Circulating CD26 Is Negatively Associated with Inflammation in Human and Experimental Arthritis

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Dipeptidyl peptidase IV (DPPIV, CD26), a protease-cleaving N-terminal X-Pro dipeptide from selected proteins including some chemokines, is expressed both as a soluble form in plasma and on the cell surface of various immune and nonimmune cell types. To gain insights into the pathophysiological role of CD26 in arthritis, we explored DPPIV/CD26 expression during murine antigen-induced arthritis (AIA), an experimental model of arthritis. AIA induction led to reduced plasma DPPIV activity. In CD26-deficient mice, the severity of AIA was increased as assessed by enhanced technetium uptake and by increased histological parameters of inflammation (synovial thickness and exudate). We demonstrated that CD26 controls the *in vivo* half-life of the intact active form of the proinflammatory chemokine stromal cell-derived factor-1 (SDF-1). CD26-deficient mice exhibited increased levels of circulating active SDF-1, associated with increased numbers of SDF-1 receptor (CXCR4)-positive cells infiltrating arthritic joints. In a clinical study, plasma levels of DPPIV/CD26 from rheumatoid arthritis patients were significantly decreased when compared to those from osteoarthritis patients and inversely correlate with C-reactive protein levels. In conclusion, decreased circulating CD26 levels in arthritis may influence CD26-mediated regulation of the chemotactic SDF-1/CXCR4 axis. (*Am J Pathol* 2005, 166:433–442)

CD26, also known as dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.5), is a multifunctional type II transmembrane glycoprotein.¹ This protein is expressed constitutively on

epithelial cells, several types of endothelial cells and fibroblasts, as well as leukocyte subsets such as T, B, and natural killer lymphocytes and macrophages. A soluble form of CD26, lacking the cytoplasmic tail and transmembrane region is also found in plasma and other biological fluids.

The enzyme activity of CD26 is capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine residues in the penultimate position. Distinct enzymes with similar DPP specificity have also been described.² Several cytokines, hematopoietic growth factors, neuropeptides, and hormones share the X-Pro or X-Ala motif at their N-terminus, including substance P, neuropeptide Y, endomorphin-2, GLP-1 (glucagon-like peptide), GIP (glucose-dependent insulinotropic polypeptide), RANTES (regulated on activation normal T cell expressed and secreted), eotaxin, MDC (monocyte-derived chemokine), and SDF-1 (stromal-derived factors).² In many cases, CD26/DPPIV-mediated truncation of natural substrates has drastic effects on the biological activity or function. For instance, SDF-1 is a proinflammatory chemokine that stimulates chemotaxis in leukocytes by binding to its receptor CXCR4 (being itself only recognized by SDF-1).³ *In vitro*, N-terminal processing of SDF-1 by CD26 reduced lymphocyte chemotaxis and CXCR4-signaling properties.⁴ However, it is not known whether CD26 or other enzymes may truncate and inactivate SDF-1 *in vivo*, and if so what the functional consequences may be.

CD26 may also potentially modulate immune responses by directly regulating lymphocytes.^{5,6} On human T cells, CD26 exhibits a co-stimulatory function;⁷ furthermore, CD26 plays an important role in the immune system via its ability to bind adenosine deaminase⁸ and mediates signaling by direct interaction with the cytoplasmic domain of CD45.⁹ Finally, CD26 was also involved in interactions with the extracellular matrix proteins, collagen and fibronectin.¹ The relative contribution of these effects of CD26 on lymphocytes, versus indirect effects

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because of possible cleavage of immunoregulating chemokines or cytokines, is not clear.

In rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease,¹⁰ CD26 may have a dual role, on the one hand stimulating cellular immunity, and on the other hand inhibiting chemokine function. It has been previously reported that in RA patients the number of peripheral blood T lymphocytes expressing CD26 is increased.^{11–13} By contrast, in serum as well as in synovial membrane from RA patients, DPPIV activity or CD26 antigen levels were reduced compared to controls.^{14–16} These studies in RA patients, and similar ones in experimental mouse models of RA¹⁷ suggested that decreased levels of CD26 are associated with severity of inflammation in RA. Paradoxically, it has been reported that pharmacological inhibition of DPPIV enzyme activity could reduce the progression of arthritis in an experimental rat model of RA,^{18,19} suggesting that decreases in DPPIV-activity may alleviate inflammation under some circumstances, and based on these results it was proposed that DPPIV-inhibitors may be useful as treatment of RA.²⁰ However, these inhibitors may have inhibited not just CD26, but also other enzymes that exhibit similar enzyme activity. Thus, the relative roles of CD26 and other related enzymes in RA are not clear. It is also not clear whether the levels of circulating DPPIV-enzyme activity may itself influence the extent of inflammation in RA, or whether expression of this enzyme is down-modulated in inflammatory arthritis. Here we address these questions by analyzing the progression of antigen-induced arthritis (AIA) in CD26 gene knockout mice and wild-type control animals. AIA is a commonly used murine RA model, which is T- and B-cell-dependent, providing an ideal opportunity to dissect the potential role of CD26 in regulating the immune responses and/or in modulating through its peptidase activity crucial substrate for RA. We have finally extended the study of CD26 to patients with RA, in which chronic synovitis is prominent, and with osteoarthritis (OA), in which there is limited synovial inflammation.²¹

