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A CD26-Controlled Cell Surface Cascade for Regulation of T Cell Motility and Chemokine Signals

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Chemokines are key regulators of cell trafficking, and dipeptidyl peptidase IV/CXCR12 (CD26) inactivates chemokines. Here we show that the CD26-processed chemokines SDF1α/CXCL12 and RANTES/CCL5, in contrast to a control chemokine not processed by CD26, are potent inducers of cell surface expression of thrombospondin-1 (TSP-1) in T lymphocytes through a CD26-controlled mechanism and that TSP-1 stimulates expression of lipoprotein receptor related protein/CD91. Accordingly, intact TSP-1 and a peptide mimetic of a sequence in TSP-1 were sufficient to stimulate CD91 expression. The chemokine-induced expression of TSP-1 and CD91 was mimicked by inhibitors of CD26 and CXCL12 and CCL5 as well as inhibitors of CD26 stimulated polarized cytoplasmic spreading and migration through TSP-1. Silencing of CD26 using small interfering RNA or Ab-induced modulation of CD26 also increased TSP-1 expression and enhanced cytoplasmic spreading and T cell migration markedly. These results indicate that CD26 is an endogenous inhibitor of T cell motility through inhibition of TSP-1 expression and that chemokines stimulate cell polarity and migration through abrogation of the CD26-dependent inhibition. This suggests that T cell motility is regulated by a cascade of interacting cell surface molecules. The Journal of Immunology, 2009, 183: 0000–0000.

The chemokines are a superfamily of small peptide molecules (8–20 kDa) produced by various cell types including leukocytes, epithelial cells, perivascular myofibroblasts (1), and tumor cells (2). Chemokines regulate the migration of inflammatory cells, stem cells, and cancer cells (3–5) to specific destinations within the organism (6) and determine the extravasation as well as the migration within target organs (7). In addition, chemokines induce release of proteinases from leukocytes, control angiogenesis (8), and regulate T cell differentiation and function as scavenger molecules (9). The chemokine superfamily includes >50 ligands and 20 receptors and is currently classified into 4 groups based on the amino acid motif that occurs within the first 2 N-terminal cysteines (10, 11). Chemokines bind to G-protein-coupled surface receptors on target cells (12), and almost all chemokine receptors recognize more than one chemokine (12, 13). Chemokines have been shown to be important orchestrators of the capacity of lymphocytes to extravasate and migrate that makes it possible for these cells to reposition themselves between a free-floating vascular state and active migration in tissues (14, 15). Although chemokines thus play a critical role for lymphocyte adhesion and migration, and hence for immune surveillance against pathogens, altered self-components, and cancer throughout the organism, the mechanisms via which chemokines regulate adhesion and migration are only partly known (16–20). Understanding of this regulation therefore requires deeper mechanistic insights and perhaps even identification of additional regulatory factors and pathways. This lack of understanding applies particularly to the formation of active cell edges formed by adhering and migrating cells that probably senses the environment and is critical for recognition, a key feature of the adaptive immune system.

Thrombospondin-1 (TSP-1)3 is a 420-kDa glycoprotein composed of three identical disulfide-linked polypeptide chains that display binding sites for various cell surface receptors including glycosaminoglycans; the integrins α6β1, α4β1, α6β1, α3β1 and αvβ3; calreticulin (CRT); CD91; CXCL3; and CD47 (21–29). T lymphocytes express endogenous TSP-1 associated with CD91 and CRT that seem to function as a cell surface ligand for regulation of T cell adhesion and migration (30–32). In vivo findings further suggest that TSP-1 has a role in immune regulation. Thus, TSP-1-deficient mice show inflammatory disturbances in lungs and other organs and TSP-1 or TSP-2 as well as CD47-deficient mice show defective delayed-type hypersensitivity with prolonged inflammation (33–35). Deficiency of TSP-1 also reduces Th17 differentiation and attenuates experimental autoimmune encephalomyelitis (36). The starting point for the present investigation was a working hypothesis, based on preliminary evidence that chemokines may influence T cell TSP-1, that the chemokine receptor system may cross-talk with the cell surface receptors that bind TSP-1. We further hypothesized that such a cross-talk might be important for regulation of T cell functions such as environmental recognition by active cell edges, adhesion, and migration, and perhaps contribute to the extensive lymphocyte infiltration seen in autoimmune and allergic inflammatory diseases. It is interesting in this context that the chemokine CXCL12, which is expressed in many tissues (37), binds to the chemokine receptor CXCR4, which is associated with the membrane-bound ectopeptidase dipeptidyl

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3 Abbreviations used in this paper: TSP-1, thrombospondin-1; CD26, dipeptidyl peptidase IV/CXCR12; CRT, calreticulin; siRNA, small interfering RNA; 4N1K; KRFTV-YMVWKK; Sc4N1K, KVFREKWYVMR (scrambled 4N1K).

