10′ *Escherichia coli* and purified by MiniPrep (Qiagen, Valencia, CA). The DNA content of the standards was determined spectrophotometrically on a GeneQuant (GE Healthcare, Freiburg, Germany).

Real-time PCR was performed according to ABI protocols on ABI7000 (Applied Biosystems, Darmstadt, Germany). A reaction mixture containing Taq, cDNA, SYBR Green, and the specific primers was prepared. After initial activation at 95°C for 15 min, 40 cycles followed with denaturation at 94°C, annealing for 30 s at 60°C, and elongation at 72°C for 30 s. All samples were analyzed in triplicate. To exclude any possible cross-contaminations, samples containing water instead of cDNA were included in every measurement. Following real-time PCR, the PCR products were checked on agarose gels. RNA levels were normalized to the β-actin level as a measure for the cDNA content of the sample.

Determination of the 50% inhibition concentration (IC_{50}) values of the inhibition of attractin and DP IV with Lys[Z(NO_2)]-thiazolidide

For inhibitor kinetics, we used human attractin purified from plasma by a two-step procedure including ethanol precipitation and cationic exchange chromatography [23] and human recombinant DP IV produced by a *Pichia pastoris* expression system [24]. IC_{50} determinations were performed using the chromogenic substrate Gly-Pro-*p*NA (0.4 mM) in HEPES buffer, pH 7.6 (40 mM HEPES, I=0.12 by KCl). Measurements were carried out at 30°C. Release of *p*NA was monitored continuously at 405 nm using a micro plate reader, and activity was calculated from the slope of the time-response curves.

IC_{50} values were determined using one substrate concentration (0.4 mM) and 11–15 serial inhibitor dilutions starting with 0.1 mM and calculated from nonlinear regression to a four-parameter equation (Prism 4.0, GraphPad Software Inc., San Diego, CA).

Attractin-catalyzed hydrolysis of interleukin (IL)-2(1–12)

Purified attractin (see above) was preincubated with 10 μM Lys[Z(NO_2)]-thiazolidide for 30 min at 37°C in 10 mM Na-phosphate, pH 7.5, or buffer alone, respectively. The enzymatic reaction was started by addition of 400 μM IL-2(1–12) substrate. Samples were incubated for 60 min at 37°C. The reaction was stopped by addition of 30 mM phosphoric acid. Degradation of IL-2(1–12) was measured by capillary electrophoresis using a P/ACE MDQ capillary system (Beckman, Krefeld, Germany). Separations were performed with a self-assembled polyacrylamide-coated silica capillary (BioRad, München, Germany, ID 50 nm, length 30 cm) working at a constant high voltage of 17 kV positive-to-negative, as reported earlier [25]. For quantification of the residual substrate, histidine as an internal standard was added.

Determination of cytokine concentrations in monocyte culture supernatants

For generation of cell culture supernatants, 10^6 monocytes per ml were seeded in serum-free AIM-V medium (Gibco-BRL, Invitrogen) for 2 h in 24-well plates. Nonadherent cells were removed, and the remaining cells were preincubated for 30 min with 50 μM Lys[Z(NO_2)]-thiazolidide in AIM-V medium prior to addition of lipopolysaccharide (LPS) to a final concentration of 1 μg/ml. Cell culture supernatants were collected after 24 h. Concentrations of IL-1 receptor antagonist (IL-1ra), tumor necrosis factor α (TNF-α), IL-6, and latent TGF-β1 were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Wiesbaden, Germany, and Diaclone, Besancon, France), according to the recommended protocols. For an additional control, the noninhibitory compound Lys[Z(NO_2)]-OH was added instead of Lys[Z(NO_2)]-thiazolidide.

Adhesion assay

Freshly prepared peripheral blood monocytes were preincubated for 30 min at 37°C in the presence or absence of 50 μM Lys[Z(NO_2)]-thiazolidide. Parallel samples were incubated with 1 mM manganese (II)-chloride ($MnCl_2$) to activate integrins. Thereafter, the cells were transferred to fibronectin (Roche Penzberg, Germany)-coated culture dishes (Falcon, VWR Darmstadt, Germany). Unspecific binding sites were blocked with 0.15% polyvinyl alcohol for 3 h at room temperature or at 4°C overnight. After 30 min adhesion to fibronectin at 37°C, nonadherent cells were washed away, and adherent cells were fixed with 1% paraformaldehyde in PBS, and three representative fields from 100 were counted at 200-fold magnification (×20 objective and ×10 ocular E-PI with 10×10 reticule). For an additional control, cells were preincubated with the noninhibitory compound Lys[Z(NO_2)]-OH instead of Lys[Z(NO_2)]-thiazolidide.