Materials and Methods

Animal Studies

Mice

CD26-deficient mice on a C57BL/6 genetic background have been generated before.²² All experiments were performed on females between 8 to 10 weeks of age at the start of the experiment. Age-matched wild-type female C57BL/6 mice were used as controls.

Induction of Experimental Arthritis

AIA was established as previously described.²³ Briefly, mice were immunized at day 0 and 7 with 100 μ g of mBSA (Sigma Chemical Co., Buchs, Switzerland) emulsified in 0.1 ml of complete Freund's adjuvant containing 200 μ g of mycobacterial strain H37RA (Difco,

Basel, Switzerland) by intradermal injection at the base of the tail. On day 0, 2×10^9 heat-killed *Bordetella pertussis* organisms (Berna, Bern, Switzerland) were also injected intraperitoneally as an additional adjuvant. Arthritis was induced at day 21 by intra-articular injection of 100 μ g of mBSA in 10 μ l of sterile phosphate-buffered saline (PBS) into the right knee, the left knee being injected with sterile PBS alone. Collagen-induced arthritis (CIA) was established as previously described.²⁴ Institutional approval was obtained for these experiments.

Isotopic Quantification of Joint Inflammation

Joint inflammation was measured by ^{99m}Tc-Technecium (Tc) uptake in the knee joint, as previously described.²³ Briefly, mice were first anesthetized by methoxyflurane and then injected subcutaneously in the neck region with 10 μ Ci ^{99m}Tc. The accumulation of the isotope in the knee was determined by external γ counting after 15 minutes. The ratio of ^{99m}Tc uptake in the inflamed arthritic knee versus ^{99m}Tc uptake in the contralateral control knee was calculated. A ratio higher than 1.1 indicated joint inflammation.

Histological Grading of Arthritis

At least 12 mice per group were sacrificed, the knees dissected and fixed in 10% buffered formalin for 7 days. Fixed tissues were decalcified for 3 weeks in 15% ethylenediaminetetraacetic acid, dehydrated, and embedded in paraffin. Sagittal sections (5 μ m) of the whole knee joint were stained with safranin-O and counterstained with fast green/iron hematoxylin. Histological sections were graded independently by two observers unaware of animal genotype using the following parameters. Synovial membrane thickness, which reflects the degree of synovial inflammation and hyperplasia, was scored on a scale of 0 to 6 (0 = normal thickness to 6 = maximum thickness). Synovial cell exudate was scored from 0 to 6 according to the amount of inflammatory cells in synovial fluid (0 = no cells, 6 = maximal cell amount). Cartilage proteoglycan depletion, reflected by loss of safranin-O staining intensity, was scored on a scale of 0 (fully stained cartilage) to 6 (totally unstained cartilage) in proportion to severity. For each histopathological parameter, the score (mean $\pm$ SEM) of all slides was calculated.

In Vitro T-Cell Proliferation Assay

Mice were sacrificed according to the experimental protocol. Inguinal lymph nodes and spleen were removed, and single cell suspensions were incubated in RPMI supplemented with β -mercaptoethanol, penicillin, streptomycin, and 1% autologous serum. Lymph node cells and spleen cells (2×10^5 /200 μ l/well) were plated in 96-well flat-bottom plates and stimulated with 0, 10, 50, and 250 μ g/ml mBSA (Sigma). The cells were incubated at 37°C in 5% CO₂ for 48 hours, then 1 μ Ci/well of [³H]-thymidine was added in cultures for 18 hours. The cells were harvested and [³H]-thymidine uptake was measured using a β -scintillation counter.

Determination of Interferon (IFN)- γ Production in Vitro

Lymph node and spleen cells were isolated and cultured with or without the presence of 0 to 250 $\mu\text{g/ml}$ of mBSA. The culture supernatants were harvested after 72 hours for determination of IFN- γ levels. Quantification of cytokine levels production was performed by an enzyme-linked immunosorbent assay (ELISA) for murine IFN- γ (Amersham Pharmacia, Dubendorf, Switzerland).

