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CD43 Plays Both Antiadhesive and Proadhesive Roles in Neutrophil Rolling in a Context-Dependent Manner

Masanori Matsumoto,*† Akiko Shigeta,* Masayuki Miyasaka,† and Takako Hirata2*

As the first step in the recruitment of neutrophils into tissues, the cells become tethered to and roll on the vessel wall. These processes are mediated by interactions between the P- and E-selectins, expressed on the endothelial cells of the vessel wall, and their ligands, expressed on the neutrophils. Recently, we reported that CD43 on activated T cells functions as an E-selectin ligand and thereby mediates T cell migration to inflamed sites, in collaboration with P-selectin glycoprotein ligand-1 (PSGL-1), a major P- and E-selectin ligand. Here, we examined whether CD43 on neutrophils also functions as an E-selectin ligand. CD43 was precipitated with an E-selectin-IgG chimera from mouse bone marrow neutrophils. A CD43 deficiency diminished the E-selectin-binding activity of neutrophils when PSGL-1 was also deficient. Intravital microscopy showed that the CD43 deficiency significantly increased leukocyte rolling velocities in TNF-α-stimulated venules blocked with an anti-P-selectin mAb, where the rolling was mostly E-selectin dependent, when PSGL-1 was also absent. In contrast, in venules with trauma-induced inflammation, where the rolling was largely P-selectin dependent, the CD43 deficiency reduced leukocyte rolling velocities. Collectively, these observations suggest that CD43 generally serves as an antiadhesive molecule to attenuate neutrophil-endothelial interactions, but when E-selectin is expressed on endothelial cells, it also plays a proadhesive role as an E-selectin ligand. *The Journal of Immunology, 2008, 181: 3628–3635.

The recruitment of neutrophils from the blood into tissues is a multistep process regulated by a cascade of molecular interactions between neutrophils and endothelial cells. This process is initiated by the tethering and rolling of neutrophils on endothelial cells, which are primarily mediated by selectins. Both P-selectin (CD62P) and E-selectin (CD62E) are expressed on endothelial cells during inflammation, and they interact with P- and E-selectin ligands expressed on the neutrophil surface (1, 2).

The major P-selectin ligand on neutrophils is P-selectin glycoprotein ligand-1 (PSGL-1; CD162), a sialomucin expressed on most leukocytes (3). In PSGL-1-deficient (PSGL-1−/−) mice, leukocyte rolling in cremaster muscle venules after trauma-induced inflammation is markedly decreased at early time points, when the rolling is largely dependent on P-selectin, indicating that PSGL-1 plays a critical role as a P-selectin ligand (4). PSGL-1−/− mice also show reduced leukocyte rolling in TNF-α-stimulated venules blocked with an anti-P-selectin mAb, where the rolling is mostly mediated by E-selectin, indicating that PSGL-1 also functions as an E-selectin ligand (5). However, some PSGL-1−/− leukocytes can still roll in an E-selectin-dependent manner, suggesting that E-selectin ligands other than PSGL-1 mediate the residual rolling.

E-selectin recognizes sialylated and fucosylated carbohydrate structures such as sialyl LewisX (sLeX) presented on certain core molecules (6). Besides PSGL-1, several glycoproteins that bind E-selectin have been reported. CD44, a hyaluronan-binding cell-surface glycoprotein, was reported to bind E-selectin through N-linked glycans and to mediate the E-selectin-dependent rolling of neutrophils (7). However, mice deficient in both PSGL-1 and CD44 still exhibit only a partial defect in neutrophil rolling and migration, suggesting that still other functional E-selectin ligands exist on neutrophils. E-selectin ligand-1 (ESL-1) is a transmembrane glycoprotein that was identified using a recombinant E-selectin-IgG chimera as the major E-selectin ligand on mouse neutrophils (8). The specific glycoform of ESL-1 expressed on myeloid cells also carries sLeX on N-linked glycans. A recent report showed, using RNA interference specific to ESL-1, that ESL-1 serves as a major physiological E-selectin ligand on mouse neutrophils (9). Additionally, in humans, L-selectin on neutrophils is capable of binding E-selectin (10–12), although whether L-selectin supports physiologically relevant interactions with E-selectin remains unknown.