Statistics

Statistics were calculated with Instat 3 for Macintosh (GraphPad Software Inc.) choosing nonparametric Wilcoxon matched-pairs signed-ranked test for calculating two-tailed *P* values.

RESULTS

Determination of DP IV-like surface activity of monocytes

To investigate the DP IV-like surface activity of monocytes, we prepared human peripheral blood monocytes by counter-current elutriation from PBMC. Gly-Pro-pNA hydrolysis of peripheral blood monocytes was found to be 1.5 ± 0.5 pkat/10^6 cells (mean±SD, seven experiments) compared with 4.0 ± 0.5 pkat/10^6 cells PBMC under serum-free conditions. These data indicate the presence of at least one DP IV-like ectoenzyme on these cells. Monocytes cultured for 48 h and 72 h showed slightly increased Gly-Pro-pNA hydrolysis as compared with freshly prepared monocytes. At both time-points, we did not

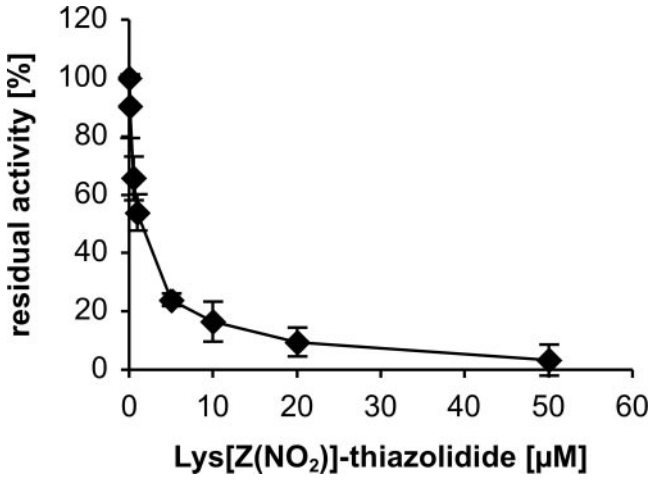

Fig. 1. Dose-dependent inhibition of the Gly-Pro-pNA-hydrolyzing activity of monocytes by Lys[Z(NO_2)]-thiazolidide. Monocytes were prepared as described in Materials and Methods and seeded serum-free in 48-well plates. After 1 h, nonadherent cells were removed, and the remaining cells were preincubated for 10 min with Lys[Z(NO_2)]-thiazolidide in the concentrations indicated. In the absence of inhibitor, monocytes hydrolyze 1.5 ± 0.5 pkat/10^6 cells Gly-Pro-*p*NA corresponding to 100%. Shown are the mean ± SD of five independent experiments each carried out in triplicate.

observe differences between the DP IV-like activities of unstimulated monocytes and monocytes activated with 1 μg/ml LPS (data not shown). The DP IV-like activity of freshly prepared monocytes was inhibited completely by preincubation with 50 μM Lys[Z(NO_2]-thiazolidide (**Fig. 1**). This inhibitor also completely inhibited Gly-Pro-pNA hydrolysis of LPS-stimulated monocytes. Cell viability was not impaired by Lys[Z(NO_2]-thiazolidide in concentrations up to 200 μM, as confirmed by a LDH release assay (data not shown).

Moreover, we prepared cell membranes from peripheral blood monocytes by differential centrifugation. We treated the membrane fraction with hypo-osmolar buffer to remove loosely attached activities and activities enclosed in vesicles. From the total Gly-Pro-pNA-hydrolyzing activities of monocyte lysates, more than 80% were soluble and were detected in the cytosolic fraction. Additional soluble activities (3.8±0.9%, mean of two independent experiments) were found in the hypo-osmolar supernatant. However, 12.1 ± 1.2% of Gly-Pro-pNA-hydrolyzing activities were measured in the membrane fraction indicating the presence of membrane-bound DP IV-like activities.

Identification of the DP IV-like ectoenzyme attractin on monocytes

To elucidate the nature of this DP IV-like activity of monocytes, we first analyzed surface expression of the two well-characterized DP IV-like ectopeptidases, DP IV (CD26) and attractin. Flow cytometric analysis revealed that 92 ± 9% (n=7) of monocytes were positive for the DP IV-like enzyme attractin monocytes. CD26 immunoreactivity could not be observed (**Fig. 2, A** and **C**). Meanwhile, lymphocytes used as a positive control for the CD26 staining were negative for attractin surface staining (Fig. 2, B and D). Furthermore, fluorescence microscopy clearly showed attractin surface staining on peripheral blood monocytes (**Fig. 3**).

