The Neutrophil-specific Antigen CD177 Is a Counter-receptor for Platelet Endothelial Cell Adhesion Molecule-1 (CD31)*

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Human neutrophil-specific CD177 (NB1 and PRV-1) has been reported to be up-regulated in a number of inflammatory settings, including bacterial infection and granulocyte-colony-stimulating factor application. Little is known about its function. By flow cytometry and immunoprecipitation studies, we identified platelet endothelial cell adhesion molecule-1 (PECAM-1) as a binding partner of CD177. Real-time protein-protein analysis using surface plasmon resonance confirmed a cation-dependent, specific interaction between CD177 and the heterophilic domains of PECAM-1. Monoclonal antibodies against CD177 and against PECAM-1 domain 6 inhibited adhesion of U937 cells stably expressing CD177 to immobilized PECAM-1. Transendothelial migration of human neutrophils was also inhibited by these antibodies. Our findings provide direct evidence that neutrophil-specific CD177 is a heterophilic binding partner of PECAM-1. This interaction may constitute a new pathway that participates in neutrophil transmigration.

CD177 (NB1 and PRV-1) is a 58- to 64-kDa glycosylphosphatidylinositol-anchored glycoprotein expressed exclusively by neutrophils, neutrophilic metamyelocytes, and myelocytes, but not by any other blood cells (1, 2). We and others elucidated its primary structure by sequencing the NB1 and PRV-1 genes, which later turned out to be two alleles of a single CD177 gene (3–5). The surface expression of CD177 is unique in that only a subpopulation of neutrophils expresses this protein on the cell surface, with the mean size of the CD177-positive subpopulation ranging from 45% to 65% (2, 6).

CD177 has been well studied as a target antigen in immune-mediated disorders. During pregnancy, women with a CD177 null phenotype are prone to produce alloantibodies against CD177 that cross the placenta, react with fetal neutrophils, and cause neutropenia of the newborn. This mechanism led to the initial discovery of the NB1 antigen in 1971 (7). Alloantibodies to CD177, present in blood products obtained from immunized donors, have also been implicated as mediators of transfusion-related acute lung injury (8).

Although well characterized as an immunotarget, the function of CD177 is largely unknown. It has been reported that CD177 is up-regulated on the neutrophil surface upon stimulation, including during severe bacterial infections, and following granulocyte-colony-stimulating factor treatment (9). In addition, antibody-mediated clustering of CD177 primes the N-formyl-methionyl-leucyl-phenylalanine (fMLP)3-activated respiratory burst reaction of the neutrophil (8). Taken together, these observations make it reasonable to suppose that CD177 may be involved in processes of neutrophil-mediated host defense. One preliminary study suggests a participation of CD177 in neutrophil-endothelial cell interaction (10). The latter observation is in line with the fact that CD177, as a member of the leukocyte antigen-6 superfamily, shares a similar structure with the urokinase plasminogen activator receptor (11). Urokinase plasminogen activator receptor is expressed on numerous cell types and plays an important role in cell-extra-cellular matrix and cell-cell interaction by binding of vitronectin, and by regulating β1 and β2 integrin-dependent adhesion of leukocytes (12).

Our understanding of the role that CD177 might similarly play in mediating neutrophil-endothelial cell interactions is limited, however, by lack of an identifiable counter-receptor on the endothelial cell surface. A complex molecular crosstalk is known to be responsible for the interaction between neutrophils and endothelial cells (13, 14). Whereas the initiating step of rolling and subsequent firm leukocyte adhesion have been well characterized (15), less is known about the mechanisms that mediate the migration of leukocytes through the endothelium. A number of adhesion molecules have been implicated in this process, including β2 integrins, ICAM-1 (intercellular adhesion molecule-1), junctional adhesion molecules (JAMs), CD99, and platelet endothelial cell adhesion molecule-1.

