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Inhibition of CD26/Dipeptidyl Peptidase IV Enhances CCL11/Eotaxin-Mediated Recruitment of Eosinophils In Vivo

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Chemokines mediate the recruitment of leukocytes to the sites of inflammation. N-terminal truncation of chemokines by the protease dipeptidyl peptidase IV (DPPIV) potentially restricts their activity during inflammatory processes such as allergic reactions, but direct evidence in vivo is very rare. After demonstrating that N-terminal truncation of the chemokine CCL11/eotaxin by DPPIV results in a loss of CCR3-mediated intracellular calcium mobilization and CCR3 internalization in human eosinophils, we focused on the in vivo role of CCL11 and provide direct evidence for specific kinetic and rate-determining effects by DPPIV-like enzymatic activity on CCL11-mediated responses of eosinophils. Namely, it is demonstrated that i.v. administration of CCL11 in wild-type F344 rats leads to mobilization of eosinophils into the blood, peaking at 30 min. This mobilization is significantly increased in DPPIV-deficient F344 rats. Intradermal administration of CCL11 is followed by a dose-dependent recruitment of eosinophils into the skin and is significantly more effective in DPPIV-deficient F344 mutants as well as after pharmacological inhibition of DPPIV. Interestingly, CCL11 application leads to an up-regulation of DPPIV, which is not associated with negative feedback inhibition via DPPIV-cleaved CCL11(3–74). These findings demonstrate regulatory effects of DPPIV for the recruitment of eosinophils. Furthermore, they illustrate that inhibitors of DPPIV have the potential to interfere with chemokine-mediated effects in vivo including but not limited to allergy. The Journal of Immunology, 2008, 181: 1120–1127.

Chemokines represent a family of small cytokines that attract and activate leukocytes, resulting in a pivotal role in various diseases (1, 2). In the case of allergic diseases it can be expected that the local concentration of chemokines such as CCL11/eotaxin and the expression of its corresponding receptor, the CCR3 determine to a large extent their biological effects, for example the recruitment of eosinophils. N-terminal truncation of chemokines by peptidases such as dipeptidyl peptidase IV (DPPIV), regulating their biological activity, adds another level of complexity to their local action at inflammatory sites, including but not limited to allergy-like reactions of the skin.

DPPIV is outstanding within the group of serine proteases, because it is abundantly distributed, being enzymatically active in both soluble and membrane-bound forms (3). The principle of the inhibition of the serine protease DPPIV to block the degradation of the insulinotropic peptide-hormone GLP-1 has opened up new avenues for the treatment of diabetes type 2 (4). Because DPPIV is not specific for insulinotropic hormones such as GLP-1 and also exerts a modulating effect on a broad range of substrates significantly involved in the regulation of body homeostasis, unwanted effects cannot be excluded (5, 6). Substrates of DPPIV such as neuropeptide Y and substance P are involved in cardiovascular regulation, feeding regulation, and mediation of pain (7, 8). Moreover, DPPIV enzymatic activity potentially modulates immune functions in particular via the processing of chemokines (9).

Thus, in the context of allergic diseases, it is of primary importance to evaluate in vivo, whether the modulation of the local activity of the CCR3 ligand CCL11/eotaxin is affected upon systemic inhibition of DPPIV. Continuous blockade of DPPIV might prolong the activity of locally accumulated CCR3 ligands, ultimately leading to an exacerbation of inflammatory processes. Our data indicate that inhibition of DPPIV increases the mobilization and recruitment of eosinophils thereby promoting allergy-like responses mediated by CCL11 in vivo and give warning of immuno-regulatory dysfunctions in patients receiving DPPIV inhibitors.

Materials and Methods

Animals

A DPPIV-mutant F344 rat strain from Charles River Sulzfeld/Germany [F344/Crl(GER)] (DPPIV<sup>−/−</sup> rats), and a wild-type-like substrain derived from Charles River Portage/USA [F344/CrlPor] (DPPIV<sup>+/+</sup> rats) were obtained from a breeding colony kept in barrier-reared conditions as previously described at the Central Animal Laboratory at Hannover Medical School, Germany (10). All research and animal care procedures were approved by the Lower Saxony state district government (Hannover, Germany).