CD43 is a major sialomucin expressed by most leukocytes (13). Two major glycoforms, of 115 and 130 kDa, have been identified for both human and mouse CD43 (13, 14). We and others recently showed that the 130-kDa glycoform of CD43 expressed on mouse Th1 cells and human T lymphoblasts binds E-selectin (15, 16). In vivo, CD43 mediates T cell migration into inflamed skin, a P- and E-selectin-dependent process, in collaboration with PSGL-1 (17). Therefore, the contribution of CD43 to T cell migration was most apparent in the absence of PSGL-1. CD43 is also expressed on neutrophils (18), raising the possibility that CD43 on neutrophils functions as an E-selectin ligand as well. The functional roles of CD43 in neutrophil trafficking, however, are controversial. In one

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study, CD43-deficient (CD43−/−) neutrophils exhibited enhanced interactions with the vessel wall in vivo and immobilized E-selectin under flow conditions in vitro, suggesting an antiadhesive function of CD43 in neutrophil–endothelial cell interactions (19). Paradoxically, the same study showed that neutrophil migration into the inflamed peritoneum is significantly reduced in CD43−/− mice, implicating CD43 in facilitating the migration of neutrophils into tissues. In contrast, another study showed that CD43−/− neutrophils are recruited into the inflamed peritoneum with an efficiency comparable to wild-type (WT) neutrophils, using competitive migration assays (20).

To determine the role of CD43 on neutrophils, particularly its role as an E-selectin ligand, we investigated in vivo the impact of CD43 deficiency on neutrophil trafficking in the absence of the major P- and E-selectin ligand PSGL-1, using PSGL-1 and CD43 double-knockout (DKO) mice. We found that a CD43 deficiency significantly increased leukocyte rolling velocities in venules with TNF-α-induced inflammation blocked with an anti-P-selectin mAb, where the rolling was largely mediated by E-selectin, when PSGL-1 was also absent. In contrast, the CD43 deficiency significantly reduced leukocyte rolling velocities in venules with trauma-induced inflammation, where P-selectin-dependent rolling was observed. Taken together, our results suggest that CD43 generally functions as an antiadhesive molecule to attenuate neutrophil–endothelial cell interactions, but in the venules where E-selectin is expressed, it also plays a role as an E-selectin ligand.

Materials and Methods

Mice

C57BL/6J (B6) mice were purchased from CLEA Japan. PSGL-1−/− mice on a B6 background were provided by B. Furie (Harvard Medical School, Boston, MA). CD43−/− mice on a B6 × 129S4/SvJae background were purchased from The Jackson Laboratory. PSGL-1−/− mice were intercrossed with CD43−/− mice, and the resulting double heterozygotes were bred to yield WT, CD43−/−, PSGL-1−/−, and DKO mice as described previously (17). All the mice used were 6–10 wk old. The mice were housed at the Institute of Experimental Animal Sciences at Osaka University Medical School. All studies and procedures were approved by the Ethics Review Committee for Animal Experimentation of the Osaka University Graduate School of Medicine.

Chimeric proteins

The expression plasmids for mouse P- and E-selectin-IgM chimeric proteins were provided by J. Lowe (University of Michigan Medical School, Ann Arbor, MI). COS-7 cells were transfected with the plasmids using DEAE-dextran. Mouse P- and E-selectin-IgG chimeric proteins were prepared as described previously (21).

Flow cytometry

mAbs used for flow cytometric analyses included anti-CD44-FITC (IM7; BD Biosciences), anti-CD11a-FITC (M17/4; BD Biosciences), anti-CD11b-FITC (M1/70; BD Biosciences), anti-IFN-γ/PE (Cl.A3-1; Caltag Laboratories), anti-Gr-1-FITC and anti-CD11b (RB6-8C5; BioLegend), anti-CD43 mAbs eBioR2/60-FITC (eBioscience), S7-FITC (BD Biosciences) 1B11-PE (BD Biosciences), and anti-PSGL-1-PE (2PH1; BD Biosciences). Blood was collected from the tail vein of 8–10-wk-old male mice and stained with mAbs for 30 min at room temperature. The erythrocytes were then lysed with FACS lysis solution (BD Biosciences). The remaining leukocytes were washed and analyzed on a FACScalibur (BD Biosciences). To assess the selectin-Gr-1 binding, blood leukocytes were prepared by red cell lysis with ACK buffer (168 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). The cells were incubated with a COS-7 supernatant containing P-selectin-IgM or E-selectin-IgM. Nonspecific staining was determined by the addition of 5 mM EDTA. The cells were then washed and stained with FITC-labeled sheep anti-human IgM (The Binding Site) and anti-Gr-1allophycocyanin. The cells were analyzed on a FACScalibur.