To combine attractin immunoreactivity with the Gly-Pro-pNA-hydrolyzing activity of monocyte cultures, we examined attractin immunoprecipitates from monocyte lysates for Gly-Pro-pNA hydrolysis. With an anti-attractin rabbit antiserum prepared by Duke-Cohan et al. [7], we obtained immunoprecipitates, which were capable of hydrolyzing Gly-Pro-pNA (0.12±0.01 pkat/10^6 monocytes from two different volunteers).

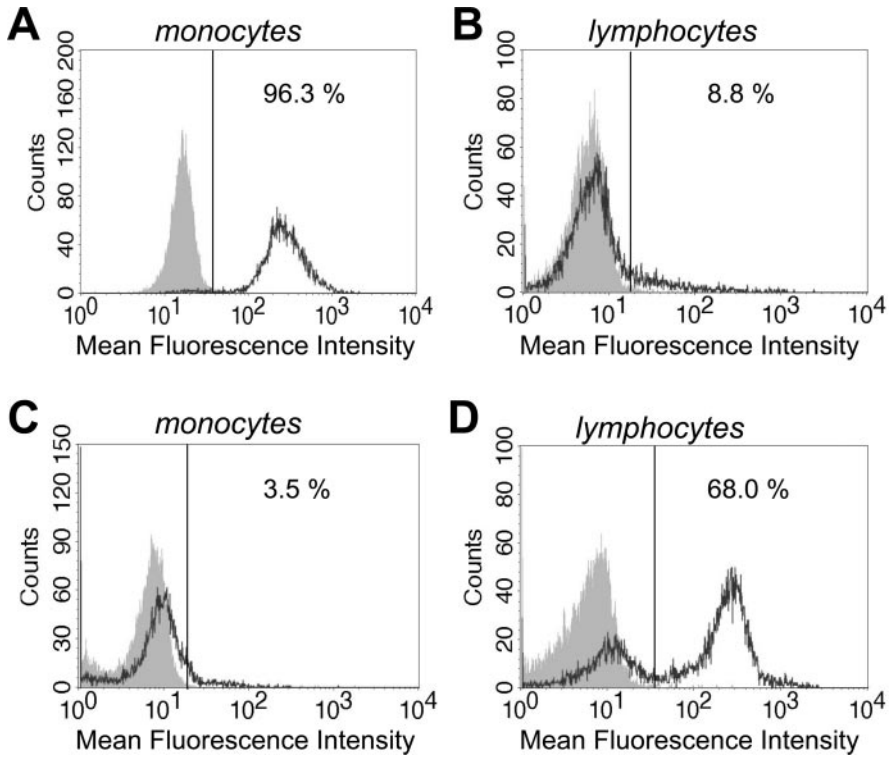

Fig. 2. Flow cytometric analysis of attractin (A, B) and DP IV/CD26 (C, D) surface expression on purified peripheral blood monocytes and lymphocytes. The cell preparations were stained for CD26 or for attractin as described in Materials and Methods. Controls (filled histograms) were performed with the appropriate IgG control antibodies and for attractin, with the irrelevant unconjugated primary antibody, followed by the secondary antibody. (A, C) Peripheral blood monocytes purified by counter-current elutriation. (B, D) PBMC gated on lymphocytes. The histograms show one representative of seven experiments.