† The abbreviations used are: fMLP, N-formyl-methionyl-leucyl-phenylalanine; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; sr, soluble recombinant; SPR, surface plasmon resonance; IL, interleukin; BCECF, 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; JAM, junctional adhesion molecule; PECAM-1, platelet endothelial cell adhesion molecule-1; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

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**CD177 Is a Counter-receptor for PECAM-1**

(PECAM-1) (16, 17). PECAM-1 is a constitutively expressed, abundant component of endothelial cell junctions at all levels of the vascular tree (18, 19). Homophilic PECAM-1-PECAM-1 interaction as part of a sensing and activating process of neutrophils plays a central role in leukocyte migration (20). In addition, a number of heterophilic binding partners for PECAM-1 have been described, including CD38, αβ3, and glycosaminoglycans (21–23), but the relevance of these partners in leukocyte transmigration is currently not well established (24–27).

In this study, we demonstrate that neutrophil-specific CD177 can directly bind PECAM-1 and that CD177 and PECAM-1 constitute a heterophilic ligand pair with contributions to neutrophil-endothelial cell interactions.

**EXPERIMENTAL PROCEDURES**

**Cells**—U937 cells were obtained from DSMZ, Braunschweig, Germany, and maintained in α-minimal essential medium and RPMI 1640 medium (Invitrogen). HEK cell lines were kindly provided from Dr. B. Nieswandt (Würzburg, Germany) and grown in Dulbecco’s modified Eagle’s medium/high glucose medium (PAA Cell Culture Co., Göttingen, Germany). All media were supplemented with 10% fetal calf serum (Seromed, Berlin, Germany) and 1% penicillin/streptomycin (PAA Cell Culture Co.). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord and cultured as previously described (28). HUVECs were cultured in MCDB131 medium (Invitrogen) supplemented with 1% penicillin/streptomycin (PAA Cell Culture Co.).

**Monoclonal Antibodies**—Hybridoma producing mAb 7D8 against CD177 was kindly provided by Dr. D. Strokopce (Dept. of Transfusion Medicine, NIH, Bethesda, MD). mAb MEM166 specific for CD177 was purchased from Serotec (Düsseldorf, Germany). mAb Gi18 directed against the first two domains of PECAM-1 was produced and characterized in our laboratory (29). mAbs PECAM-1.1 and PECAM-1.2 recognizing domains 5 and 6 of PECAM-1, respectively, have been previously described (24, 30). mAbs 68-5H11 and AK-4 against E- and P-selectin, respectively, were purchased from BD Pharmingen (Heidelberg, Germany). mAb Gi11 specific for JAM-C was produced in our laboratory (31). All antibodies are of IgG1 isotype, except for PECAM1.1 (IgG2a).

**Recombinant Proteins**—Soluble recombinant (sr) PECAM-1 derived from PECAM-1-stable transfected Chinese hamster ovary cells has been previously described (32). All recombinant proteins were purified by affinity chromatography before use. Fc protein as control was purchased from R&D Systems (Wiesbaden, Germany).

**Generation of CD177-Fc Construct**—Full-length CD177 cDNA (3) was amplified by PCR using forward primer 5’-CGACTCTGCTCAGGACGCTCTGGAC-3’ and reverse primer 5’-CTGCACTCATCGTCTCGAC-3’. Aliquots of 1 μl of CD177 construct were diluted with 10X PCR buffer, 0.5 μM of each primer, 2.5 μM dNTP, 2.5 units of cloned Pfu DNA polymerase (Stratagene, Heidelberg, Germany) in a total volume of 50 μl. PCR amplification was performed for 32 cycles. Each cycle consisted of denaturation for 1 min at 95 °C, annealing for 1 min at 60 °C, and extension for 1 min at 72 °C. In the final cycle, the sample was kept at a temperature of 72 °C for 10 min and chilled to 4 °C. The PCR product was purified from a 1% agarose gel by using QIAquick (Qiagen), subcloned into the EcoRV cloning site of Signal piplgus plasmid (kindly provided by Dr. B. Nieswandt, Würzburg, Germany), and then transformed into DH5α competent *Escherichia coli* (Invitrogen). Positive clones were screened by PCR using CD177 and T7 primer pairs as described above. A plasmid from selected positive clones was validated by nucleotide sequence analysis on an ABI Prism Genetic Analyzer 3100 (Applied Biosystems, Weiterstadt, Germany).