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Materials

Human CCL11 was obtained from PeproTech and n-nonanoyl (NNY)-CCL14 was prepared as previously described (11). CCL11,3–74, was prepared as follows: CCL11 (0.5 mg) was dissolved in 1.5 ml of 0.01 M Tris-HCl buffer (pH 7.4). DPPIV (0.1 U/µl) was added and the reaction was conducted at 37°C. After 2 h the reaction was quenched with 50 µl of trifluoroacetic acid (TFA) and CCL3–74 was isolated using C18 chromatography. The resulting peptide was analyzed using reverse phase HPLC, electrospray mass spectrometry (MS; purity >90%). The reversible, competitive transition-state analog inhibitor of DPPIV Ile-thia has been previously described (4).

Peelution of CCL11 or NNY-CCL14, each at a concentration of 10⁻³ M, was performed for 24 h before biological assays as described previously (12). In addition, in several experiments, the DPPIV inhibitor Ile-thia (1.25 × 10⁻⁴ M) was added to samples containing either porcine kidney DPPIV or human serum.

Cells

Eosinophils were purified from the venous blood of normal non-atopic healthy or atopic volunteers as described previously (13), all having given their informed consent according to the local research ethics committee at Hannover Medical School.

For the preparation of T cells, PBMCs were isolated from blood by Ficoll gradient centrifugation. PBMCs were then washed and finally re-suspended at 7 × 10⁶ cells/ml and cultured as previously described (14). Stably transfected murine pre-B 300.19 cells expressing human CCR3 (15) were obtained from B. Moser (Theodor Kocher Institute, University of Berne, Switzerland).

Normal human epidermal keratinocytes were obtained from Promocell and cultured in the appropriate keratinocyte growth medium with supplement mix (Promocell).

Cell-based assays

Analysis of intracellular Ca²⁺ fluxes in stably transfected murine pre-B 300.19 cells expressing human CCR3 was performed on a fluorescence image plate reader (FLIPR) system (Molecular Devices) as previously described (16). CCR3 internalization in eosinophils and flow cytometric measurements were performed as described elsewhere (13, 17). Quantification of CD26 gene expression on keratinocytes using real-time PCR was performed as described previously (18).

Mass spectrometry analysis of CCL11 processing by human T cells

To analyze the processing of the naturally occurring chemokine CCL11, 10 µg of CCL11 were incubated with 1–5 × 10⁵ cultured human T cells (1 × 10⁶ cells/100 µl) in Dulbecco’s PBS (pH 7.5) at 37°C and 5% CO₂. After 1 and 24 h, samples of 20 or 50 µl were taken and the degradation was stopped with 2 or 5 µl of TFA and frozen to −20°C immediately. Cleavage products of CCL11 were determined offline with a MALDI-MS (Voyager DE-Pro) in linear positive ion mode, as described recently (11).

Intravenous application of CCL11 and measurement of blood eosinophils

In the first set of in vivo experiments, 10 nmol/kg body weight (BW) hCCL11 in 200 µl of saline was injected into the lateral tail vein of DPPIV⁺⁺⁺ rats (n = 13) vs DPPIV⁻⁻⁻ rats (n = 12). Blood was taken from the retroorbital sinus of isoflurane anesthetized rats at 1 min before and at 10, 30, and 120 min after injection of CCL11. For the quantification of eosinophils, 200 µl of blood/time point were lysed, leukocytes counted, and unfixed cytopsins stained by a combined May-Grünwald/Giemsa staining. As previously described, at least 1000 cells were differentiated on each slide by two independent observers (19).