In Vivo Lymph Node Cell Proliferation

Mice were injected intraperitoneally with 0.2 ml of 5 mg/ml of bromodeoxyuridine (BrdU, Sigma Chemical Co.) 1 hour before sacrifice. At the end of the experiment, inguinal lymph nodes were dissected. Lymph node paraffin sections were subjected to BrdU immunohistochemistry. Briefly, paraffin sections, treated with 3% H_2O_2 to block endogenous peroxidase and incubated with 1 N HCl for 30 minutes at 42°C, were incubated for 60 minutes with a mouse anti-BrdU biotinylated primary antibody (anti-BrdU, Zymed, Basel, Switzerland) at room temperature. The slides were washed in PBS and incubated for 30 minutes with avidin-biotin-peroxidase complex (Vector Laboratories, Seruion, Switzerland) at room temperature. After washing, diaminobenzidine was added as a chromogen, and the slides were then counterstained with hematoxylin. Proliferating cells, having incorporated BrdU, had brown-stained nuclei. Quantification of stained lymph node cells was performed by morphometry. Briefly, noncounterstained tissue sections, magnified 61 times through a microscope (Reichert Jung, Germany), were scanned using a color Kappa CF15/3 camera (Kappa, Germany) and a Semper 6P image analysis software (Synoptics, UK). The results were expressed as the ratio between the number of pixels associated to immunoreactive regions and to the total area examined.

Measurement of Serum Levels of Anti-mBSA Antibodies

For determination of anti-mBSA IgG, 96-well plates (Maxisorp-Nunc; Life Technologies AG, Basel, Switzerland) were coated overnight at 4°C with 1% bovine serum albumin (BSA) in PBS. After four washings with TTBS (50 mmol/L Tris, pH 7.4, 140 mmol/L NaCl, containing 0.05% Tween 20), 100 μl of serum, serially diluted in 1% gelatin/PBS (final dilutions 1/100, 1/200, 1/400) were incubated for 2 hours at room temperature. Wells were washed four times. Then, 100 μl /well of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co.) diluted 1/500 in TTBS was added for 30 minutes. After four washings with TTBS, color was developed with 100 μl /well of *p*-nitrophenylphosphate (Sigma Chemical Co.) and the reaction stopped by adding 25 μl /well of 3 mol/L NaOH. Plate reading was performed at 405 nm and results calculated according to a standard curve with a reference serum.

Mass Spectrometric Analysis of SDF-1 in Plasma

Plasma pooled from wild-type and CD26-/- mice were analyzed by antibody-capture surface enhanced laser desorption ionization time-of-flight-mass spectrometry (SELDI-MS). The anti-SDF-1 monoclonal antibody MAB310 (R&D Systems, Minneapolis, MN) was coupled to a PS20 chip (Ciphergen, Dumbarton Circle, CA) at a concentration of 200 ng/ml in 0.01 mol/L PBS, pH 7.4. The total volume on each spot was 4 μl . After the coupling the chip was treated with ethanolamine to block remaining activated coupling sites. The chip was subsequently incubated overnight with mouse plasma diluted 1:1 in a PBS buffer, pH 7.4 with fish gelatin and bovine IgG as protein stabilizing additives. The total volume of plasma/buffer mixture was 60 μl . After this incubation, chips were washed and covered with matrix (α -cyano-cinnamic acid saturated in 0.1% trifluoroacetic acid/50% acetonitrile). The chips were subjected to MS analysis on the Ciphergen PS-II protein chip system. Mass spectra were obtained with a laser intensity of 230, a sensitivity of 10, and an average of 90 shots. The PBS II system was calibrated with LMW calibrator kit obtained from Ciphergen. Positive controls consisted of mouse plasma spiked with recombinant SDF-1 α (460-SD, R&D Systems), and analyzed immediately or after a 1-hour incubation at room temperature.

Assessment of CXCR4 by Immunohistochemistry

Paraffin knee joint sections were deparaffinized, saturated with 5% BSA and 20% normal rabbit serum for 1 hour at room temperature, and treated with 3% H_2O_2 for 10 minutes to block endogenous peroxidases. The slides were then incubated overnight with a primary goat anti-mouse CXCR4 antibody at 6.6 $\mu\text{g/ml}$ (anti-CXCR4; Abcam, Cambridge, UK) at 4°C. Bound antibodies were visualized using a secondary anti-goat biotinylated antibody followed by avidin-biotin-peroxidase complex (Vector Laboratories) at room temperature. After washings, diaminobenzidine was added as a chromogen, and CXCR4 positivity graded independently by two observers unaware of animal genotype on a scale of 0 to 6 (0 = no staining at all to 6 = maximum of staining).