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peptidase IV/CD26 (CD26), a cell surface protease with a widespread organ distribution, composed of two identical 110-kDa subunits, that inactivates chemokines and other peptides by selective removal of the N-terminal proline or alanine in the second position (38–40). CD26 affects T cell activation, proliferation, and cytokine production (41–44), and CD26 inhibitors introduced into animal models have demonstrated a role for CD26 in the development of autoimmune diseases (45). However, the severity of autoimmune diseases including rheumatoid arthritis and experimental autoimmune encephalomyelitis is increased in CD26-deficient mice indicating that CD26 rather exerts a protective effect against disease development (46, 47). Our results show that CXCL12 and CCL5, which both are processed by CD26, are potent inducers of cell surface expression of TSP-1 and that TSP-1 induces expression of CD91 and CRT and stimulates cytoplasmic spreading and migration. Small interfering RNA (siRNA) silencing of CD26, inhibitors of CD26 as well as Ab-induced modulation of CD26 also stimulate this chemokine-induced molecular cascade, thus implicating CD26 as a suppressor factor for T cell formation of cytoplasmic projections and migrations.

Materials and Methods

Chemicals and Abs

Human plasma fibronectin and rat tendon collagen type I were purified and prepared as described elsewhere (48, 49). Polyclonal anti-CD26 antibodies (Sigma-Aldrich, Sc4N1K) at 50 μg/ml were used for Western blot analysis. Anti-CD26 (clone TA5.9) has been described previously (50). Anti-CD26 (clone BA5) was obtained from Santa Cruz Biotechnology. CD26-siRNA (human) and control-siRNA were prepared as described elsewhere (48, 49). Poly-L-lysine (molecular mass, 5300) was purchased from Miles-Yeda. IL-2 and IL-4 were from Genzyme Diagnostics. Brefeldin A was fromSigma-Aldrich, and ICAM-1 was from R&D Systems. Human recombinant CD26 was obtained from Alexis Biochemicals. Anti-fibronectin (clone IST1, IgG1) was obtained from Sera-Lab. Anti-CD3 (clone SK7, IgG1) and anti-CD4 (clone SK3, IgG1) were obtained from BD Biosciences. Dynabeads were from Dynal. Mouse IgG and anti-CD8 (C8/144B, IgG1) were from Dako. Goat anti-mouse Ab was obtained from Dakopatts. Anti-TSP-1 clone MBC 200.1 (also called TSP-Ab-9, IgG1), clone A6.1 (also called TSP-Ab-4, IgG1) and clone C 6.7 (also called TSP-Ab-3, IgG1), were from NEO-MARKERS. Anti-CD91 (clone A2Mr2, IgG1) was from Santa Cruz Biotechnology. Anti-CD26 (clone TA5.9) has been described previously (50). Anti-CD26 (clone BA5) was obtained from Dako. Anti-CRT (clone FMC75, IgG1) was from Biosite. Anti-CD3 (clone B6H12.2, IgG1) was from NEO-MARKERS. Biotinylated peroxidase and avidin were from Vector Laboratories. The peptides KRFYVVMWKK (4N1K) and KVFVRKYVKM (scrambled (Sc) 4N1K) were synthesized with Tri pep (Novum Research Park). Protease inhibitors were used GM6001, a metalloprotease inhibitor (Chemicon), Ile-Glu-Thr-Asp aldehyde, an inhibitor of granulocyte B, Z-Vala/Ala/Asp fluoromethyl ketone, a caspase inhibitor (Calbiochem), and dipeptide A and KR 62436, two inhibitors of CD26 (Sigma-Aldrich). The CD26 inhibitor vildagliptin was custom synthesized by GLSynthesis. Serum-free AIM-V medium was obtained from Invitrogen, and 10× RPMI 1640 was from Life Technologies.