**Production of Recombinant CD177-Fc Fusion Protein**—HEK cells were grown in 24-well plates and were transfected with 0.2 μg of CD177 construct in 350 μl of Opti-Mem medium (Invitrogen) by the use of Effectene (Qiagen). Transfected cells were selected with increasing concentration of Geneticin (40–800 μg/ml, PAA Cell Culture Co.). Culture supernatants from stable cell lines were then tested by enzyme-linked immunosorbent assay. In brief, microtiter wells were coated with 50 μl of donkey anti-human Fc (diluted 1:250, Dianova, Hamburg, Germany) overnight at 4 °C. After washing three times with 100 μl PBS, wells were blocked with 2% BSA in PBS for 30 min at 4 °C. An aliquot of 50 μl of supernatant was added, and the mixture was incubated for 30 min at 37 °C. Wells were washed twice, and bound Fc fusion protein was detected with 50 μl of peroxidase-labeled donkey anti-human Fc (diluted 1:3000, Dianova) and ortho-phenylenediamine (Dako, Hamburg, Germany) as substrate. Reaction was stopped after 15 min with 50 μl of H2SO4 and was read on an enzyme-linked immunosorbent assay reader at 405 nm. CD177-Fc fusion protein was isolated from 1 liter of culture supernatant and purified by the use of a protein G column. Purified protein was analyzed on 7.5% SDS-PAGE by silver staining and verified by immunoblotting (see below).

**Analysis of CD177-Fc Binding to Endothelial Cells by Flow Cytometry**—Aliquots of 4 × 10^6^ HUVECs were fixed with 1% formaldehyde for 5 min and washed twice with PBS. Subsequently, cells were incubated with 4 μg of CD177-Fc or Fc alone for 1 h at 37 °C, washed with 0.02% BSA in PBS buffer, and then labeled with 40 μl of fluorescein-conjugated donkey anti-human IgG (diluted 1:800, Dianova). After washing, cells were resuspended in 500 μl of 0.2% BSA for flow cytometry analysis (FACSCalibur, BD Biosciences, Heidelberg, Germany).

**Antigen Capture Assay**—Microwell filters were coated with 100 μl of donkey anti-human Fc (see above) overnight at 4 °C. Wells were washed extensively three times with 0.2% BSA and blocked with 2% BSA for 30 min at 4 °C. Aliquots of 250 ng of CD177-Fc fusion protein or Fc alone (as control) were added, and the mixture was incubated for 1 h at 37 °C. Wells were washed twice and then incubated with 100 μl of endothelial cell lysates (1 μg) for 30 min at 37 °C. Bound protein was detected by the addition of mAbs against PECAM-1, E-selectin, and P-selectin (1:500 dilutions) at 37 °C for 30 min. After washings, 100 μl of peroxidase-labeled donkey anti-mouse IgG (diluted 1:800, Dianova) was added for 1 h at 37 °C. Bound antibodies were measured on an enzyme-linked immunosorbent assay reader as described above.

**Affinity Isolation of the CD177 Binding Partner**—HUVECs were labeled with 2 ml of 5 mM NHS-LC-Biotin (Pierce) and
lysed in 1 ml of lysis buffer containing protease inhibitors as previously described (33). After preclearing with protein-G beads (Amersham Biosciences), cell lysates were stored at −70 °C until use. Aliquots of 100 μl of Protein-G beads were coupled with 2.5 μg of CD177-Fc fusion protein or Fc control for 1 h at 4 °C. 300 μl of labeled cell lysates were added to the beads and were rotated overnight at 4 °C. After washings with immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100), bound proteins were eluted by adding SDS buffer for 5 min at 100 °C. Eluates were analyzed on 7.5% SDS-PAGE under reducing conditions. Separated proteins were transferred onto nitrocellulose membranes and developed with peroxidase-labeled streptavidin and a chemiluminescence system (ECL, Amersham Biosciences). After stripping with Restore™ Western blot (Pierce), the membrane was redeveloped with selected mAbs against endothelial cell markers (20 μg/ml). Bound mAbs were visualized with peroxidase labeled rabbit anti-mouse IgG (diluted 1:100,000, Dianova) and the ECL system.