Human Studies

Patients

Plasma were collected from patients with diagnosis of OA ($n = 26$, 15 females and 11 males; mean age, 63.2 ± 3.7 years) and RA ($n = 41$, 26 females and 15 males; mean age, 58.9 ± 2.2 years). Synovial fluids were collected from patients with OA ($n = 22$, 14 females and 8 males; mean age, 71.7 ± 2.48 years) and RA ($n = 17$, 9 females and 8 males; mean age, 56 ± 3.28). OA patients were diagnosed by clinical and radiological criteria and RA patients fulfilled at least four of the seven American Rheumatism Association revised criteria for RA.

Plasma and Synovial Fluid Sampling

Plasma and synovial fluid samples were collected into citrated Sarstedt Monovette tubes (Numbrecht, Germany). Anti-coagulated plasma and synovial fluid were centrifuged at $2000 \times g$ for 15 minutes and the supernatants aliquoted and stored at -80°C until assayed.

Laboratory Parameters

C-Reactive protein (CRP) was measured by nephelometry.

CD26 ELISA

A sandwich ELISA, recognizing human CD26 (Bender Medsystems, Vienna, Austria) was used.

Determination of DPPIV Activity

DPPIV activity was determined according to Scharpé and colleagues²⁵ with the following modifications: DPPIV activity was determined on 1-, 2.5-, and 5- μl samples (plasma, synovial fluid, or tissue extracts) fluorometrically using Gly-Pro-AMC (Novabiochem, Lucerne, Switzerland) at 5 mmol/L final concentration for 60 minutes at 37°C under agitation in an Eppendorf thermomixer in 25 μl of 100 mmol/L Tris-HCl, pH 8. The reaction was stopped by the addition of 2.5 μl of pure acetic acid. The incubation mixture was recovered in 3 ml of water. A blank value was obtained by incubating the substrate in the absence of the enzyme. A standard curve was determined by using AMC fluorescence measurement on a Perkin-Elmer LS-5 fluorometer (λ excitation, 370 nm; λ emission, 460 nm). DPPIV activities were expressed as nmol of substrate converted per minute per ml.

Statistical Analysis

Data are reported as mean values $\pm$ standard error of the mean (SEM). The Wilcoxon/Kruskal-Wallis (rank sum test) for unpaired variables was used to compare differences between groups with non-Gaussian distribution. The unpaired Student's *t*-test was used to compare the groups with normally distributed values. A level of $P < 0.05$ was considered as statistically significant. Correlation between parameters were analyzed by linear regression. All statistical calculations were performed using the JMP package (JMP version 4.02; SAS Institute, Cary, NC).

Results

Decreased Plasma DPPIV Activity in Murine Models of Arthritis

AIA and CIA are recognized to be murine models that recapitulate some of the features of RA. We asked if in these animal models there was a decrease in DPPIV activity associated with the establishment of arthritis. Plasma from naive mice (before immunization) and of

Figure 1. Plasma DPPIV activity in experimental models of arthritis. Plasma levels of DPPIV activity in AIA (a) and CIA (b) arthritic mice compared to nonimmunized, nonarthritic (naive) controls. For the AIA model, citrated plasmas were prepared from arthritic mice at day 3 after arthritis induction. For the CIA model, citrated plasmas were prepared from arthritic mice 3 days after clinical signs of arthritis. Values are the mean $\pm$ SEM of at least four different samples from four different mice. *, $P < 0.05$ arthritic versus nonarthritic values.

arthritic mice were collected and DPPIV activity measured. In both AIA and CIA there was a small but significant reduction in DPPIV activity in the diseased state (Figure 1, a and b, respectively). Most of this peptidase activity is accounted for by CD26 because we observed a reduction of more than 80% in plasma from CD26-deficient mice (results not shown).

Effect of CD26 Deficiency on the Severity of AIA

To explore whether CD26 deficiency had an effect on the course of AIA, we measured the levels of knee joint inflammation according to the ratio of $^{99\text{m}}\text{Tc}$ uptake in the inflamed arthritic joint over that of the nonarthritic contralateral knee joint at different time points up to day 14 after the onset of arthritis (Figure 2). The results showed that the levels of $^{99\text{m}}\text{Tc}$ uptake on days 1, 3, 7, and 14 were higher in CD26 $^{-/-}$ mice than in CD26 $^{+/+}$ mice, but only results on day 7 reached a significant difference (day 7, 1.42 ± 0.06 versus 1.63 ± 0.07 ; $P < 0.05$). This

Figure 2. Time course of knee joint inflammation in CD26-deficient mice with AIA. Time course of knee joint inflammation in CD26-deficient mice with AIA was measured by external γ counting of $^{99\text{m}}\text{Tc}$ uptake on days 1, 3, 7, and 14 after antigen-challenge into the right knee. Results are expressed as the ratio of $^{99\text{m}}\text{Tc}$ uptake in the right (R) arthritic knee joint over the left (L) noninflamed knee joint. For each time point, mean of ratios is represented by a horizontal bar. Joint inflammation was significantly increased at day 7 in CD26 $^{-/-}$ mice versus the control group ($P < 0.05$). Control mice at days 1, 3, 7 ($n = 22$), and at day 14 ($n = 6$), and CD26-deficient mice at days 1, 3, 7 ($n = 24$), and at day 14 ($n = 11$).