Precipitation with E-selectin-IgG

Bone marrow (BM) cells were flushed from the femurs of WT and CD43−/− mice with cold PBS and then depleted of erythrocytes with ACK buffer. The cells were enriched for neutrophils by centrifugation on 62%/18% Percoll (GE Healthcare) (22). The cells at the interface were collected, washed three times with PBS, and surface-biotinylated in PBS containing 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce) (2.5 × 107 cells/ml) at room temperature for 30 min. The cells were then washed three times with PBS and lysed at a density of 3 × 107 cells/ml in cold lysis buffer (1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM CaCl2, and protease inhibitor cocktail (complete, EDTA-free; Roche)) for 30 min. Insoluble materials were pelleted at 15,000 × g for 20 min. The supernatant was aliquoted, and a fraction corresponding to 1 × 107 cells was incubated for 4 h with 50 μl of packed protein A-Sepharose (GE Healthcare). After removal of the Sepharose beads, the lysate was incubated in the presence of 1 mM CaCl2 with 20 μl of protein A-Sepharose preloaded for 4 h at 4°C with 50 μg of E-selectin-IgG or human IgG. After a 4-h incubation, the beads were washed with wash buffer (0.1% Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM CaCl2). Proteins bound to E-selectin-IgG were eluted with elution buffer (5 mM EDTA, 50 mM Tris (pH 7.4), and 0.1% Triton X-100). Eluted materials were separated by SDS-PAGE under nonreducing conditions and transferred to an Immobilon-P membrane (Millipore). Membranes were blotted with HRP-conjugated streptavidin (SA-HP; Zymed). The membranes were also blotted with a polyclonal anti-mouse PSGL-1 Ab (23), an anti-mouse CD43 mAb 1B11 (BD Biosciences), or a polyclonal anti-mouse E-1L-1 Ab (provided by Dr. B. Furie), followed by the appropriate HRP-conjugated secondary Abs (all from American Qualex).

Peripheral blood counts

Blood was collected from the tail vein of 8-wk-old male mice of each genotype. The blood was diluted in Türk’s stain solution (Nacalai Tesque) and the total leukocyte counts were determined with a hemocytometer. Flow cytometry of whole blood was performed to determine the fraction of each subset. Neutrophils were determined as Gr-1highF4/80−/− cells.

Thioglycollate- and oyster glycogen-induced peritonitis models

All mice were used at 7–8 wk of age. Mice were injected i.p. with 1 ml of 4% Brewer’s thioglycollate (Difco) or 1% oyster glycogen (Sigma-Aldrich). The mice were killed 8 h (thioglycollate) or 4 h (oyster glycogen) after injection, and 8 ml of PBS containing 1% BSA, 0.5 mM EDTA, and 10 U/ml heparin was injected into the peritoneal cavity. After the peritoneal wall was gently massaged, the injected wash was withdrawn. Total cell numbers in the peritoneal lavage were determined with a hemocytometer. The peritoneal cells were stained with anti-Gr-1-allophycocyanin and anti-F4/80-PE, and the percentage of neutrophils (Gr-1highF4/80low) was determined by flow cytometry. From the total cell count in the peritoneal lavage and the percentage of neutrophils, the absolute number was calculated. The percentage of neutrophils was also determined from cytospin preparations stained with May-Grünwald and Giemsa solutions (both from Wako Pure Chemicals). Both methods yielded essentially identical percentages.