Expression of CD177 in U937 Monocyte Cell Line—U937 cells were grown in RPMI medium containing 10% fetal calf serum and 0.5% penicillin/streptomycin. An aliquot of 250 μl of cell suspension (2 × 10⁷ cells/ml) was transfected with 20 μg of CD177 in pcDNA3 vector (3) using electroporation technique for 40 ms (Bio-Rad GenePulser). After 10-min incubation on 4 °C, cells were seeded in 10 ml of RPMI medium. The next day, cells were washed and selected for stable expression by the use of RPMI medium containing Genetecin (1 mg/ml). Transfectants expressing recombinant CD177 were identified by flow cytometry analysis using mAb 7D8 as described above.

Phenotyping of Neutrophils—Neutrophils were phenotyped for CD177 expression by flow cytometry as previously described (9). Briefly, granulocytes were isolated from EDTA-anti-coagulated blood by dextran sedimentation and gradient centrifugation. Indirect immunofluorescence was performed by flow cytometry using mAb 7D8. Five thousand cells were analyzed. The size of the positive subpopulation was calculated from the histogram employing CellQuest software. To investigate the influence of sfpMF (Sigma) or IL-8 (Immuno Tools, Friesoythe, Germany) on protein surface expression, granulocytes were isolated as described and incubated with 5 μg/ml cytochalasin B (Sigma) for 30 min at room temperature. Subsequently, cells were incubated for 30 min at room temperature in the absence or presence of 10 nm sfpMF or 100 ng/ml IL-8, respectively. Indirect immunofluorescence was performed as outlined above using mAbs 7D8 and Gi18.

Isolation of CD177 Proteins from Human Neutrophils—Granulocyte concentrates were obtained from healthy volunteers by a standard leukapheresis procedure (34). Twelve hours prior to apheresis, all volunteers received 7.5 μg per kilogram bodyweight of human recombinant granulocyte-colony-stimulating factor (ChugaiPharma, Frankfurt am Main, Germany), as approved by the local ethics committee. Granulocyte concentrates (medium volume, 230 ml) were diluted in PBS (1:5). Aliquots of 15 ml were layered onto Ficoll gradient and then centrifuged for 20 min at 800 × g. Cell pellets were resuspended into 12 ml of ammonium chloride for 5 min on ice to lyse erythrocytes. After washing twice, 10⁶ cells were solubilized in 1 ml of lysis buffer (20 mM Tris-buffered saline, pH 7.4. 0.25% Triton-X, 100 μl of protease inhibitor mixture, 100 μl of 5% EDTA) for 30 min. Cell lysates were then centrifuged for 30 min at 800 × g at 4 °C and pooled for purification by affinity chromatography as previously described (3). Isolated CD177 protein was then intensively dialyzed against PBS, and the remaining trace of Triton X-100 was removed by filtration through YM-10 column (Amicon, Witten, Germany). Platelet-derived αββ3 protein as a control was isolated from outdated platelet concentrates by mAb Gi5 affinity column under identical conditions. The purity and identity of both proteins were proved by silver staining and immunoblotting analysis, and protein concentration was determined by BCA™ (Pierce). Aliquots of purified proteins were stored at −80 °C until use.

Surface Plasmon Resonance—Direct protein interaction between PECA-1 and CD177 was examined in real-time with a surface plasmon resonance (SPR) technique on a BLAcore 2000 (Biacore AB, Freiberg, Germany). Purified srPECAM-1 diluted in 10 mM acetate buffer (pH 4.0) to a concentration of 60 μg/ml was directly immobilized on a CM5-sensor chip via amino coupling as recommended by the manufacturer. Aliquots of 20 μl of purified CD177 or BSA (Pierce) as control were injected at a flow rate of 15 μl/min at different concentrations as indicated. In some experiments, 2 mM CaCl₂, 25 μM ZnSO₄, or 10 mM EDTA were added into CD177 analyte prior to analysis. For blocking studies, 50 μg of mAbs was added in selected experiments. Running buffer was PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) containing 0.005% surfactant P20 (Biacore AB). The sensor chip was regenerated with 25 mM NaOH.