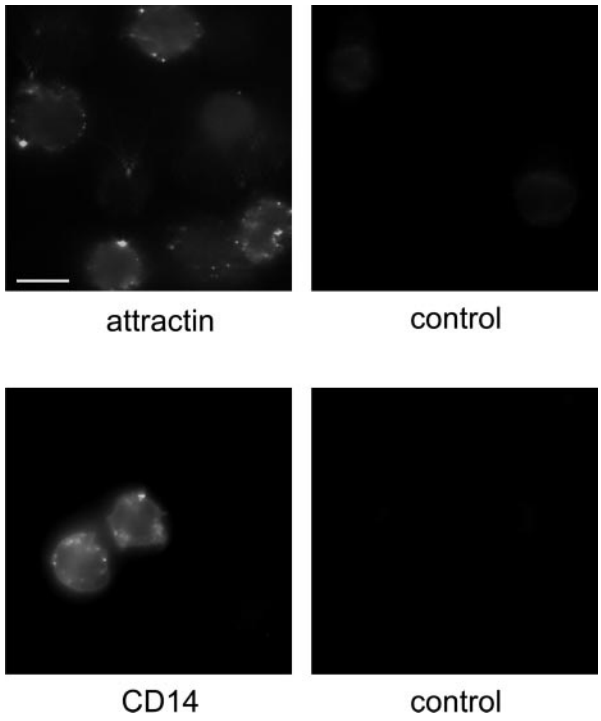

Fig. 3. Cell surface expression of attractin on peripheral blood monocytes. The cell preparation was stained with monoclonal anti-attractin antibodies (Clone 41) followed by donkey anti-mouse FITC-conjugated antibodies and with anti-CD14 monoclonals, according to the staining procedure for flow cytometry. Thereafter, the cells were seeded on poly-L-lysine-coated coverslips, fixed, and examined by fluorescence microscopy (original bar, 10 μm). For the controls, parallel samples were incubated with irrelevant antibodies of the corresponding isotypes.

In contrast, precipitates obtained with the appropriate preserum did not hydrolyze Gly-Pro-pNA.

Attractin expression of monocytes could be confirmed on a transcript level by quantitative PCR. The relative transcript levels of attractin related to β-actin (attractin/β-actin ratio) were in each of the five examined monocyte preparations, approximately 10 times higher than the relative attractin transcript levels of PBMC (**Fig. 4**). Moreover, quantitative PCR revealed two orders of magnitude lower relative DP IV levels (DP IV/β-actin ratios) in monocyte preparations compared with relative DP IV levels of PBMC, which were used for positive control. After 48 h of PHA stimulation, relative DP IV transcript levels of PBMC were increased up to three times the level of freshly prepared PBMC, whereas relative attractin transcript levels of PBMC were decreased 24 and 48 h after PHA stimulation (Fig. 4).

Lys[Z(NO_2)]-thiazolidide strongly inhibits human plasma attractin

Our data suggest the ability of the peptidase inhibitor Lys[Z(NO_2)]-thiazolidide, originally regarded as specific for DP IV, to also inhibit attractin. Thus, we examined inhibitor kinetics with attractin purified from human plasma and recombinant human soluble DP IV (hrecDP IV). Gly-Pro-*p*NA hydrolysis by human plasma attractin was strongly inhibited by Lys[Z(NO_2)]-thiazolidide. It is remarkable that the IC_{50} for the inhibition of attractin with Lys[Z(NO_2)]-thiazolidide was more than one magnitude lower than that for DP IV inhibition (8.45×10^{-9} M for human plasma attractin compared with 2.22×10^{-7} M for hrecDP IV). With the aid of capillary electrophoresis, it could be demonstrated that purified plasma attractin is capable of cleaving IL-2(1–12), a more physiological substrate than chromogenic *p*-nitroanilides, which consists of the N-terminal 12 amino acids of IL-2, carrying proline in the second position. This cleavage was also strongly inhibited by Lys[Z(NO_2)]-thiazolidide (**Fig. 5**).

Functional relevance of inhibition of the monocytic DP IV-like activity

We observed that Lys[Z(NO_2)]-thiazolidide inhibits Gly-Pro-*p*NA hydrolysis in monocyte cultures. Thus, we addressed the question whether Lys[Z(NO_2)]-thiazolidide influences crucial functions of monocytes.

We stimulated peripheral blood monocytes from seven healthy blood donors for 24 h with LPS. Lys[Z(NO_2)]-thiazolidide-treated monocytes showed significantly enhanced release of the cytokines IL-6 and latent TGF-β1 into the culture supernatants and significantly reduced release of IL-1ra (**Fig. 6A**). Release of the proinflammatory cytokine TNF-α, however, was not altered significantly by Lys[Z(NO_2)]-thiazolidide. In all cases, the effects of Lys[Z(NO_2)]-thiazolidide on the cytokine release of nonstimulated monocytes were not significant (Fig. 6A). In additional experiments, we used Lys[Z(NO_2)]-OH as a control substance, which does not inhibit DP IV activity [15]. In contrast to Lys[Z(NO_2)]-thiazolidide, Lys[Z(NO_2)]-OH, given in the same concentration as Lys[Z(NO_2)]-thiazolidide, did not influence cytokine release significantly. In cultures cultivated