Cell culture

Human peripheral T cells were purified as previously described and stimulated with anti-CD3 in the presence of IL-2 and IL-4 for 3–5 days before the experiments (51). The birch (Bet v 1)-specific T cell clone AF 24 was kindly provided by Dr. J. van Neerwen (ALK). This cell line was regularly stimulated with anti-CD3 and cultured in the presence of IL-2 and IL-4 for 5–12 days before the experiments. All cells were cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 0.16% sodium bicarbonate, 10,000 U/ml benzylpenicillin, 100,000 μg/ml streptomycin, and 10% FCS or in serum-free AIM-V medium. Peptides, brefeldin A, and mAbs when applied to study the influence on Ag expression or adhesion were added immediately before the experiments. To induce down-modulation of CD26, the lymphocytes were incubated with anti-CD26 (TA5.9) for 4 h (50).

siRNA-mediated CD26 silencing

The expression of CD26 was suppressed using the human T cell Nucleofector kit (Lonza) and a Nucleofector device (Amaka Biosystems) as previously described (52). CD26-siRNA (human) and control-siRNA were obtained from Santa Cruz Biotechnology.

Biotinylation and immunoprecipitation of cell surface proteins

Anti-CD3-activated cells were biotinylated in solution, 3 × 10^6/well, and after adhesion to tissue culture dishes (Falcon 35 3001), 5 × 10^6/well, coated overnight at 4°C with fibronectin (20 μg/ml) using reagents for biotinylation from the Cellular Labeling and Immunoprecipitation Kit (Roche). The reaction was stopped and cells were washed and lysed with 1 ml of lysis buffer on ice for 30 min, followed by centrifugation at 12,000 × g for 10 min at 4°C. The cells were allowed to adhere for 30 min. Where stated, cells were treated with 4N1K at 50 μM or scrambled 4N1K (Sc4N1K) at 50 μM or inhibitor of CD26, dipeptide A, at 50 μM for 30 min before biotinylation. Adherent cells were washed and biotinylated while remaining attached (0.5 ml of biotinylation buffer/well). Adherent cells were washed off the plate, washed, lysed, and centrifuged as above. For preclearing, each tube with lysate was incubated with protein G agarose at 4°C overnight. Agarose beads were discarded and lysates mixed with 1 μg of TSP-1 Ab or CD91 Ab and incubated at 4°C overnight. Protein G agarose was added to each tube followed by incubation at 4°C overnight. Beads were washed, and 20 μl of reducing sample buffer were added followed by boiling for 4 min. Proteins were separated on 6% SDS-PAGE gels.

Quantitative immunocytochemistry

Glass chambers were coated with ICAM-1 (2 μg/ml) or fibronectin (10 μg/ml) overnight at 4°C and washed. Anti-CD3 activated cells in serum-free AIM-V medium (15 μl of 1 × 10^6 cells/ml per position) were allowed to adhere to the coated surface for 30 min at 37°C followed by fixation with 2% paraformaldehyde at 4°C for 20 min. For detection of CD26, the cells were subsequently permeabilized using 0.1% saponin. Mouse anti-human Abs to TSP-1, CD91, or ICAM-1, and the added, followed by detection of the primary Ab with the biotin-avidin-HRP-based Vectastain ABC Kit (Vector Laboratories) and a peroxidase substrate (3-amino-9-ethylcarbazole). Pictures were taken of each slide using the program NIKON ACT-1 and a NIKON Eclipse E1000M microscope at ×600. Mean staining intensity (arbitrary units) was determined using the Image J picture analysis program.

Cell migration

Collagen type I was diluted in serum-free RPMI 1640 and H 2O (8:1:1), applied in plastic petri dishes (30 mm; BD Biosciences), and allowed to polymerize at room temperature. The chemokines were dissolved in the gel, whereas Abs and peptides were present with the cells in migration experiments. A total of 1.0 × 10^6 cells in AIM-V medium was added to each well and allowed to migrate for different times. The cells were fixed in 2.5% glutaraldehyde and washed twice with PBS. Cell morphology and cell migration were routinely, unless otherwise stated, evaluated in nine fixed positions in each well and at 50-μm intervals throughout the gel by the use of an inverted microscope (Nikon Eclipse TE300) and a digital depth meter (Heidenheim ND221). The results are given as maximal infiltration depth or mean number of infiltrating cells/field (×20 objective) per infiltration depth (50 μm for the first two layers immediately beneath the gel surface and 100 μm for other layers further down). The infiltrating cells were identified in situ in the collagen gels using immunocytochemistry after fixation in parafomaldehyde.