Cell Adhesion Assay—Cell adhesion to PECA-1-coated wells was tested as described previously (35). Briefly, microtiter plates were coated with 50 μl of Fc-PECAM-1 or Fc alone as control (10 μg/ml in PBS) and blocked with 3% BSA solution. Nontransfected or CD177-transfected U937 cells were incubated with 5 μl of 2',7'-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein (BCECF, Molecular Probes, Leiden, The Netherlands) for 30 min in the dark. Cells were washed twice in serum-free RPMI, and 10⁵ cells/well were plated onto the precoated wells at 37 °C for 60 min. For inhibition studies, cells were incubated with 10 μg/ml mAbs specific for CD177 (MEM166) and PECA-1 (Gi18, PECA1.1, and PECA1.2) or with 50 μl of purified CD177 (10 μg/ml) during the adhesion period. After the incubation period, the wells were washed gently, and adhesion was quantified using a fluorescence microplate reader Flx-800 (Biotek, Neufahrn, Germany).

Transmigration Assay—Briefly, transmigration assays were performed using 6.5-mm Transwells with an 8-μm pore size (Costar, Bodenheim, Germany). Inserts were coated with gelatin (Sigma). HUVECs were seeded on Transwell filters 2 days prior to the assay and grown for 48 h in a humidified atmosphere (37 °C and 5% CO₂). The integrity of HUVEC monolayers was assessed microscopically. At the beginning of the transmigration assay, HUVECs in the upper chamber were washed with serum-free RPMI, the lower chamber was filled with serum-free RPMI medium containing 10 nm sfpMF (Sigma) or 100 ng/ml IL-8 (Immuno Tools). 1 ml of neutrophils (5 × 10⁶ cells/ml) was labeled with 5 μl of BCECF as described above. An
CD177 Is a Counter-receptor for PECAM-1

FIGURE 1. Analysis of purified CD177-Fc. A, silver staining and immunoblotting analysis. Affinity-purified CD177-Fc was run on 7.5% SDS-PAGE under reducing condition. It was visualized by silver staining (lane 2) in comparison to indicated molecular weight standards (lane 1). CD177-Fc was then transferred onto nitrocellulose membrane and stained with mAb 7D8 and peroxidase-labeled donkey anti-mouse IgG (lane 1), peroxidase-labeled donkey anti-human IgG (lane 2), or peroxidase-labeled donkey anti-mouse alone (lane 3). Bound antibodies were detected using the chemiluminescence system. B, binding analysis of CD177-Fc with HUVECs by flow cytometry. HUVECs were labeled with CD177-Fc, Fc alone, mAb 7D8 (anti-CD177), and mAb Gi18 (anti-PECAM-1). After washings, bound Fc proteins and bound mAbs were detected with fluorescein-labeled donkey anti-human IgG or fluorescein-conjugated donkey anti-mouse IgG. In the control experiments, mAb Gi18 specific for PECAM-1 reacted strongly with HUVECs. In contrast, mAb 7D8 against CD177 did not react with endothelial cells.

FIGURE 2. Analysis of the CD177-Fc binding partner. A, antigen capture assay: CD177-Fc was immobilized to microtiter wells via donkey anti-human IgG and incubated with HUVEC lysates. After washings, bound endothelial proteins were incubated with different mAbs as indicated and detected by the use of peroxidase-labeled donkey anti-mouse IgG and ortho-phenylenediamine as substrate. Reaction was measured on an enzyme-linked immunosorbent assay reader at 405 nm (A405). Data represent means ± S.D. from n = 3 independent experiments. B, immunoprecipitation and immunoblotting: Lysates of biotin surface-labeled HUVECs were precipitated with Fc alone, CD177-Fc, or mAb 7D8 coupled to Protein G beads. After washings, bound proteins were separated on a 7.5% SDS-PAGE under nonreducing conditions. Proteins were then transferred onto polyvinylined difluoride membrane and were visualized by the use of streptavidin and the chemiluminescence detection system (ECL). Subsequently, the membrane was stripped and redeveloped with mAb Gi18 against PECAM-1 and visualized with peroxidase-labeled rabbit anti-mouse IgG and the ECL system. CD177 CR, CD177 counter-receptor.