Figure 3. Histological features of knee joints from control and CD26-deficient mice with AIA. **a:** Histologies of whole knee joint sections of control wild-type (WT) and of CD26^{-/-} (KO) mice with AIA. Safranin-O-stained sections of arthritic knee joints were performed at day 20 after arthritis induction. Note the difference of thickness of synovial membrane, which is thicker in CD26^{-/-} mice than in the control arthritic mice. **b:** Synovial membrane thickness, cell exudate, and cartilage damage were scored histologically using an arbitrary scale from 0 to 6, at day 20 after arthritis induction. Results are expressed as mean ± SEM (WT mice, *n* = 18; CD26^{-/-} mice, *n* = 21). Statistical significance was tested by the Wilcoxon rank sum test. *, *P* < 0.05 was considered significant.

increased joint inflammation at day 7 was associated with an increase in serum amyloid A, an acute-phase response protein, which was more elevated in serum of CD26-deficient mice ($499 \pm 151 \mu\text{g/ml}$ versus $350 \pm 59 \mu\text{g/ml}$ in control mice), although this increase was not statistically significant.

We then examined the histological features of arthritic knee joints from CD26^{-/-} and CD26^{+/+} mice at day 10 and 20 after arthritis onset (Figure 3, only results at day 20 are shown). In both mouse strains, overt signs of synovitis were seen in the joints injected with mBSA. In contrast, no signs of inflammation were present in contralateral knees injected with PBS (data not shown). The

synovial thickness was graded independently by two observers unaware of animal genotype. At day 10 of AIA there was a trend toward a higher synovial inflammation score in CD26^{-/-} mice as compared with controls, although results did not reach statistical significance (4.37 ± 0.34 versus 3.62 ± 0.31 , *P* = 0.07). At day 20, there was a clear difference in the thickness of synovial membrane, which was more inflamed in CD26^{-/-} than in CD26^{+/+} mice [Figure 3, 3.4 ± 0.08 in CD26^{-/-} mice (*n* = 21) versus 2.2 ± 0.1 in control mice (*n* = 18), *P* = 0.03]. In addition, the abundance of inflammatory synovial fluid (exudates) in the joints of CD26-deficient mice was significantly increased (2.38 ± 0.08 versus 0.94 ± 0.09 , *P* = 0.01). The effect of AIA on articular cartilage degradation was evaluated according to the proteoglycan content, as demonstrated by safranin-O staining. In contrast to the results on synovial inflammation, cartilage damage scorings were not significantly different in CD26^{-/-} compared to control mice. These results suggested that CD26 deficiency exacerbates inflammation in AIA.

Effect of CD26 Deficiency on Humoral and Cellular Responses of AIA

To investigate potential mechanisms whereby CD26 deficiency may contribute to inflammation in AIA, we first examined the humoral immune response by measuring the serum levels of total immunoglobulins and of specific anti-mBSA antibodies by ELISA in naive mice and in immunized mice at day 10 and 20 after the onset of arthritis. Anti-mBSA antibodies were undetectable in nonimmunized mice (data not shown). In arthritic mice, the circulating levels of anti-mBSA antibodies were similar in CD26^{-/-} and CD26^{+/+} mice [46.8 ± 9 in CD26^{-/-} mice (*n* = 21) versus 50.5 ± 10.1 in control mice (*n* = 18)].

The role of CD26 in cell-mediated immune responses was examined by isolating inguinal lymph node cells and spleen cells from mice with AIA. Proliferation assays, as assessed by [³H]-thymidine uptake of lymph node cells (Figure 4a) and of splenocytes (not shown) from immunized mice revealed the presence of T cells reactive to mBSA in both knockout and wild-type mice and a similar proliferation rate for CD26-deficient and for wild-type immune cells.

Proliferation of immune cells from CD26-deficient or wild-type mice (with AIA, Figure 4b; or naive mice, results not shown) with concanavalin A, used for nonspecific activation as a control, was also comparable. Finally, *in vivo* lymph node cell proliferation determined by intraperitoneal injection of BrdU 1 hour before sacrifice of mice and followed by BrdU immunohistochemistry on lymph node sections, revealed no significant difference between CD26^{-/-} and CD26^{+/+} mice (results not shown).