Cell adhesion

To study cell adhesion, plastic petri dishes (90 mm; Heger) were coated with ICAM-1 (2 μg/ml) or fibronectin (10 μg/ml) and extensively washed before use. The cells (10,000/position) in AIM-V medium were incubated on the substrates in a humified CO2 incubator at 37°C for 15 or 30 min. Cells were fixed in 2.4% cold glutaraldehyde for 10 min, and the unbound cells were removed by gentle aspiration. The number of adherent cells per microscopic field (>20 objective) was counted. Chemokines, dipeptide A, and peptides were present with the cells in adhesion experiments. A total of 1.0 × 10^6 cells in AIM-V medium were added and allowed to migrate for different times. The cells were fixed in 2.5% glutaraldehyde, except for immunocytochemistry, for which 2% paraformaldehyde was used, and washed twice with PBS. Cell adhesion was routinely, unless otherwise stated, evaluated in five fixed positions.

Statistical analysis

Staining intensity in immunocytochemistry experiments is presented as mean ± SD. For analysis of differences between groups or for analysis of adhesion and spreading, mean values ± SD are shown. For determination of differences between inhibitor-treated lymphocytes and control lymphocytes, the paired
Results

CXCL12 and CCL5 induce cell surface expression of TSP-1 and CD91

To study the possible influence of chemokines on the cell surface expression of TSP-1, we exposed anti-CD3-activated lymphocytes (generally >92% CD3 positive cells) in suspension and on a fibronectin (FN)-coated plastic surface (Fig. 1) to CXCL12 and CCL5, two chemokines generated by CD26 (53, 54). The cells were cultured in serum-free medium to exclude interference of exogenous TSP-1. Anti-CD3-activated cells were used because we intended to examine cell migration and formation of pseudopodia that develop poorly in lymphocytes fresh from the blood (55). Both chemokines were potent inducers of cell surface expression of TSP-1, CD91, and CRT as determined by SDS-PAGE of immunoprecipitated biotinylated cell surface components from cells in suspension (Fig. 1A) and quantitative immunocytochemistry of adherent cells fixed in situ to prevent Ab-induced perturbations of Ag expression and turnover of Ags during the detection procedure (Fig. 1, C–G). In contrast, CXCL12 and CCL5 (not shown) did not affect the cell surface expression of CD3 and CD4 (Fig. 1H). Brefeldin A inhibited the cell surface expression of TSP-1 induced by CXCL12.
indicating that the chemokines induced TSP-1 transport
to the cell surface from an intracellular source. The effect of
CXCL12 on the expression of TSP-1 was concentration dependent
with a peak at 50–200 ng/ml (Fig. 1D). Immunoprecipitation of
surface-biotinylated cells demonstrated intact 175-kDa TSP-1 as
well as 130- and 115-kDa fragments that also could be identified
as TSP-1 using Western immunoblotting (Fig. 1B). It is also evi-
dent from Fig. 1B that CXCL12 increased the amount of intact
175-kDa TSP-1 in comparison with control cells. In contrast, these
showed a prominent 115-kDa TSP-1 band that was weak in cells
cultured in the presence of CXCL12. In addition, there were mul-
tiple minor bands immunoprecipitated with anti-TSP-1 Abs as re-
vealed by the gradient gel in Fig. 1A. It is possible that some of the
multiple bands seen on the gel in Fig. 1A represent components
that associate with TSP-1, such as CRT or CD47. CXCL12 also
markedly increased the cell surface expression of TSP-1 in a T cell
clone as revealed by SDS-PAGE gels of immunoprecipitated bi-
otinylated cell surface components (results not shown). In contrast
to CXCL12 and CCL5, CXCL8, which binds to and induces func-
tional responses in activated T cells (56) but is not processed by
CD26 (57), did not affect TSP-1 expression (Fig. 1C). The results
in Fig. 1 indicate that CD26-processed chemokines induce cell
surface expression of TSP-1 through a brefeldin A-sensitive trans-
port mechanism and also expression of CD91 and its coreceptor
CRT, which has been shown to coprecipitate with cell surface
TSP-1 in T cells (32).