RESULTS

Production and Characterization of CD177-Fc Fusion Protein—To determine the binding partner of CD177, we first established stably transfected mammalian HEK cells producing soluble recombinant CD177-Fc fusion protein. Supernatants were collected after 3 days of culture and purified by the use of a protein G column. The purified protein was analyzed by silver staining to document its purity (Fig. 1A, left panel) and by immunoblotting to prove the immunoreactivity of both CD177 and the Fc portion (Fig. 1A, right panel). Purified CD177-Fc showed positive reactions with mAb 7D8 specific for CD177, as well as with donkey anti-human Fc (lanes 1 and 2). In the control experiment, donkey anti-mouse Fc did not show any reaction (lane 3). These results demonstrate that both portions, CD177 and Fc, of our fusion protein are immunoreactive.

Aliquot of 100 μl of untreated cells (5 × 10⁶ cells/ml) or cells preincubated with mAbs (10 μg/ml, 15 min, room temperature) was added to the upper chamber on top of the endothelial monolayer. After incubation for 90 min at 37 °C, the number of transmigrated cells in the lower chamber was measured using a fluorescence reader as above.

Binding of CD177-Fc Fusion Protein to HUVECs—The reactivity of the CD177-Fc fusion protein with HUVECs was tested by flow cytometry (Fig. 1B). Isolated HUVECs were incubated with CD177-Fc or Fc alone as a control, and a specific reaction with CD177-Fc only was observed. These results indicate that endothelial cells carry a binding partner for CD177.

To further characterize the binding partner of CD177, an antigen capture assay was performed using CD177-Fc fusion protein immobilized on microtiter wells. After adding HUVEC lysates, bound protein was screened with different mAbs against endothelial proteins. As shown in Fig. 2A, a positive reaction was observed with anti-PECAM-1, whereas no reaction was detectable with mAbs against E-selectin, P-selectin, and against JAM-C, indicating that PECAM-1 may represent the binding partner of CD177.

Immunoprecipitation was then performed with surface biotin-labeled HUVECs to confirm this finding. Labeled cell lysates were precipitated with Fc alone, CD177-Fc, and mAb 7D8 coupled to Protein G beads. Immunoprecipitates were analyzed by immunoblotting using a peroxidase-labeled streptavidin system (Fig. 2B, left panel). In comparison to con-
Concentrations (0.5–2.0 M) were affinity-purified before use and verified by silver staining or were transferred onto nitrocellulose membrane for immunoblotting (Fig. 3). SrPECAM-1 was immobilized onto the sensor surface, and the binding of CD177 at various concentrations (0.5–2.0 M) was measured. As shown in Fig. 4B, addition of 2 mM Ca^{2+} increased the binding of CD177 to PECAM-1 significantly. In the control experiment, the presence of 25 μM Zn^{2+}, which increased the binding of urokinase plasminogen activator receptor to vitronectin and fibrinogen (38), did not have any impact on CD177/PECAM-1 interaction (Fig. 4B). In the presence of 10 μM EDTA, the interaction between PECAM-1 and CD177 was nearly abolished, underlining the importance of divalent calcium cation for the heterophilic binding.

Finally, to confirm the existence of PECAM-1/CD177 heterophilic binding, inhibition studies were performed with CD177-specific mAbs 7D8 and MEM166. As shown in Fig. 4C, both mAbs were able to inhibit the PECAM-1/CD177 interaction to different degrees, whereas mAb Gi5 specific for the αιβ3 integrin (as a control) failed to block this binding. Competitive binding studies showed that mAb 7D8 and MEM 166 bind different epitopes on CD177 (not shown). These results demonstrate that CD177 can directly and specifically interact with PECAM-1.

**Heterophilic Interactions between CD177 and PECAM-1 Mediate Cell Adhesion**—To determine whether the heterophilic interaction of CD177 with PECAM-1 functions to mediate cell adhesion, we first generated U937-transfected cells expressing CD177. Analysis by flow cytometry showed that CD177 transfectants express CD177 (Fig. 5). Curiously, loss of PECAM-1 expression was observed in CD177-transfected cells. In contrast, nontransfected U937 cells express PECAM-1, but not CD177, on their surface. This observation was confirmed by immunoblotting (Fig. 5). Thus, although the adhesion of nontransfected U937 cells (PECAM-1-positive and CD177-negative) and CD177-transfected U937 cells (PECAM-1-negative and CD177-positive) cannot be compared quantitatively, both cells represent an appropriate system in which to study homophilic interactions of PECAM-1, and heterophilic interactions between PECAM-1 and CD177, respectively.