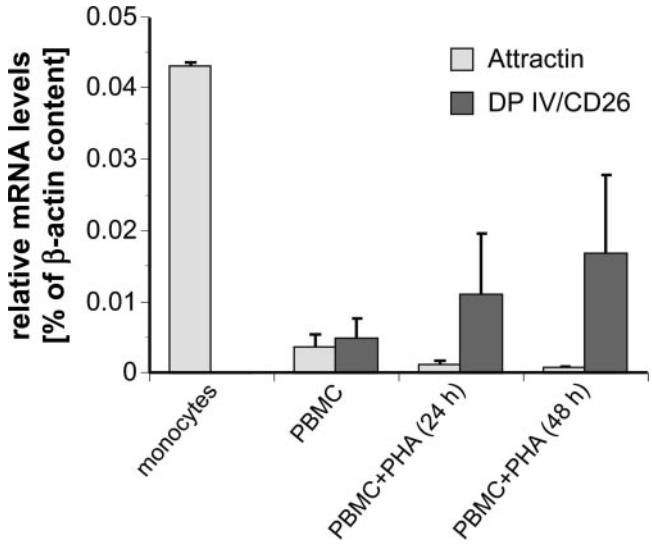

Fig. 4. Determination of attractin and CD26 transcript levels in peripheral blood monocytes compared with PBMC. Transcript levels were analyzed in triplicates by real-time PCR and quantified, according to the relative standard curve method. Transcript amounts were normalized to β-actin transcript levels. The figure shows relative CD26 and attractin transcript levels of monocytes and of freshly prepared PBMC and PBMC stimulated with 1 μg/ml phytohemagglutinin (PHA) for the periods of time indicated in percent of the β-actin levels. Values represent the mean ± SEM of three PBMC preparations and five monocyte preparations.

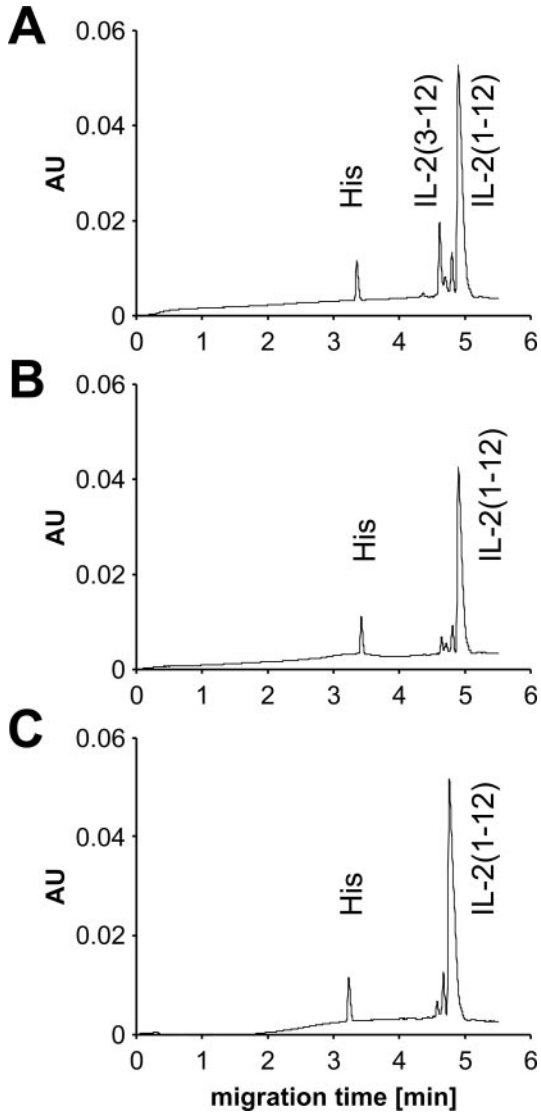

Fig. 5. Inhibition of attractin-catalyzed IL-2(1–12) hydrolysis by Lys[Z(NO_2)]-thiazolidide. (A) The substrate peptide IL-2(1–12) was incubated with purified human plasma attractin. The substrate was separated from IL-2(3–12) by capillary electrophoresis. Remaining substrate was quantified by normalization to histidine (His), added as internal standard. (B) Degradation of IL-2(1–12) by attractin in the presence of 10 μM Lys[Z(NO_2)]-thiazolidide. (C) For the control, buffer was added instead of enzyme. The experiment was performed twice with similar results. The electropherograms represent one representative of two experiments carried out in duplicate. AU, Absorption units.

for 24 h in the presence of Lys[Z(NO_2)]-OH and in the absence of LPS, we measured for TNF-α, 140 ± 35%, for IL-6, 112 ± 2%, for IL-1ra, 115 ± 5%, and for TGF-β1, 73 ± 26% of the basal levels without effector addition. In LPS-stimulated cultures, Lys[Z(NO_2)]-OH treatment resulted in 118 ± 12% TNF-α, 103 ± 12% IL-6, 97 ± 6% IL-1ra, and 80 ± 12% TGF-β1. All values are related to samples without Lys[Z(NO_2)]-OH (mean±SEM of four independent experiments). In the concentration used, Lys[Z(NO_2)]-OH was not cytotoxic for monocytes.