We also measured the levels of IFN- γ in lymph node cell supernatants, as a typical Th1-cytokine. Both specific T-cell activation with mBSA and nonspecific activation with concanavalin A led to markedly increased levels of IFN- γ in lymph node cell supernatants from CD26^{-/-} mice (Figure 4, c and d). Taken together, these results suggest that CD26 deficiency may selectively modulate

Figure 4. Lymph node cell immune response. Inguinal lymph nodes were removed at day 20 of AIA. Lymph node cell proliferation in the presence or absence of mBSA (**a**) or concanavalin A (**b**) was determined by ³H-thymidine incorporation. The results of one representative experiment of three is shown. IFN-γ production by lymph node cells was measured in cell culture supernatants by ELISA (**c**, **d**). In cells from CD26^{-/-} mice, IFN-γ production was significantly higher as compared to CD26^{+/+} mouse cells.

particular functions of certain lymphocytes, such as Th1-mediated production of proinflammatory IFN-γ, which may contribute to the progression of arthritis in AIA.

Effect of CD26 Deficiency on Stromal Cell-Derived Factor 1 (SDF-1/CXCL12) Functionality in AIA

In addition to direct effects on lymphocytes, CD26 may potentially influence inflammation by cleaving chemokines that regulate recruitment or activity of lymphocytes at sites of inflammation. One possible substrate of CD26 in this category is SDF-1, an important chemokine in arthritis pathogenesis. Intact SDF-1 (1-68) was incubated in serum from CD26^{-/-} mice (Figure 5A) or from wild-type controls (Figure 5B) and analyzed by mass spectrometry. After a 1-hour incubation in serum from wild-type mice, the intact form 1-68 was cleaved at the C-terminus to generate SDF-1 1-67. A substantial part of these 1-68 and 1-67 forms were also N-terminally truncated to inactive SDF-1 (3-68) and (3-67), respectively. In contrast, incubation of intact SDF-1 in serum derived from CD26^{-/-} mice resulted exclusively in C-terminal degradation, with no N-terminal truncation. Similar results were obtained when analyzing endogenous SDF-1 in arthritic sera from CD26^{-/-} (Figure 5C) and in matched control mice (Figure 5D). In serum from CD26-deficient mice, SDF-1 was exclusively C-terminally truncated leading to one single peak (form 1-67) whereas in wild-type mice SDF-1 was degraded at both C- and N-terminus leading to the inactive 3-67 truncated form of SDF-1.

Because virtually all SDF-1 is inactivated *in vivo* by CD26-mediated cleavage, we asked if this inactivation of SDF-1 could have functional repercussions in the recruitment of SDF-1-responding cells in arthritic synovial mem-

Figure 5. Detection by surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry of intact and truncated SDF-1α in mouse sera. **A:** Incubation of recombinant SDF-1α in serum from CD26^{-/-} mice resulted in two peaks of 7971 and 7842 d, the former corresponding to the predicted molecular weight of intact (1-68) mouse SDF-1α, the second representing a truncated form (1-67) at the carboxy-terminus of SDF-1α. **B:** Incubation of recombinant SDF-1α in serum from CD26^{+/+} mice led to four peaks. Two peaks (7970 and 7841 d) correspond to the ones seen in serum from CD26^{-/-} mice. Cleavage of these by CD26 in wild-type serum removed the N-terminal lysine and proline resulting in the peaks at 7744 (SDF-1α 3-68) and 7616 (SDF-1α 3-67), respectively. Therefore, the species of 7970 and 7842 d both contain intact N-termini that can be cleaved by CD26. **C:** Analysis of endogenous SDF-1α in sera from CD26^{-/-} mice revealed one peak at 7856, corresponding to N-terminally intact, C-cleaved SDF-1α 1-67. **D:** In sera from wild-type mice, endogenous SDF-1α was mostly converted to truncated SDF-1α both at the N and the C terminus (SDF-1α 3-67).

brane. To test this hypothesis, we analyzed, by immunohistochemistry, the expression of CXCR4, the unique receptor of SDF-1 in arthritic knee joints from CD26^{-/-} and wild-type mice (Figure 6). We found widespread expression of CXCR4-positive cells in CD26^{-/-} tissues (in perivascular aggregates, in the sublining layer on cells with macrophagic and fibroblastic appearance) whereas the expression in CD26^{+/+} ones was more scanty as determined by immunohistological scoring [Figure 6; wild-type 1.33 ± 0.202 ($n = 6$), CD26 knockout 2.16 ± 0.114 ($n = 6$), $P < 0.01$].