To study cell adhesion, we allowed nontransfected U937- and CD177-transfected U937 cells to adhere to immobilized srPECAM-1. As shown in Fig. 6, both cell types adhered to immobilized PECAM-1. The adhesion of nontransfected U937 cells to immobilized PECAM-1 could be inhibited by mAb Gi18 against the first domain of PECAM-1, which interferes with the homophilic interaction of PECAM-1, but not by mAbs PECAM1.1 and PECAM1.2 specific for the fifth and sixth immunoglobulin domains of PECAM-1, respectively. Additionally, neither mAb MEM166 nor purified CD177 interfered with the adhesion of nontransfected U937 cells. The opposite inhibition pattern was observed when CD177-transfected U937 cells were used. The adhesion of these cells (CD177-positive and PECAM-1-negative) to PECAM-1 was inhibited by mAb MEM166, purified CD177, as well as by mAb PECAM1.2, but not by mAb Gi18.

Differences in the adhesion of nontransfected or CD177-transfected U937 cells to immobilized fibrinogen or JAM-C (mediated by β2 integrins) and fibronectin (mediated by β1 integrins) were not observed (data not shown), suggesting that the presence of CD177 on U937 cells specifically influenced
CD177 Is a Counter-receptor for PECAM-1

The Role of CD177 in Neutrophil Transmigration—To demonstrate the participation of CD177 in neutrophil recruitment, we first assessed the influence of two important chemoattractants, fMLP and IL-8, on CD177 surface expression. Stimulation of neutrophils for 30 min with fMLP increased the surface expression of CD177 by 2.75-fold (95% confidence interval, 2.21- to 3.29-fold). In contrast, PECAM-1 surface expression was down-regulated in the presence of fMLP (mean decrease, 0.33-fold; 95% confidence interval, 0.18- to 0.48-fold). Comparable results were obtained after stimulation with IL-8, which increased the surface expression of CD177 by 3.1-fold (95% confidence interval, 2.47- to 3.73-fold) and down-regulated PECAM-1 surface expression 0.32-fold (95% confidence interval, 0.18- to 0.46-fold). Fig. 7A shows one example from a representative experiment.

Next, we tested the migration of neutrophils through endothelial cells toward the chemoattractants fMLP and IL-8 (Fig. 7B). Significant inhibition of neutrophil migration was observed with mAb MEM166 specific for CD177 and mAb PECAM1.2 against the sixth domain of PECAM-1. In contrast, mAb PECAM1.1 was unable to interfere significantly with neutrophil transmigration toward fMLP or IL-8. A monoclonal antibody against the first domain of PECAM-1 (Gi18), which is known to inhibit PECAM-1/PECAM-1 homophilic interactions, did interfere with the migration of neutrophils. Thus, CD177-mediated neutrophil transmigration occurs via heterophilic interaction with PECAM-1 and can be blocked partially in the presence of mAbs against CD177 or against a heterophilic domain of PECAM-1. Besides this heterophilic interaction, PECAM-1/PECAM-1 homophilic interaction is significantly involved in transendothelial migration.

Finally, to address the question whether CD177-positive and CD177-negative neutrophils behave equally in transmigration, we assessed the size (amount) of each neutrophil subpopulation after 90 min of transmigration by analyzing neutrophils that had transmigrated and those which remained in the upper chamber of the transmigration system (Fig. 7C). Whereas prior to transmigration nearly 50% of all neutrophils were CD177-positive and 50% were CD177-negative, the proportion of CD177-positive neutrophils decreased to roughly one-third of the population of cells that did not transmigrate (upper chamber). In contrast, the proportion of CD177-positive neutrophils among those which had migrated through the endothelium was roughly two-thirds of all cells (lower chamber). Apparently, CD177-positive neutrophils tended to transmigrate more readily...
than CD177-negative neutrophils. Taken together, our data indicate that the heterophilic interaction between CD177 and PECAM-1 participates in transendothelial migration of neutrophils.