Intradermal application of CCL11 and quantification of eosinophil infiltration

CCL11 was administered intradermally at a dose of 0, 10 (0.084 µg), 100 (0.84 µg), and 1000 (8.4 µg) pmol of 100 µl of saline in DPPIV⁺⁺⁺ (n = 6/dose) and DPPIV⁻⁻⁻ (n = 5/dose) rats with two injection sites of the same dose, one on either side of the shaved back-side, respectively. Vehicle (saline) and non-injected skin served as controls. Within the same experimental series, a combined solution of 1000 pmol of CCL11 and the DPPIV inhibitor Ile-thia at a dose of 30 µg (MW: 260.4 g/mol) was injected intradermally using DPPIV⁻⁻⁻ rats (n = 5). Punch biopsies of 8-mm diameter were always taken under isoflurane anesthesia 4 h after injection of CCL11. Biopsies were either fixed in formalin, embedded in paraffin, and 4-µm sections were prepared or they were snap-frozen and further processed for immunohistochemistry. Paraffin sections were stained with Giemsa solution, and the eosinophil infiltrates were evaluated. In each section, eosinophils located in the dermis were counted in 50 randomly selected grids, and the number of positive cells (i.e., eosinophils) per cm² was calculated as previously described (20).

DPPIV immunohistochemistry

Immunohistochemistry by avidin-biotin complex (ABC) and alkaline phosphatase-anti-alkaline phosphatase (APAAP) methods were performed to provide morphological information on expression and localization of DPPIV protein in snap-frozen skin biopsies derived from naive, vehicle (saline), and CCL11 (1000 pmol) experimental conditions. Sections were processed by incubating for 4 h with anti-rat DPPIV/CD26 mAb (clone 5E8, 1:500, Cell Science) followed by incubation with secondary Abs (1: 200 in 5% normal goat serum) either for ABC or APAAP stains as previously described (20, 21). Leukocyte subpopulations recruited to the skin were characterized by double APAAP stainings and quantified as previously described (20). Control sections were included, in which primary Ab was omitted. APAAP stainings appeared to be specific while endogenous peroxidase activity interfered with ABC stainings. Sections from different levels of the APAAP-stained sections were evaluated by two independent observers blinded to the treatment as previously described (20).

DPPIV enzymatic assays

Effect of CCL11,3–74, on DPPIV-catalyzed hydrolysis of glycyrl-prolyl-7-amido-4-methylcoumarin (GP-AMC) was analyzed using recombinant human DPPIV expressed in Pichia pastoris (22). Activity of DPPIV was monitored by the release of AMC measured at an excitation/emission of 380/460 nm. Assays were done at 30°C using HEPES buffer pH 7.6 (40 mM) using a total volume of 30 µl in a 384-well microplate. Substrate concentration in the assay was 10 µM and CCL11,3–74, was used in final concentrations of 10⁻⁸ to 10⁻⁴ M.

Statistical analysis

For the in vivo study on eosinophil mobilization into blood, the data obtained at different time points were subjected to analysis by ANOVA for repeated measurements using Statview 5.0 software (SAS Institute) with treatment being the between groups factor and repeated measures of cell numbers being the within subject factor. Afterwards, data were analyzed by one-way ANOVAs split by time, if appropriate. For the in vivo study on eosinophil infiltration into skin, one-way ANOVA was performed with either genotype or dosages being the factors. The Fisher protected least significant difference was used for post hoc comparisons. Significant differences (p < 0.05) between controls and test groups are indicated by asterisks with * < 0.05, ** < 0.01, and *** < 0.001. All data are given as arithmetic mean ± SEM.

Results

Purified DPPIV abrogates CCL11-induced CCR3 activation

The reduction of DPPIV-pretreated CCL11-induced calcium mobilization is completely reversed by coinoculation of CCL11 with DPPIV and the DPPIV inhibitor isoleucine thiazolidide (Ile-thia) (Fig. 1A). In contrast to this, 24-h incubation of NNY-CCL14 with DPPIV at 37°C does not affect its ability to mobilize intracellular calcium per se (data not shown). Incubation of CCL11 for 24 h with DPPIV, which results in the truncated form CCL11(3–74), strongly reduces its capacity to internalize the principal CCL11 receptor CCR3 (Fig. 1B, left middle panel, black bold line). However, a residual activity remains. Again, inhibition of Ile-thia-mediated CCL11 processing by DPPIV prevented the loss of biological activity (Fig. 1B, left lower panel, black bold line) whereas the activity of NYY-CCL14 is not affected by DPPIV pretreatment (Fig. 1B, right middle panel).