**Figures**

**Figure 2.** A TSP-1-CD47-CD91 cascade on the T cell surface. A–C. The influence of TSP-1 (5 μg/ml), the CD47-binding site in TSP-1, 4N1K, and a scrambled control peptide, Sc4N1K, on the cell surface expression of CD91 (A and B) and CD3 (C) as showed by quantitative immunocytochemistry (A and C) and immunoprecipitation of biotinylated cell surface components (B). One representative experiment of two to six independent experiments is shown.

**Figure 3.** CD26 inhibitors increase the cell surface expression of TSP-1. A–C. Quantitative immunocytochemistry showing that incubation for 30 min in the presence of diprotin A (50 μM), KR 62436 (50 μM), or vildagliptin (100 μM), and modulation by the anti-CD26 Ab TA5.9 (5 μg/ml) increases the cell surface expression of TSP-1 (A) and CD91 (B). C. Comparison of the influence of CXCL12 and diprotin A on TSP-1 expression in cells adherent to fibronectin. D. SDS-PAGE gel analysis (6% and 4–12% gels) showing immunoprecipitated biotinylated cell surface proteins from lymphocytes allowed to adhere to fibronectin for 30 min in the presence of diprotin A (50 μM) or GM6001 (10 μM). Diprotin A enhances expression of intact 175-kDa TSP-1, 130- and 115-kDa proteins as well as several other coprecipitated components and/or fragments of TSP-1. One representative experiment of three to four independent experiments is shown. Ac-IETD-CHO, Ile-Glu-Thr-Asp aldehyde.

**TSP-1 induces cell surface expression of CD91**

TSP-1 and a peptide mimetic of a sequence in TSP-1, 4N1K, were
found to be inducers of cell surface expression of CD91 whereas
they did not affect CD3 expression (Fig. 2). In contrast, a scram-
bled control peptide, Sc4N1K, did not induce CD91 expression.
This suggests that the chemokine-induced cell surface expression
of CD91 demonstrated in Fig. 1 is dependent on TSP-1.

**CD26 influences cell surface expression of TSP-1**

The results in Fig. 1 suggested that chemokines metabolized by
CD26, such as CXCL12 and CCL5 (53, 54, 58), that cleave N-
terminal dipeptides from various chemokines regulate the cell sur-
face expression of CD91 and TSP-1 through CD26. Therefore, we
examined the possible influence of the CD26 inhibitors diprotin A,
KR 62436, and vildagliptin on the cell surface expression of TSP-1
in anti-CD3-activated lymphocytes using quantitative immunocy-
tochemistry and SDS-PAGE of immunoprecipitated biotinylated
cell surface proteins (Fig. 3), Diprotin A, KR 62436, and vildagliptin increased the cell surface expression of TSP-1 and CD91 (Fig. 3, A and B). The fact that vildagliptin has a high specificity for CD26 (59, 60) indicated that CD26 counteracts the expression of these components. In contrast, an inhibitor of granzyme B (Ile-Glu-Thr-Asp aldehyde), another protease expressed by T cells, did not increase TSP-1 and CD91 expression. A CD26-modulating mAb to the ADA-binding site in CD26 (Fig. 3, A and B) similar to CXCL12 and CLL5 as demonstrated in Fig. 1. The 130- and 115-kDa bands were identified as TSP-1 using Western immunoblotting with an anti-TSP-1 Ab (TSP-4; Fig. 1B). The other bands may represent degradation products of TSP-1 or coprecipitated components, such as CRT (70 kDa) or CD47 (50 kDa). Thus, inhibition of CD26 and chemokines processed by CD26 had virtually the same effect on the expression of TSP-1 and CD91. This indicates that CD26 suppresses the cell surface expression of TSP-1 and CD91 and that chemokines interacting with CD26 abrogate this suppression. GM6001 decreased the intensity of intact TSP-1, enhanced the 115-kDa band, and induced a 70-kDa band (Fig. 3D). This points to the possibility that a metalloprotease also influences TSP-1 expression, and further suggestive evidence for involvement of several proteases is provided by the loss of cell surface TSP-1 when GM6001 was combined with diprotin A.