Croton oil-induced acute cutaneous inflammation

Mice were painted with 20 μl of 0.8% croton oil (Sigma-Aldrich) in acetone on the left ear (10 μl per side). The right ear was painted with acetone alone. Ear swelling responses were measured using a dial thickness gauge (Mitutoyo). Skin-infiltrating cells were isolated via enzyme digestion. Briefly, ears taken 4 h after painting were separated into ventral and dorsal sheets. The sheets were cut into small pieces and incubated in RPMI 1640 containing 10% FCS, 400 U/ml collagenase (Roche), and 10 μg/ml DNase 1 (Roche) with continuous stirring at 37°C for 60 min. The resulting cell population was filtered through a 100-μm strainer (BD Falcon) and then centrifuged to discontinuous Percoll density gradient (40 and 75%). The cells were stained with anti-Gr-1-FITC and anti-F4/80-PE and then analyzed on a FACScalibur.

Intravital microscopy

Mice were anesthetized with an i.p. injection of a mixture of 60 mg/kg α-chloralose (Sigma-Aldrich) and 600 mg/kg urethane (Sigma-Aldrich). To maintain a neutral fluid balance, 1 ml of saline was administered i.p. The cremaster muscle was prepared for intravital microscopy as described by Ley et al. (24). The cremaster preparation was superfused with thermocontrolled (37°C) and aerated (5% CO2, 95% N2) bicarbonate-buffered saline throughout the experiment. The cremaster exteriorization surgery.
was typically accomplished in 4–7 min. Intravital microscopy was conducted using a microscope (BX50; Olympus) with a water immersion objective (×40, 0.8 numerical aperture). The microscope was equipped with a CCD camera (ICD-878; Ikegami) connected to a Panasonic video recorder (DMR-E250V). After the start of the cremaster surgery, data were acquired for 50 min. In some experiments, mice were injected intracranially with murine TNF-α (1 µg in 300 µl PBS; R&D Systems) 2.5 h before exteriorization of the cremaster muscle, and data were acquired between 150 and 200 min after the administration of TNF-α. Blood samples were taken from the tail vein at the end of the experiment to analyze systemic leukocyte counts. Centerline blood flow velocity was measured using a dual photodiode and a digital online cross-correlation program (Microvesel Velocity OD-RT; CircuSoft Instrumentation). In some experiments, the mice received 30 µg of the anti-P-selectin mAb RB40.34 (BD Biosciences) just before exteriorization of the cremaster muscle. Video recordings from intravital microscopy experiments were analyzed as described previously (24, 25). Leukocyte rolling velocities were calculated by measuring the time necessary to travel a distance of 100 µm. Adherent cells were defined as leukocytes that did not move for at least 30 s. The total number of adherent cells was measured for each venule and expressed per unit area of inside surface area of the venule. The surface area was calculated from diameter and length assuming cylindrical geometry of the venule.

Cell adhesion assays under flow conditions

Cell adhesion assays under flow conditions were performed according to the method of Nandi et al. (26) with slight modifications. P-selectin-IgG (0.2 µg/ml) or E-selectin-IgG (0.025 µg/ml) was immobilized on the inside walls of glass capillaries (inner diameter, 0.69 mm; Drummond Scientific) at 4°C overnight. The capillaries were then blocked with 1% BSA for 1 h at room temperature. The capillaries were mounted on the stage of an inverted microscope (Diaphot 300; Nikon) with a ×4 objective. At this magnification, all cells rolling at a fixed position of the capillaries could be monitored. Mouse BM cells were resuspended at 1 × 10^6 cells/ml in HBSS containing either CaCl_2 or EDTA and infused into the capillaries at a shear force of 1 dyn/cm². The rate of flow was controlled by a PHD 2000 syringe pump (Harvard Apparatus). Five minutes after the start of infusion, cell images were recorded with a cell-viewing system (SRM-100; Nikon) and video recorder (BR-S600; Victor), and the number of rolling cells within a fixed field was counted. The cells that rolled stably along the wall of the glass capillary tube for at least 3 s were considered to be rolling cells in this assay.

Statistical analysis

Data are presented as the means ± SEM. Statistical analyses were performed using the two-tailed unpaired Student’s t test.