Effects of CCL11 are down-regulated by human serum containing DPPIV activity

As DPPIV activity might significantly contribute to processing and subsequent inactivation of circulating chemokines in vivo, the ability of human serum to process CCL11 was studied. The
FIGURE 1. Preincubation of CCL11 with DPPIV as well as with serum reduces its biological activity and processing of CCL11 by T cells primarily depends on DPPIV. Human chemokines were incubated overnight with purified DPPIV in the presence or absence of Ile-thia. Overnight incubation with buffer served as control. The effects of chemokine processing and inhibition thereof were monitored with different cellular assays. A. Intracellular calcium mobilization was measured by fluorescence image plate reader in CCR3-transfected cells loaded with Fluo-4 upon stimulation with differentially pretreated CCL11. DPPIV treatment of the resistant NNY-CCL14 did not affect its biological activity (data not shown). B. Internalization of CCR3 from human eosinophils was analyzed by flow cytometry. Cells were treated for 30 min with the indicated differentially pretreated chemokines. Thereafter, cells were stained with anti-CCR3 mAb, measured with the FACSCalibur, and histogram analysis was performed. The gray-filled curve displays anti-CCR3 staining without chemokine stimulation; broken line, isotype control; bold line, anti-CCR3 staining after chemokine treatment. C. Human chemokines were incubated overnight with human serum in the presence or absence of Ile-thia. Overnight incubation with buffer served as control. Serum was used for processing of chemokines instead of purified DPPIV. Data are presented as original plots of one representative experiment out of four. D. MALDI-MS analysis of CCL11 degradation. One representative experiment out of five is shown; 10 μg of CCL11 were incubated with 5 × 10⁶ T cells for 1 h (top panel) and 24 h (bottom panel). Calculating area under curve, around 30% of CCL11(1–74) was processed to CCL11(3–74) within 24 h assuming that the cleaved uncharged N-terminal dipeptide Gly-Pro does not significantly influence the ionization and thus the detection of an 8-kDa chemokine.

Cultured CD26⁺ T cells cleave the N-terminal dipeptide of CCL11

CD26 on the surface of T cells is another DPPIV source, which might contribute to processing of CCL11 in vivo. To investigate the degradation of CCL11 by cell surface-expressed DPPIV, recombinant CCL11 was incubated with cultivated human T cells, expressing CD26/DPPIV. After 1 and 24 h, the reaction was stopped by addition of 2% TFA and analyzed offline using a MALDI-MS. After 1 h, no processing could be observed (Fig. 1D, upper panel) but after 24 h (Fig. 1D, lower panel) a main cleavage product of CCL11 (Mᵦ 8360.84 Da) with a molecular mass of 8207.97 Da corresponding to CCL11(3–74) (Mᵦ 8206.68 Da) was
detected. In control experiments, a 0.9% sodium chloride solution was added instead of CCL11 and MALDI-MS analysis was conducted. No molecular masses in the range of CCL11 were detected, demonstrating that endogenous CCL11 is not produced under the conditions used. In the presence of the specific DPPIV inhibitor Ile-thia, the degradation of CCL11 to CCL11(3–74) was completely abrogated (data not shown). These data demonstrate that CCL11 can be cleaved to CCL11(3–74) by cultivated human T cells and that this processing is blocked by the DPPIV-specific inhibitor Ile-thia.