Other critical properties of monocytes are the extravasation into inflamed tissue through the endothelium, the migration toward regions of inflammation, and phagocytosis of pathogens. These processes require firm adhesion of monocytes to endothelial cells, to extracellular matrix molecules, and to pathogens. As shown in Figure 6B, we observed that monocytes preincubated with Lys[Z(NO_2)]-thiazolidide adhere clearly lesser to fibronectin-coated plates than control cells placed in medium without inhibitor. Pretreatment of the cells with 1 mM $MnCl_2$, inducing direct integrin activation, enhanced adhesion to fibronectin, indicating that integrins are involved in adhesion of activated monocytes to fibronectin. Lys[Z(NO_2)]-thiazolidide significantly reduces basal as well as $MnCl_2$-induced adhesion. In contrast, Lys[Z(NO_2)]-OH, given in the same concentration as Lys[Z(NO_2)]-thiazolidide, did not influence monocyte adhesion to fibronectin significantly (three independent experiments). Basal adhesion increased slightly in the presence of Lys[Z(NO_2)]-OH, up to 140 ± 13% (mean±SD). $MnCl_2$-induced adhesion remained unchanged (97±9%).

DISCUSSION

In the present study, we examined the nature of a DP IV-like cell surface enzyme of peripheral blood monocytes. We clearly demonstrated expression of the DP IV-like enzyme attractin on transcript and protein levels. Furthermore, we first presented data suggesting the influence of Lys[Z(NO_2)]-thiazolidide, which was regarded formerly as a specific DP IV inhibitor, on different monocyte functions.

In agreement with Bauvois et al. [16], we were able to measure a DP IV-like surface activity on human peripheral blood monocytes, which did not bind the monoclonal anti-DP IV/CD26 antibody L272, indicating that monocytes did not express DP IV on the cell surface. This confirms previous findings demonstrating that the monoclonal anti-CD26 antibodies TA1 and CB.1 neither bind monocytes nor inhibit the DP IV-like activities of monocytes [16]. Moreover, we did not observe DP IV/CD26 expression in monocytes on the transcript level. From the DASH proteins with DP IV-like enzyme activity, besides DP IV, only attractin and FAP-α occur as membrane-bound ectoenzymes and could be the source for the DP IV-like cell surface activity of monocytes. The enzymatically active DP IV-like enzymes DPP8, DPP9, and DP II are soluble, intracellular proteins, which should not have access to extracellularly given substrates. As selective inhibitors for DPP8 and DPP9 do not exist, we could not completely exclude that the intracellular, localized DP IV-like enzymes somehow participate in the degradation of Gly-Pro-pNA given into the supernatant. Moreover, we identified DPP8 and DPP9 mRNA in peripheral blood monocytes (S. Wrenger, unpublished results). However, the identification of a DP IV-like activity in the membrane fraction of peripheral blood monocytes indicates that at least a part of the DP IV-like surface activity of peripheral blood monocytes is membrane-bound. In accordance with our results, it was shown previously that the DP IV-like surface activity of the monocytic cell line U937 also did not bind to anti-CD26 antibodies. For this activity, the association to the plasma membrane was demonstrated clearly by the analysis of plasma membrane fractions of U937 cells [16].