Results

Neutrophils deficient in both PSGL-1 and CD43 show a slightly greater decrease in E-selectin binding than do cells deficient in PSGL-1 alone

Two major forms of mouse CD43, a 115-kDa and 130-kDa glycoform, are recognized by the anti-CD43 mAbs S7 and 1B11, respectively (14). Flow cytometric analyses showed that both the S7 and 1B11 epitopes of CD43 are expressed on mouse neutrophils (Fig. 1). To examine whether CD43 on mouse neutrophils binds E-selectin, we performed precipitation experiments using an E-selectin-IgG chimera from mouse BM neutrophils prepared by density gradient centrifugation. The purity of the isolated neutrophils was >95% as determined by staining with anti-Gr-1 (Fig. 2A). BM neutrophils were surface-biotinylated, and detergent extracts of these cells were incubated with E-selectin-IgG bound to protein A-Sepharose. The proteins that bound to E-selectin-IgG were eluted with EDTA and subjected to Western blotting with SA-HRP. Four major bands, which migrated around 90–100, 130–140, 160–180, and 270 kDa under nonreducing conditions, were precipitated with E-selectin-IgG in the presence of calcium (Fig. 2B). No bands were detected when detergent extracts were incubated with control human IgG in the presence of calcium or with E-selectin-IgG in the presence of EDTA, confirming the specificity of the binding. Previous studies have indicated PSGL-1 and ESL-1 as major E-selectin ligands on mouse neutrophils (5, 9, 27). In-deed, 270- and 140-kDa bands were detected by Western blotting the E-selectin-IgG precipitate using anti-PSGL-1 Abs (Fig. 2C), suggesting that 270- and 140-kDa components represent dimeric and monomeric forms of PSGL-1, respectively. Additionally, a 140-kDa band was detected in the E-selectin-IgG precipitate using anti-ESL-1 Abs (Fig. 2D). Furthermore, a 130-kDa band was detected by Western blotting the E-selectin-IgG precipitate from WT BM neutrophils, but not from CD43−/− cells, using the anti-CD43 mAb 1B11 (Fig. 2E). These biochemical analyses indicate that CD43 is one of the E-selectin-binding proteins expressed on mouse neutrophils.

To investigate the role of CD43 on neutrophils as an E-selectin ligand, we examined the selectin-binding activities of peripheral
blood neutrophils from WT, CD43\(^{-/-}\), PSGL-1\(^{-/-}\), and DKO mice. The expression level of PSGL-1 on CD43\(^{-/-}\) neutrophils and CD43 on PSGL-1\(^{-/-}\) neutrophils was comparable to that on WT neutrophils (Fig. 3A). The expression of other adhesion molecules, such as CD44, CD11a, and CD11b, on neutrophils was also comparable among the four genotypes (Fig. 3A). As reported previously (5), flow cytometric assays using selectin-IgM chimeric proteins showed that WT neutrophils strongly bound P- and E-selectin-IgM, whereas PSGL-1\(^{-/-}\) neutrophils did not bind P-selectin-IgM at all and bound E-selectin-IgM less well than did WT cells (Fig. 3B). CD43\(^{-/-}\) neutrophils bound P- and E-selectin-IgM about as well as WT cells (Fig. 3B), suggesting that CD43 does not play a significant role as an E-selectin ligand. Since PSGL-1 is one of the major E-selectin ligands, we next examined the effect of CD43 deficiency on selectin-binding activities in the absence of PSGL-1. DKO neutrophils showed reduced E-selectin-IgM binding compared with WT cells (Fig. 3B). Comparison of the mean fluorescence intensity of E-selectin binding of neutrophils from the four genotypes indicated that DKO neutrophils bound E-selectin-IgM slightly less well than PSGL-1\(^{-/-}\) cells (Fig. 3C). These results suggest that CD43 plays a role as an E-selectin ligand in the absence of PSGL-1.