CCL11-induced eosinophil mobilization into blood is augmented in DPPIVneg rats

As the in vitro experiments have shown that both soluble DPPIV present in human serum as well as membrane-bound DPPIV expressed on the surface of T cells are processing CCL11 and thereby impairing its biological activity, we were interested to study whether this might be of relevance in the mobilization of eosinophils in vivo. For this purpose, two Fischer 344 (F344) rat substrains, one expressing high (DPPIVpos) and the other negligible DPPIV-like activity (DPPIVneg), were used in different experimental settings (10). First, the mobilization of eosinophils into the blood as an initial key step in allergic inflammation was studied to determine a possible role of DPPIV in the regulation of CCL11 participating in this process of inflammation.

The eosinophil fraction of leukocytes in the peripheral blood was determined directly before the i.v. application of CCL11 (10 nmol per kg of body weight) and 10, 30, and 120 min thereafter. The basal eosinophil blood count before injection was 1.0–1.5%. After an initial slight decrease at 10 min after CCL11 injection, the eosinophil numbers increased to a maximum at 30 min in both groups. A significant increase of the eosinophil blood count above the basal eosinophil count was observed in the DPPIVneg compared with DPPIVpos rats at 1000 pmol (Fig. 2, A and B). To confirm the findings in the genetic model, we additionally performed an experiment within a pharmacological setting, as a lifelong deficiency of DPPIV is not directly comparable to a limited pharmacological intervention. Fig. 3 summarizes the results of an experiment on the CCL11-dependent recruitment of eosinophils to skin using DPPIVneg and DPPIVpos rats, the latter treated either with saline or with the DPPIV inhibitor Ile-thia along with an intradermal CCL11 application. Compared with DPPIVpos rats receiving saline plus CCL11 injections (Fig. 3A; see also eosinophils in a representative skin section in Fig. 3B), application

The DPP IV inhibitor Ile-thia gives rise to increased intradermal eosinophil recruitment

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**FIGURE 2.** Mobilization of eosinophils into the blood and recruitment into the skin by CCL11 is modulated DPPIV. A, Effect of i.v. applied CCL11 on eosinophil mobilization into blood. Blood was taken from the retroorbital sinus of the rats 1 min before and 10, 30, and 120 min after injection of CCL11. Percentage of eosinophils is displayed in black (wild-type rats, DPPIVpos; n = 12) and white (DPPIV-deficient rats, DPPIVneg; n = 13) columns. Data represent means ± SEM and the asterisk (*, p < 0.05) indicates significant differences between wild-type and DPPIV-deficient F344 rat substrains. B, Dose-dependent recruitment of eosinophils into the skin following intradermal injections of CCL11 and NNY-CCL14. Chemokines were injected at a dose of 10, 100, and 1000 pmol (n = 5–6/dose) and the number of eosinophils per cm² was quantified. Data represent means ± SEM and the asterisks (*, p < 0.05; **, p < 0.01) indicate significant differences between wild-type and DPPIV-deficient F344 rat substrains.
of the DPPIV inhibitor Ile-thia plus CCL11 resulted in significantly increased numbers of intradermal eosinophils (Fig. 3A and example in Fig. 3C), which were similar to those found in DPPIVpos rats.

**Intradermal injection of CCL11 induces DPPIV expression in the skin and leukocyte recruitment**

As local CCL11 application and consecutive inflammatory processes may interact with the expression level of DPPIV, immunohistochemistry on snap-frozen skin biopsies as well as CCL11 co-incubation with cultured keratinocytes were performed to study whether and how DPPIV expression is induced in the skin (Fig. 4). There is a low expression of DPPIV in biopsies of untreated and vehicle (saline)-treated skin (Fig. 4A, B, D, and F). Fig. 4A provides an overview of the epidermis, dermis, and s.c. tissue at low magnification of untreated rat skin while Fig. 4B, D, and F illustrate DPPIV immunoreactivity of vehicle-treated skin at higher magnifications of the boxed areas. Interestingly, intradermal injection of CCL11 up-regulates the DPPIV-like immunoreactivity (Fig. 4C, E, and G). DPPIV immunoreactivity was primarily located in the epidermis, at hair follicles, and at the fascia between dermis and subcutis (Fig. 4C, E, and G, see arrows), illustrating that local expression of DPPIV is up-regulated by the CCL11-induced inflammatory process in the area surrounding the intradermal injection site. Relative DPPIV gene expression in cultured keratinocytes is not induced by coinoculation with CCL11 alone across time but does increase after stimulation with IFN-γ, TNF-α, and IL-1β (Fig. 4H). Apart from eosinophils, also DPPIV/CD26 positive T cells, monocytes, and NK cells are time-dependently