Decreased Plasma CD26/DPP-IV Activity Levels in RA

We investigated the activity levels of DPP-IV and of CD26 antigen in patients with RA and OA in both plasma and synovial fluids (Figure 7). In these two compartments, there was a high correlation ($r > 0.7$, $P < 0.0001$) between DPP-IV activity and CD26 antigenic levels, thus

Figure 6. Assessment of CXCR4 by immunohistochemistry. **a:** Immunohistochemical analysis of CXCR4 expression in arthritic knee joints from CD26^{-/-} (KO) and wild-type (WT) mice. Knee joint sections were examined at day 20 of AIA using a goat anti-mouse CXCR4 antibody. CXCR4-positive cells were more abundantly expressed in CD26^{-/-} synovial membranes compared to their expression in synovial membranes from wild-type mice. **b:** Immunohistological scoring of CXCR4 expression. CXCR4 positivity was graded independently by two observers unaware of animal genotype on a scale of 0 to 6 (0 = no staining at all to 6 = maximum of staining). Data are expressed as mean $\pm$ SEM. The *P* value between CD26-deficient mice and wild-type mice was assessed using Student's *t*-test.

Figure 7. DPPiV activity and CD26 antigen measurements in OA and RA patients. Plot distribution of DPPiV activity and CD26 antigen in two different patient groups, OA and RA. Plasma and synovial fluid samples (**a**, **b**) were analyzed by fluorogenic assay (for DPPiV activity) and by ELISA (for CD26 antigenic determination). Horizontal bars represent the mean. The asterisk denotes significant differences between groups by Student's *t*-test ($P < 0.05$). Among all patients (OA + RA), DPPiV activity and CD26 antigen were significantly correlated both in plasma (**c**) and synovial fluid (**d**).

indicating that most, if not all of the DPPiV activity is accounted for by CD26 (Figure 7, c and d). In addition, DPPiV activity and CD26 antigenic levels were significantly decreased in RA compared to OA plasma samples. By contrast, no differences were observed in synovial fluids between patient groups.

Negative Correlation between CD26/DPPiV Activity Levels and Inflammation

When analyzing patients with OA and RA together, the levels of plasma DPPiV activity and of CD26 antigen negatively correlated with the levels of CRP ($r = -0.5$, $P < 0.0001$ and $r = -0.45$, $P < 0.0029$, respectively). The RA group was further stratified in inflammatory rheumatoid arthritis (IRA) with CRP higher than 20 mg/L and in noninflammatory rheumatoid arthritis (NIRA) with CRP lower than 20 mg/L. Circulating DPPiV activity and CD26 antigen were significantly decreased in IRA compared to NIRA patients (Figure 8, a and b), further strengthening the negative relationship between CD26 and CRP levels.

Discussion

Our data demonstrate that during RA there is a selective decrease of plasma CD26 at both the activity and the antigenic level compared to OA plasma and to a small group ($n = 8$) of nondiseased control plasma (results not shown). In agreement with this clinical study, we found that the activity of DPPiV in plasma of animals with experimental arthritis was also decreased compared with

Figure 8. Association between DDPIV activity, CD26 antigen, and CRP levels in plasma of OA and RA patients. Patients in the RA group were separated in inflammatory RA (IRA) with CRP > 20 mg/L and in noninflammatory RA (NIRA) with CRP < 20 mg/L. DDPIV activity (**a**) and CD26 antigen (**b**) were significantly decreased in IRA compared to NIRA plasma samples.

control animals. This down-modulation occurred only in plasma because CD26 expression was similar in synovial fluid from RA and OA patients. These findings corroborate reports in which circulating DPPIV activity levels¹⁴ or antigenic levels¹⁵ were decreased. Similarly, in clinical studies on systemic lupus erythematosus²⁶ and inflammatory bowel disease,²⁷ serum DPPIV/CD26 was also significantly decreased. The precise source for plasma CD26 remains unclear. Many cell types express CD26. Cleavage of CD26 from the cell surface has been proposed and inhibition of CD26 shedding during inflammatory diseases was suggested.^{15,27} This mechanism could account for the increased number of CD26-positive cells in peripheral blood from RA patients.^{11–13} Finally, CD26 synthesized and shed by hepatocytes could behave as a negative acute phase protein. Finally, increases in the clearance rate of circulating CD26 could be responsible for the decreased CD26 levels measured in RA compared to OA. In this context, it was reported that sera from patients with RA and systemic lupus erythematosus contained high titers of anti-DPPIV autoantibodies and that there was a good correlation between high titers of DPPIV autoantibodies and an augmented clearance of the enzyme from the circulation.²⁸

We found that CD26 levels negatively correlated with the levels of CRP taking collectively OA and RA samples ($r = -0.5$, $P < 0.0001$ for CD26 activity levels and $r = -0.45$, $P < 0.0029$ for CD26 antigenic levels). Moreover the negative association between CD26 and inflammation was further underlined by reduced CD26 levels in IRA compared to NIRA patients. In other immunological disorders, the decreased activity of circulating CD26 was also correlated with the severity of the disease. For instance, in inflammatory bowel disease CD26 activity in serum showed an inverse correlation with known disease activity scores as well as with the concentrations of CRP and orosomucoid in serum.²⁷ Moreover, CD26 activity was significantly lower in patients with an active Crohn's disease, as was their subjective health status.²⁹ Nevertheless, although the link between reduced circulating CD26 and promotion of inflammation seems established at least in RA and inflammatory bowel diseases, the exact role of CD26 remains obscure.