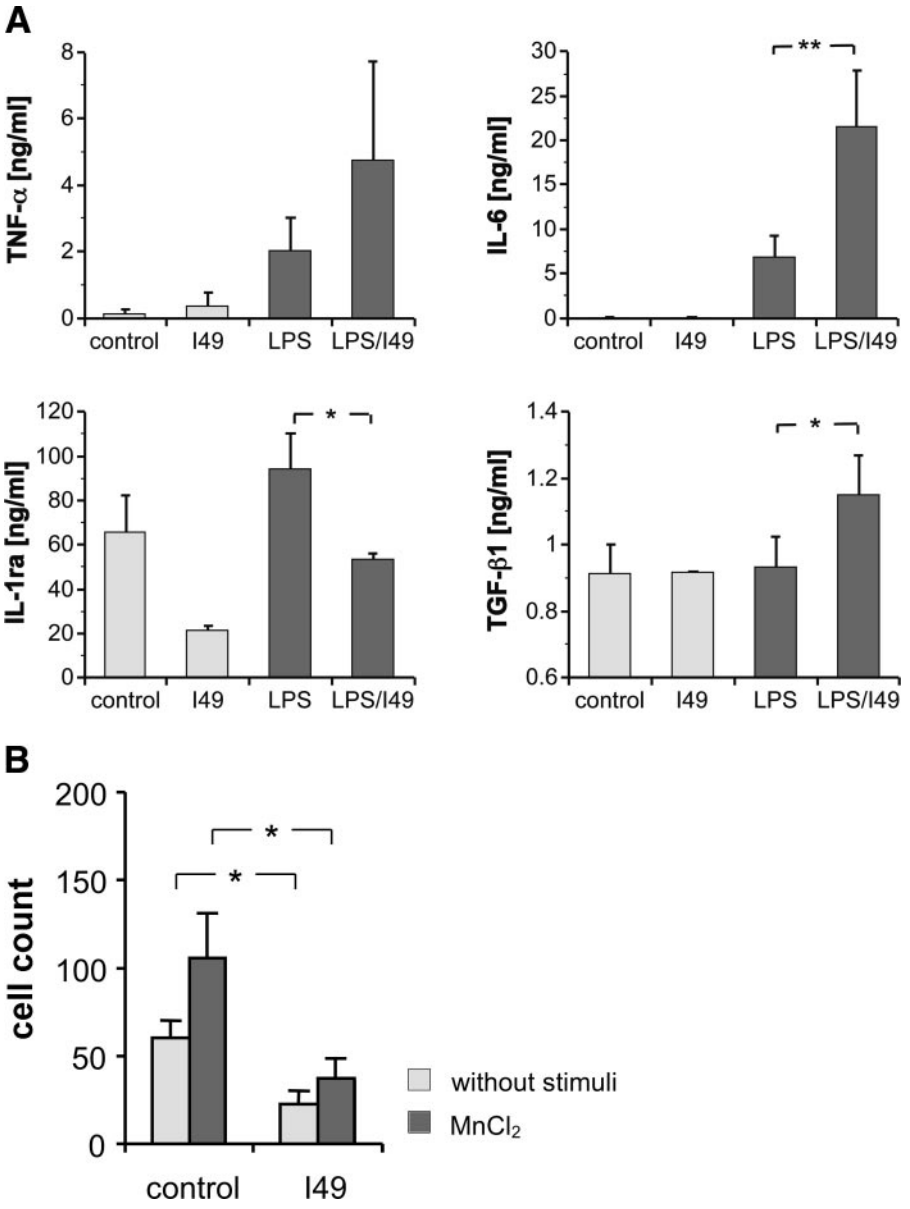

Fig. 6. Effects of the protease inhibitor Lys[Z(NO_2)]-thiazolidide (I49) on monocyte functions. (A) Peripheral blood monocytes were stimulated with 1 μg/ml LPS and incubated with 50 μM Lys[Z(NO_2)]-thiazolidide. "Control" specifies cells incubated with medium only. After 24 h, cell culture supernatants were collected, and cytokines were determined by ELISA. Original bars indicate mean values ± SEM of seven independent experiments for TNF-α, IL-6, and IL-1ra, each carried out in duplicate. For latent TGF-β1, data are shown as mean ± SEM of six independent experiments, each carried out in duplicate (*, $P<0.05$; **, $P<0.01$). (B) Adhesion of freshly prepared peripheral blood monocytes to fibronectin-coated culture dishes after 30 min of preincubation with 50 μM Lys[Z(NO_2)]-thiazolidide (I49). Control cells were incubated in the absence of inhibitor. To induce integrin activation, 1 mM $MnCl_2$ was added during preincubation. After 30 min adhesion to fibronectin at 37°C, nonadherent cells were removed, and remaining cells were fixed and counted. Original bars indicate mean values ± SEM of four independent experiments (*, $P<0.05$).

From the membrane-bound DP IV-like enzymatically active proteins, FAP-α had to be excluded, as FAP-α mRNA could not be amplified from monocytic RNA by PCR (data not shown). This result was expected, as for FAP-α, a tightly regulated distribution restricted to activated fibroblasts was known, whereas normal adult tissues are generally FAP-α-negative [26]. However, we observed high attractin mRNA levels and attractin cell surface immunoreactivity of monocytes. Moreover, attractin immunoprecipitates from monocyte lysates were capable of hydrolyzing Gly-Pro-*p*NA, indicating that monocytic attractin immunoreactivity has DP IV-like activity. For the first time, the nature of the DP IV-like enzyme expressed on monocytes could be, at least partly, assigned to attractin. High attractin transcript levels of monocytes confirmed these data.