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**FIGURE 3.** Recruitment of eosinophils into the skin following intradermal injection of CCL11 depends on DPPIV. CCL11 was intradermally injected at a dose of 1000 pmol either in combination with saline or the DPPIV inhibitor Ile-thia in DPPIVpos vs DPPIVneg rats and eosinophil recruitment quantified in samples collected 4 h later (A). Data represent means ± SEM and significant differences in comparison with DPPIVpos rats receiving CCL11 are indicated by asterisks (**, p < 0.01; ***, p < 0.001) (n = 5–6/group). B and C provide representative May-Grünwald/Giemsa-stained sections of rat skin. Eosinophils are primarily located close to small vessels.

**FIGURE 4.** Intradermal injections of CCL11 up-regulates DPPIV immunoreactivity in the skin and is associated with recruitment of DPPIV-positive leukocyte subpopulation mediating cytokine-induced expression of DPPIV on keratinocytes. Immunohistochemistry for DPPIV-like immunoreactivity on skin biopsies of untreated, vehicle-treated, and CCL11-treated F344 rats is presented. A, Overview of the epidermis, dermis, and s.c. tissue of untreated rat skin at low magnification; B, D, F, DPPIV immunoreactivity of vehicle-treated skin at higher magnification; C, E, G, Photographs from a representative section of CCL11-treated skin at higher magnification. Arrows indicate DPPIV immunoreactivity/protein in the epidermis, at hair follicles, and at the fascia between dermis and subcutis. Relative DPPIV gene expression in cultured keratinocytes after coincubation with CCL11 across time and/or stimulation with IFN-γ, TNF-α, and IL-1β is provided (H), and cell numbers of DPPIV/CD26-positive T cells, monocytes, and NK cells in skin biopsies across time (n = 6/time points at 0, 4, 8, 12, and 24 h) are given (I), together providing evidence for an indirect induction of DPPIV on keratinocytes via cytokines released by activated cells and not directly via the action of CCL11.
FIGURE 5. DPP IV enzymatic activity is not inhibited by the N-terminal truncated substrate CCL11(3–74). The diagram illustrates the effect of CCL11(3–74) on the activity of human recombinant DPP IV. Activity was measured by the release of amido-4-methylcoumarin (AMC) from glycyl-prolyl-7-AMC (10 μM). Activity without potential inhibitor was set to 100%. Data represent mean ± SD of three measurements.

recruited to the skin with T cells being initially present at 4 h already followed by a peak of monocytes at 8 h and a delayed peak of NK cell numbers at 12 h (Fig. 4f). Together these data provide evidence for an indirect induction of DPP IV on keratinocytes via cytokines released by activated cells and not directly via the action of CCL11/eotaxin.

No feedback inhibition of DPP IV enzymatic activity is given by N-terminal truncated CCL11(3–74)

The induction of local DPP IV expression in the vicinity of CCL11 injection sites might inhibit a further progression of the CCL11-induced inflammatory process along with eosinophil recruitment. Thus, it was of interest to establish whether DPP IV-truncated CCL11(3–74) exerts an inhibitory effect on DPP IV-like enzymatic activity, thereby leading to a feedback inhibition and consecutive aggravation of the inflammatory process. In Fig. 5 it is shown that DPP IV is not inhibited by CCL11(3–74), coincubation. No feedback regulation by CCL11(3–74) could be found even within the micromolar range. Thus, N-terminal truncated CCL11(3–74) does not exhibit feedback inhibition of DPP IV enzymatic activity, which is in contrast to many other substrates of DPP IV.