CD26 may inhibit the expression of TSP-1 and CD91 if capable of binding to TSP-1 and inhibit its stimulatory effect leading to CD91 expression. To study the possible influence of CD26 on TSP-1 the two molecules were mixed at increasing enzyme:substrate (w/w) ratios and incubated at 37°C for 15 h. CD26, at a relatively low concentration, prevented reduction of TSP-1 as showed by the persistence of a 420-kDa band. In addition, CD26 induced a 320-kDa band, most probably derived from the 420-kDa band, 140-, 90-, and 70-kDa bands (Fig. 4). Moreover, a 110-kDa band appeared, that did not seem to be CD26, although it had the same molecular mass, because it was not present in the control lane with CD26 alone. The results in Fig. 4 suggest that CD26 can associate with TSP-1, but further studies are needed to elucidate whether CD26 can cleave TSP-1.

**CD26 inhibits T cell polarity and migration through TSP-1**

In the light of the previous findings that endogenous TSP-1 participates in the regulation of T cell migration and adhesion (30, 32) together with the present evidence that chemokines and CD26 affect TSP-1 expression (Figs. 1 and 3), it seemed reasonable to assume that chemokines as well as CD26 may influence T cell adhesion and migration through TSP-1. To investigate this, we examined the influence of chemokines and diprotin A on adhesion of lymphocytes to ICAM-1 coated on a plastic surface. CXCL12 and diprotin A stimulated polarized cytoplasmic spreading with formation of pseudopodia in T cells adhering to these substrata whereas control cells exhibited a relatively uniform circumferential spreading along the cell periphery (Fig. 5). A peptide mimetic of a sequence in TSP-1 (4N1K) prevented spontaneous spreading (Fig. 5) as well as the potentiation of spreading induced by diprotin A and CXCL12 (not shown), probably through interference with the association of endogenous TSP-1, whereas a scrambled control peptide (Sc4N1K) had a negligible effect on spreading.

To analyze the influence of CD26 and chemokines on lymphocyte migration anti-CD3-activated cells were allowed to migrate into three-dimensional collagen gels, a well established test system for analysis of lymphocyte migration (30, 61–64), in the presence of CXCL12, the CD26 inhibitors vildagliptin, diprotin A, and KR 62436. In addition, the influence of modulation of CD26 by Ab to

**FIGURE 4.** CD26 affects TSP-1. Western blot (WB) showing TSP-1 mixed with CD26 at increasing enzyme-substrate (w/w) ratios and incubated at 37°C for 15 h. Lane 1, TSP only; lane 2, CD26 and TSP, 1/100; lane 3, CD26 and TSP, 1/50; lane 4, CD26 and TSP, 1/10; lane 5, CD26 only. It can be seen that CD26 influences the detectability of TSP-1. Arrows, Bands of lower molecular mass.

**FIGURE 5.** CXCL12 and diprotin A stimulate polarized cytoplasmic spreading and pseudopodia formation. T cells were allowed to adhere to a plastic surface coated with ICAM-1 in the presence and absence of CXCL12 (50 µg/ml), diprotin A (50 µM), 4N1K (50 µM), or Sc4N1K (50 µM). Control cells and cells allowed to adhere in the presence of Sc4N1K showed uniform circumferential formation of active cell edges, whereas cells allowed to adhere in the presence of diprotin A or CXCL12 showed pronounced polarization and development of pseudopodia. 4N1K inhibits cytoplasmic spreading both with and without diprotin A or CXCL12. One representative experiment of five independent experiments is shown. PBT, Peripheral blood T cells.
CD26 (TA5.9) on T cell migration was examined. Migration was determined by counting the number of cells at different depths of the collagen. It can be seen in Fig. 6A that diprotin A and vildagliptin stimulated T cell migration into collagen. The CD26-specific Ab (TA5.9) also enhanced T cell migration significantly, whereas an Ab to CD8 did not affect migration (Fig. 6A). A mAb to CD47, B6H12, previously found to block responses to CD47 (50 ng/ml) was used as a control Ab. *p < 0.05; †, p < 0.01; ‡, p < 0.001 vs control. B and C, T cells were allowed to migrate into a collagen gel for 30 min in the presence of diprotin A and mAbs to CD47, CD4, or CD8 (B). *p < 0.05 vs control; ‡, p < 0.01 vs control; †, p < 0.05 vs diprotin A, or into a collagen gel containing CXCL12 (50 ng/ml) in the presence and absence of mAbs to CD47 and CD4 (C). *p < 0.01 vs control; ‡, p < 0.05 vs CXCL12. D, Cells with or without diprotin A were allowed to migrate into a collagen gel containing CXCL12 (50 ng/ml). E, Cells were allowed to migrate into a collagen gel in the presence of various protease inhibitors. D and E, *p < 0.05 vs control; †, p < 0.01 vs control; ‡, p < 0.001 vs control. The number of cells at different depths of the collagen was determined using a depth meter. It is evident from the results that diprotin A markedly increased migration and that the anti-CD47 Ab inhibited migration, whereas the Abs to CD4 and CD8 did not. One representative experiment of three independent experiments is shown. Ac-IETD-CHO, Ile-Glu-Thr-Asp aldehyde; Z-VAD-fmk, Z-ValAlaAsp-fluoromethyl ketone.