**DISCUSSION**

Chemotactic factors and pro-inflammatory cytokines signal the recruitment of neutrophils to sites of infection by a multistep process that involves a number of surface molecules on both the endothelium and the neutrophil. CD177 is a neutrophil-specific, glycosylphosphatidylinositol-linked surface glycoprotein that becomes up-regulated during bacterial infections (9). In the present study we addressed whether CD177 participates in neutrophil recruitment. Here, we demonstrate heterophilic binding between neutrophil-specific CD177 and PECAM-1 that contributes to interactions between neutrophils and endothelial cells in the context of inflammatory cell recruitment.

First, a CD177-Fc fusion protein was demonstrated to specifically bind to endothelial cells. Immuno-precipitation studies revealed that the endothelial partner for CD177-Fc was a protein of 120 kDa, which was further identified as PECAM-1. In SPR, the CD177 protein bound to PECAM-1 in a concentration-dependent manner with a dissociation constant of $8.15 \times 10^{-7}$ M. This heterophilic interaction was dependent on divalent calcium cations and could be blocked by mAb MEM166 against CD177. In contrast to the homophilic interaction of PECAM-1, which is dependent on immunoglobulin domains 1 and 2 of the molecule, the heterophilic CD177 interaction was found to be mediated by immunoglobulin domain 6 (and probably also domain 5) of PECAM-1.

It has been established previously that PECAM-1 can mediate heterophilic, cation-dependent or homophilic, cation-independent cell adhesion (22, 23, 36, 39). Distinct domains of the PECAM-1 molecule are involved in these two types of PECAM-1-mediated interaction, with domains 5 and 6 being involved in heterophilic binding (30, 40), and domains 1 and 2 being involved in homophilic binding (40–43). Furthermore, high affinity cation binding sites involving domains 5 and 6 of PECAM-1 were demonstrated by binding studies using radiolabeled CaCl$_2$ (37). In addition, CD177-transfected U937 cells adhered to immobilized PECAM-1; this interaction could be inhibited by antibodies to CD177 as well as a mAb specific for domain 6 of PECAM-1. This indicates that the heterophilic CD177/PECAM-1 interaction may participate in adhesive interactions between the CD177-expressing cells and the endothelium.

The importance of PECAM-1 in mediating leukocyte transendothelial migration has been known for more than a decade (36, 44). The homophilic interaction of the N-terminal portion of leukocyte PECAM-1 with the same domains of
endothelial-cell PECAM-1 is crucial in transmigration: blocking of this interaction inhibits transmigration both in vitro and in vivo (40–43). The contribution of PECAM-1 as a heterophilic partner in transmigration is less well defined. The αvβ3 integrin has been extensively examined for its potential to serve as a counter-receptor for PECAM-1 (22, 25, 45, 46). αvβ3 integrin is expressed on endothelial cells and neutrophils as well as on other blood cells, and its involvement in the transmigration of leukocytes has been reported (47). However, blocking studies have argued against the possibility that αvβ3 and PECAM-1 act as counter-receptors in the regulation of leukocyte transmigration.
in vivo relevance of the CD177-dependent transmigration pathway will require further investigation. It has recently been demonstrated that CD177 + neutrophils express proteinase 3 as an additional, unique surface marker, probably defining them as a subpopulation with yet unknown functions in innate immunity (48). More importantly, a specific up-regulation of neutrophil CD177 has been demonstrated during bacterial infections, upon treatment with granulocyte-colony-stimulating factor, and during pregnancy as well as in newborns (9, 49, 50). This indicates that the CD177-dependent pathway could be utilized under certain (patho-)physiological conditions.

In summary, the findings of the present study provide evidence that neutrophil-specific CD177 is a heterophilic binding partner of PECAM-1. This interaction mediates leukocyte transmigration for a subpopulation of neutrophils. Thus, it represents a new approach to our understanding of neutrophil/endothelial cell interplay in host defense.

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CD177 Is a Counter-receptor for PECAM-1

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