Discussion

It is now generally accepted that DPP IV acts as an important regulator of multiple physiological processes. It catalyzes the release of dipeptides from the N-terminus of circulating hormones, neuropeptides, and chemokines. Moreover, DPP IV is engaged in T cell-dependent immune responses and has been associated with cell adhesion and tumor metastasis (19, 23, 24). As DPP IV appears to act at a checkpoint of blood glucose homeostasis via potentiation of GLP-1-mediated stimulation of the entero-insular-axis and concomitant release of insulin, it has emerged as a target for the treatment of type 2 diabetes (4). At present, several DPP IV inhibitors are in the late stage of clinical development and some of them have reached the market for this indication (25). However, based on its ubiquitous expression and pleiotropic functions, systemic and continuous pharmacological blockade of DPP IV might act as a double-edged sword, as not only the “beneficial” release of insulin is increased but also immune regulation might be affected in parallel.

The presented data provide direct evidence that under a status of genetically or pharmacologically induced DPP IV deficiency, a prolonged activity of non-processed CCL11 along with CCR3 activation is evident in vitro as well as in vivo, thereby illustrating a potential scenario of unwanted effects during therapy of diabetes type 2. Because CCR3 represents the major chemokine receptor of eosinophils, which themselves are centrally involved in allergic diseases, dysregulation of these cells might be a consequence and problem associated with pharmacological inhibition of DPP IV (26, 27). In fact, FDA-approval of the DPP IV-inhibitor vildagliptin was halted (see www.novartis.com/downloads_new/investors/2006.11.13%20Galvus%20US%20NDA%20Review.pdf), as there was evidence of skin lesions with blistering in one or more monkey tox studies.

As inactivation of chemokines has mostly only been shown in vitro (28), the relevance of DPP IV processing remains questionable. To address this issue in the case of CCL11, we applied an experimental approach in vivo. Recently, it has been shown that CCL11 is able to mobilize eosinophils into blood (29). Mobilization of eosinophils from the bone marrow is thought to represent an initial key step in inflammatory conditions involving eosinophils, such as allergic asthma or atopic dermatitis. The relevance of CCL11 on the mobilization of eosinophils remains contradictory as one group has shown a significant reduction of circulating eosinophils in mice with a targeted disruption of CCL11 compared with wild-type mice (30), while another group found no difference (31). Nevertheless, it is suggested that CCL11 is involved in this process together with the main player IL-5 (32). In guinea pigs, it has been shown that guinea pig CCL11 induces mobilization of eosinophils with a maximal effect 30 min after i.v. CCL11 injection (29). Later on, it has been demonstrated that human CCL11 is able to cause a dose-dependent increase of circulating eosinophils in the BN rat, which is ∼3-fold higher than the vehicle (33). The increased mobilization in F344 rats expressing DPP IV is similar, peaking 30 min after i.v. infusion. CCL11 injection and the kinetics are comparable to those in the guinea pig. In DPP IVneg F344 rats, the mobilization is significantly enhanced at the timepoint of optimal mobilization, providing a first hint that in vivo activity of CCL11 is modulated by DPP IV. The slow degradation of CCL11 by DPP IV questions the in vivo relevance of this process to some extent. However, the degradation of CCL11 has been shown to be completed in the range of one to two and a half hours (9, 11) which is clearly before the time-point (4 h) when the recruitment of eosinophils was quantified in the present experiments. Moreover, it is conceivable, that the DPP IV activity in vivo is even higher, as soluble activity in serum, cell bound activity from T cells and from endothelial cells contribute to overall DPP IV activity. Interestingly, it has been shown that DPP IV activity is found in skin at a significantly higher amount than in serum and that N-terminally truncated CCL11 (desGP) has been purified from supernatants of cultured skin fibroblasts (33, 34).