In vivo targeting of CD26 with antibodies and/or specific inhibitors, and knockout animals were used to provide additional insights into the underlying molecular mechanisms mediated by CD26. Various competitive as well as uncompetitive DPPIV inhibitors, were effective in three experimental rat models of RA (alkyldiamine-, collagen-, or adjuvant-induced arthritis^{18,19}). Similarly, in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, *in vivo* administration of a reversible DPPIV inhibitor decreased and delayed the clinical and neuropathological signs of EAE.^{30,31} However, some of these inhibitors were able to exert their actions even in DPPIV-deficient rats, suggesting that some of their effects may have been through proteins other than CD26.¹⁹

Paradoxically, although DPPIV inhibition was beneficial in experimental models of RA and multiple sclerosis, genetic deficiency of CD26 leads to exacerbation of these diseases: AIA was more severe in CD26-deficient mice (this study); similarly, EAE was exacerbated in CD26-knockout mice.³² The reasons for such discrepancy may be related to the additional effects of the inhibitors, able to act even in DPPIV-deficient animals¹⁹ suggesting that, besides DPPIV inhibition, these inhibitors may have other functional targets.

Although a lot of evidence for the importance of CD26 in immunoactivation have been reported, the molecular mechanism of this activation has not yet been elucidated. Inhibition of the enzymatic activity of CD26 by the use of DPPIV inhibitors suppresses T-cell proliferation *in vitro* and decreases antibody production in mice immunized with bovine serum albumin *in vivo*.³³ However, attenuation of T-cell activation *in vitro* was not reproduced by more selective DPPIV inhibitors³⁴ suggesting that the *in vitro* and *in vivo* immunological effects observed previously were likely because of inhibition of other dipeptidylpeptidase activities (ie, DPP8/9). In our experiments, neither *in vitro* or *in vivo* proliferation of immune cells (either lymph node cells or spleen cells) nor *in vivo* antibody production on mBSA immunization were affected by CD26 deficiency.

In line with our results, no significant differences were found between CD26 knockout and wild-type mice immune response after stimulation with pokeweed mitogen.³⁵ Moreover, similar immunological competence was observed in rats lacking active dipeptidylpeptidase IV compared to wild-type rats.³⁶ Altogether these results suggest either that CD26 may not play a pivotal role in mediating *in vivo* immune responses or that in the absence of functional CD26/DPPIV, deficient animals have developed a compensatory mechanism for T-cell activation.

A key event in the pathogenesis of synovial inflammation is the infiltration of joint space and tissue with lymphocytes and monocytes. In this context, SDF-1 and its receptor CXCR4 are thought to play a central role in inflammatory cell recruitment. SDF-1 was shown to be expressed by synovial tissues, synoviocytes, and endothelial cells, whereas CXCR4 was found on T cells and monocytes^{37,38} and SDF-1 has been shown to be involved in the migration to and retention of T cells,^{37,38} and monocytes^{39,40} in the synovium. It was also found that

SDF-1 co-stimulates CD4⁺ cells to induce cytokine production, activation markers, and proliferation.³⁷ In addition SDF-1 also modulates cytokine production by synovocytes.⁴¹ The *in vitro* modulation through CD26-mediated truncation of SDF-1 of the SDF-1/CXCR4 axis⁴ prompted us to hypothesize first that SDF-1 would not be N-terminally cleaved and inactivated in CD26-deficient mice, and secondly that CXCR4-positive cell recruitment will be favored in these deficient mice. Indeed, we found that SDF-1 in sera from CD26^{-/-} mice corresponded to the N-terminally intact, form (1-67) whereas in sera from wild-type mice it was mostly converted to the inactive N-terminally truncated form (3-67). These results are in agreement with the work of De La Luz Sierra and colleagues⁴², in which exposed to human serum, full-length SDF-1 α (1-68) undergoes processing first at the COOH terminus to produce SDF-1 α (1-67) and then at the NH2 terminus to produce SDF-1 α (3-67). In our study we show unequivocally that CD26 *in vivo* is responsible for serum cleavage of SDF-1 α at the NH2 terminus. As a matter of consequence, the active, N-terminal intact form of SDF-1 (1-67) in CD26^{-/-} mice was probably, at least in part, responsible for the increased CXCR4-positive cells in arthritic synovial membranes that we observed in these deficient mice.

In conclusion, the data presented in this study provide strong evidence that the DPPIV activity of CD26 may contribute to control of inflammatory responses, in part by regulating levels of the intact, active form of SDF-1.

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