In PBMC, the relative attractin transcript level was approximately 10 times lower than in monocytes. Moreover, we observed that the attractin transcript level in PBMC decreased 24 h and 48 h after PHA stimulation. On the protein level, Duke-Cohan et al. [27] observed a rapidly increased surface expression of attractin on PHA-stimulated T cells with a peak 24 h after PHA addition, which then decreased beginning at Day 3 after stimulation. The decreased attractin transcript levels we observed would suggest that the decreased attractin protein expression on the T cell surface 3 days after PHA treatment is a result of a diminished de novo protein biosynthesis.

Moreover, we showed that Lys[Z(NO_2)]-thiazolidide, a widely used DP IV inhibitor, is not specific for DP IV, as it also inhibited human plasma attractin in the nanomolar range. This underlines previous findings, indicating that attractin, although it is not structurally related to DP IV, was found to exert strikingly similar substrate specificities [28]. Moreover, it is notable that Lys[Z(NO_2)]-thiazolidide inhibits human plasma attractin much better than DP IV, as indicated by the lower IC_{50} value for attractin than for DP IV.

Selected inhibitors of DP IV are currently under investigation in Clinical Phase II and III trials for the treatment of Type

2 diabetes and represent a new class of drugs for the treatment of this disease. These inhibitors enhance the half-life of the insulin secretion-inducing incretins, glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1, leading to blood glucose level reduction [29]. The immunosuppressive effects of several DP IV-inhibiting compounds, which were observed in vitro and in vivo, suggest that these compounds are also suitable drugs for the treatment of autoimmune diseases, such as RA, colitis, MS, and other disorders associated with an over-reaction of the immune system [5].

In previous studies, the effects of different inhibitors of DP IV on the stimulation of PBMC, especially of T cells, were examined in detail as yet. It is evident, however, that in arthritis and in MS, not only autoreactive lymphocytes but also mononuclear phagocytes are involved in the severe tissue destructions [17, 30]. We observed that Lys[Z(NO_2)]-thiazolidide not only inhibited monocytic DP IV-like activities efficiently but also altered the release of the cytokines IL-6, IL-1ra, and TGF-β1. These are the first data indicating that monocytes could be a target for systemically administered inhibitors of DP IV, although monocytes do not express DP IV.

Moreover, Lys[Z(NO_2)]-thiazolidide decreased the adhesion of monocytes to fibronectin. Adhesion is important for monocyte function, as it is necessary for migration into regions of inflammation, for binding to pathogens, and for subsequent phagocytosis and elimination of pathogens. Cell adhesion to fibronectin is usually mediated by β1-integrins. Limited adhesion by Lys[Z(NO_2)]-thiazolidide treatment indicates a clearly reduced monocyte functionality. As attractin is composed of several variants of epidermal growth factor motifs, a CUB domain, a C-type lectin-like domain, and plexin-semaphorin-integrin motifs, it could be involved directly in cell–cell interactions and cell adhesion processes. Some years ago, plasma attractin was found to somehow mediate interactions between T cells and monocytes [7]. The processes underlying the decrease of monocyte adhesion to fibronectin mediated by Lys[Z(NO_2)]-thiazolidide remain to be addressed in future studies.

The suppression of monocyte functionality should be beneficial for the treatment of autoimmune diseases in which mononuclear phagocytes play a crucial role in the pathogenesis. Recently, we observed decreased clinical scores and a postponed disease onset of EAE in Lys[Z(NO_2)]-pyrrolidide-treated mice linked to higher myelination and decreased vacuolation [15]. These effects could be the consequence of a combined suppression of reactive T cells and attacking monocytes by the inhibitor. Moreover, the expression of DP IV on human B cells and a restricted subpopulation of cattle dendritic cells were reported, and for alveolar macrophages, DP IV-like activities were measured with DP IV substrates [31–34]. Thus, it is likewise that the inhibition of DP IV activities could also influence the function of antigen-presenting cells other than peripheral blood monocytes.

Our results presented here clearly demonstrate that monocytes express the DP IV-like protein attractin on the cell surface and that human plasma attractin is inhibited by Lys[Z(NO_2)]-thiazolidide. Thus, attractin has to be taken into account as a potential target for systemically administered drugs directed to DP IV-like enzyme activity. Moreover, altered cytokine levels in the presence of Lys[Z(NO_2)]-thiazolidide gave the first evidence that substances inhibiting DP IV and DP IV-like enzymes could influence monocytes. Reduced cell adhesion indicates that such compounds could downregulate the monocyte function.

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