**FIGURE 6.** CD26 inhibits T cell migration and adhesion dependent on CD47 and chemokines stimulate T cell migration through CD47. A, T cells were allowed to migrate into a three-dimensional collagen type I gel for 30 min in the presence of vildagliptin (200 μM), diprotin A (200 μM) and anti-CD26 TA5.9 (5 μg/ml). Anti-CD8 (5 μg/ml) was used as a control Ab. *p < 0.05 vs diprotin A, or into a collagen gel containing CXCL12 (50 ng/ml) in the presence and absence of mAbs to CD47, CD4, or CD8 (B). *p < 0.05 vs control; ‡, p < 0.01 vs control. B and C, T cells were allowed to migrate into a collagen gel for 30 min in the presence of diprotin A and mAbs to CD47, CD4, or CD8 (B). *p < 0.05 vs control; ‡, p < 0.01 vs control; †, p < 0.05 vs diprotin A, or into a collagen gel containing CXCL12 (50 ng/ml) in the presence and absence of mAbs to CD47 and CD4 (C). *p < 0.01 vs control; ‡, p < 0.05 vs CXCL12. D, Cells with or without diprotin A were allowed to migrate into a collagen gel containing CXCL12 (50 ng/ml). E, Cells were allowed to migrate into a collagen gel in the presence of various protease inhibitors. D and E, *p < 0.05 vs control; †, p < 0.01 vs control; ‡, p < 0.001 vs control. The number of cells at different depths of the collagen was determined using a depth meter. It is evident from the results that diprotin A markedly increased migration and that the anti-CD47 Ab inhibited migration, whereas the Abs to CD4 and CD8 did not. One representative experiment of three independent experiments is shown. Ac-IETD-CHO, Ile-Glu-Thr-Asp aldehyde; Z-VAD-fmk, Z-ValAlaAsp-fluoromethyl ketone.

**Discussion**

The present results unveil a previously unknown CD26-controlled intraplasmic membrane pathway for T cell regulation of adhesion, particularly cytoplasmic spreading, and migration connecting chemokines/chemokine receptors with TSP-1, CD91, including coreceptor CRT, and CD47 (31, 32, 66). CD26 thus down-regulates TSP-1 expression, cell polarization, and migration at the plasma membrane level, where environmental interactions take place. CXCL12 and CCL5 seem to abrogate this CD26-dependent negative regulation and hence are potent inducers of a molecular cascade characterized by up-regulated cell surface expression of TSP-1 that stimulates expression of CD91. This indicates that the CD26-controlled chemokine-activated TSP-1-CD91 cascade is essential for the motile capacity that enables T cells to carry out immune surveillance throughout the organism. The involvement of CD47, a TSP-1 receptor for induction of growth, apoptosis, and differentiation of lymphocytes as well as for the regulation of T cell inflammation (34, 67–70), suggests that this cascade may also have bearing on other aspects of immune regulation besides T cell adhesion and migration. Furthermore, the fact that chemokines as well as TSP-1, CD47, CD91, and CD26 are present also in non-lymphoid cells means that interactions in cis between these
components within the same plasma membrane may have implications also for other cell types.