As a next step, an influence of DPP IV activity on cutaneous CCL11-dependent eosinophil accumulation was investigated. The cutaneous recruitment of eosinophils in rats is supposed to be modulated by DPP IV, as it has been shown that DPP IV is present in rat epidermis (35). Furthermore, the capacity of human CCL11 to attract eosinophils to rat skin has been shown previously (33). A dose-dependent infiltration could be observed in DPP IVneg and DPP IVwes rats. A trend for enhanced cellular recruitment was observed already at the lower concentrations of CCL11 applied in DPP IVwes rats, which became a significant effect at the highest concentration applied, implying that a certain dose is necessary to induce effective cellular recruitment. A similar profile was derived for application of resistant NNY-CCL14 in DPP IVneg rats, substantiating the relevance of DPP IV in limiting the proinflammatory effects of CCL11. To mimic a specific pharmacological intervention and to compare with the findings based on genetic DPP IV deficiency, inhibitor treatment was combined with CCL11 application, revealing also a significant effect at the high dose applied.
These results strongly suggest that in fact DPPIV expression represents an important limiting, negative regulating factor in CCL11-induced effects, as illustrated by but not limited to eosinophil recruitment to the skin. Although the DPPIV inhibitor Ile-thia has been shown to also inhibit the intracellularly located DPPIV-related dipeptidyl peptides DP8 and DP9, the results obtained from the DPPIV<sup>−/−</sup> rats and the in vitro experiments with CCL11 and serum give strong evidence that the observed effects virtually depend on the activity of DPPIV (36).

The relevance of CD26/DPPIV for cellular homing in settings other than inflammatory responses has just recently been impressively demonstrated by Christopherson III and colleagues in the context of hematopoietic stem cell homing and engraftment (37). In the case of bone marrow transplantation, there is a need for augmented homing of cells in contrast to inflammatory disorders. In this study, it was elaborated that inhibition of CD26 on donor cells might be a relevant strategy to enhance the efficiency of transplantation. In contrast to T cells, whose recruitment to inflammatory sites is decreased by DPPIV blockade, CD26-deficient donor cells have increased homing potential. Thus, there are dramatic differences between pathologic condition and type of cells involved with respect to DPPIV activity. A good example suggesting that DPPIV inhibition aggravates eosinophilic inflammation is the observation that DPPIV activity in serum is decreased in subjects with Churg-Strauss syndrome (38). Although it is not clear whether CCL11 has a specific non-redundant role in allergic inflammation, regulating the mobilization and recruitment of eosinophils, the presented data suggest that modulating the activity of DPPIV with specific inhibitors might have significant impact on many physiological and pathophysiological processes such as inflammation, as many other chemokines and several other biologically relevant peptides are substrates of DPPIV.

Overall, we show here that the CCL11-mediated effects on two steps of eosinophil recruitment in vivo are more effective in DPPIV-deficient F344 rats as well as after pharmacological inhibition of DPPIV. In addition, we illustrate that CCL11 application into skin leads to an up-regulation of DPPIV, which is not associated with negative feedback inhibition via N-terminally DPPIV-cleaved-CCL11<sub>1-74</sub>. Together, this demonstrates that the DPPIV-mediated N-terminal truncation of CCL11 limits the recruitment of eosinophils in vivo, which in general exemplifies a critical role of DPPIV for the control of the chemokine network and specifically points to an important role for DPPIV expression and activity in limiting allergic responses.

Nevertheless, so far, the development of DPPIV inhibitors, especially for the treatment of type 2 diabetes, is very promising and the clinical trials, which have been disclosed until recently neither reported on adverse events (39–41), nor were indicative for a modulation of the chemokine system. However, the biology and the consequences of sustained DPPIV inhibition may be different in subjects with comorbidity such as immune and inflammatory disorders or atopy, which obviously have not yet been studied in sufficient detail in any clinical trials. Therefore, in the future, these aspects should be diligently observed, when DPPIV inhibitors are administered. On the one hand, a transient, intermittent treatment with DPPIV inhibitors might be favorable to avoid putative adverse events associated with the chemokine system. In contrast, the application of longer-acting GLP-1 analogues targeting one specific receptor is an appealing alternative for the treatment of type 2 diabetes (42).

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