It is well established that chemokines regulate T cell migration through G-protein-coupled receptors and that CD26 is involved in the truncation of CCL5, CCL4, and CXCL12, which is considered to inactivate or reduce their cell-stimulating capacity (53, 71, 72). However, the present results suggest that the regulatory influence of chemokines and CD26 is more complex and that chemokines stimulate T cells through abrogation of a CD26-dependent suppression. This abrogated suppression probably reflects a different handling of TSP-1 in comparison with that of cells not stimulated by chemokines. It is reasonable to assume that the CD26 control mechanism counteracts the capacity of TSP-1 to stimulate CD91 expression and as a consequence of this TSP-1 is not associated with and processed by CD91 and TSP-1 turnover maintained at a low level. CD26 may thus be postulated to have a dual role as a regulator of T cell functions being engaged either in suppression of the TSP-1-CD91 cascade or in the processing of chemokines stimulating the same cascade. Chemokines processed by CD26 may be presented to T cells associated with extracellular matrix components, on cell surfaces such as endothelial cells, or in solution, and may thus increase the locomotor capacity of T cells through TSP-1 and CD91 by acting as CD26 inhibitors.

Transient chemokine-induced inhibition of CD26 may up-regulate the motility of vascular lymphocytes as part of a commitment to extravasation. Under normal physiological conditions with respect to chemokine concentrations and endothelial presentation, CD26 probably controls the TSP-1-CD91 cascade effectively. However, inflammation-induced chemokine production provoked by microorganisms, autoantigens, or allergens may lead to persistent inhibition of CD26 and down-regulate its suppressive effect on this cascade. Thus, it is possible that persistent down-regulation of the suppressive CD26 effect on TSP-1 expression and T cell motility may account for a propensity of certain individuals to develop autoimmune and other types of T cell-dependent inflammation. Involvement in TSP-1 regulation may explain the protective effect of CD26 against autoimmunity, as demonstrated in knockout mice (46, 47). It is interesting in this context that recent studies of regulatory T cells, which are important for ensuring that the immune system does not attack self and does not overreact to external Ags, suggest that the ability of regulatory T cells to suppress bystander T cells depends on their capacity to produce TSP-1 (73). The present finding that CD26 exerts a negative control of T cell activity through TSP-1 taken together with the notion that CD26 suppresses autoimmune disease in vivo models (46, 47) suggest that TSP-1, CD91, CD47, CD26, and chemokine/chemokine receptors are components in a multimolecular network for integrated positive and negative T cell regulation of immunity.

**FIGURE 7.** Silencing of CD26 enhances cell surface expression of TSP-1 and CD91 as well as development of locomotor morphology and migration in T cells. A, Comparison of the expression of CD26, TSP-1, CD91, and CD4 in T cells after transfection with CD26 siRNA and negative controls (control siRNA and nontransfected cells). †, p < 0.001 vs negative control. CD26 was determined after permeabilization of the cells. B and C, CD26-silenced cells were allowed to migrate into a collagen gel for 30 min. B, morphological appearance of CD26-silenced cells in comparison with negative control cells. C, Depth of migration in comparison with negative control cells. One representative experiment of two independent experiments is shown.

**FIGURE 8.** Model of a chemokine-activated CD26-suppressed molecular cascade for T lymphocyte regulation. In the absence of chemokine stimulation, CD26 inhibits TSP-1-induced cell surface expression of CD91. Chemokines inhibit CD26 and abrogate its suppression of T cell motility through TSP-1. TSP-1 is transported to the cell surface through a brefeldin A-sensitive process and CD91 when present on the cell surface is postulated to drive a pathway for endocytosis of TSP-1. CD26-processed chemokines stimulate T cell polarity and migration and probably also other T cell functions through CD26 inhibition and up-regulation of the TSP-1-CD91 cascade.
In conclusion, the present investigation provides evidence for the existence of a CD26-controlled chemokine-activated cell surface cascade for regulation of T lymphocyte functions through cis receptor communication, as outlined in Fig. 8. According to this model, CD26-processed chemokines inhibit the capacity of CD26 to suppress the formation of active cell edges and motility in T cells through TSP-1 and CD91. This CD26-mediated suppression seems to target a mechanism that probably plays a pivotal role for T cell recognition and interaction with the environment and that may be important for the pathogenesis of immunological diseases. An inherent poor CD26 expression or down-regulation of the CD26-processed chemokines seems to target a mechanism that probably plays a pivotal role for regulation of T lymphocyte functions through TSP-1 and CD91. This CD26-mediated suppression of chemokines inhibits the capacity of CD26 to regulate the existence of a CD26-controlled chemokine-activated cell surface cascade for regulation of T lymphocyte functions through cis receptor communication.

Disclosures
The authors have no financial conflict of interest.

References