**CD43 deficiency does not affect neutrophil migration in vivo**

To investigate whether CD43 is involved in neutrophil trafficking in vivo, we examined the peripheral blood neutrophil counts in WT, CD43\(^{-/-}\), PSGL-1\(^{-/-}\), and DKO mice, since neutrophilia is often an indication of a neutrophil trafficking defect (28). Consistent with published results (4, 5), neutrophil counts were modestly elevated in PSGL-1\(^{-/-}\) mice (2707 ± 366 cells/μl; \(n = 9\)) compared with WT mice (1315 ± 123 cells/μl; \(n = 11\)). On the other hand, neutrophil counts in CD43\(^{-/-}\) mice (1061 ± 57 cells/μl; \(n = 9\)) were comparable to those in WT mice. DKO mice also exhibited an increase in neutrophil counts (2983 ± 204 cells/μl; \(n = 9\)), which were slightly greater than those of the PSGL-1\(^{-/-}\) mice, although this further increase did not reach statistical significance. No alterations were observed in the counts of other subsets, including lymphocyte subsets (data not shown).

To study the role of CD43 in neutrophil migration into sites of inflammation, chemical peritonitis was induced in the four genotypes by thioglycollate given as an i.p. injection. Neutrophil migration into the peritoneal cavity in this model is dependent on P-, E-, and L-selectin (29, 30). In accordance with the published results (4), the number of neutrophils migrating into the peritoneal cavity was reduced by 40% in PSGL-1\(^{-/-}\) mice 8 h after thioglycollate injection (Fig. 4A). In CD43\(^{-/-}\) mice, the number of migrated neutrophils was comparable to that in WT mice (Fig. 4A). The DKO mice showed a 40% reduction in the number of neutrophils migrating into the peritoneal cavity, which was the same as in the PSGL-1\(^{-/-}\) mice (Fig. 4A). Neutrophil migration was also examined in an oyster glycogen-induced peritonitis model, which was used previously to show a defect in neutrophil migration in vivo.

**FIGURE 3.** P- and E-selectin-IgM binding of neutrophils from WT, CD43\(^{-/-}\), PSGL-1\(^{-/-}\), and DKO mice. A, Expression of PSGL-1, CD43, CD44, CD11a, and CD11b on neutrophils from WT, CD43\(^{-/-}\), PSGL-1\(^{-/-}\), and DKO mice. Peripheral blood leukocytes were stained with anti-Gr-1-allophycocyanin and either the indicated mAbs (open histograms) or isotype controls (shaded histograms) and analyzed by flow cytometry. Cells were gated on side scatter\(^{high}\) and Gr-1\(^{high}\). B, Peripheral blood leukocytes of each genotype were incubated with P- and E-selectin-IgM in the presence of calcium (open histograms) or EDTA (shaded histograms). The cells were then incubated with FITC-labeled sheep anti-human IgM and anti-Gr-1-allophycocyanin. Cells were gated on side scatter\(^{high}\) and Gr-1\(^{high}\) to show the E-selectin-IgM binding of neutrophils shown in B. Values are means ± SEM from six to eight mice. *, \(p < 0.05\); **, \(p < 0.001\).

**FIGURE 4.** Neutrophil migration in thioglycollate- and oyster glycogen-induced peritonitis. Absolute neutrophil counts in the peritoneal exudates were determined 8 h after thioglycollate (A) and 4 h after oyster glycogen (B) injection. Data represent the average values from 8 to 12 mice. Data are presented as means ± SEM. *, \(p < 0.05\); **, \(p < 0.001\).
CD43−/− mice (19). In this model, too, no reduction in the number of neutrophils in the peritoneal cavity was observed in CD43−/− mice compared with WT mice 4 h after oyster glycogen injection, and the amount of reduction from WT levels in the PSGL-1−/− and DKO mice was similar (Fig. 4B). These results suggest that the CD43 deficiency does not affect selectin-mediated neutrophil migration into the peritoneal cavity in vivo.

We also examined the role of CD43 in neutrophil migration in a croton oil-induced acute cutaneous inflammation model. In this model, infiltrating cells in the dermis consist mostly of neutrophils, and both P- and E-selectin mediate their recruitment into the inflamed skin (31). The left ear of WT, CD43−/−, PSGL-1−/−, and DKO mice was painted with croton oil. The time course of ear swelling was not significantly different among the four genotypes (data not shown). The number of neutrophils infiltrating the skin in PSGL-1−/− mice was reduced by 50% compared with WT mice, but in CD43−/− mice no reduction was seen (Fig. 5). In the DKO mice, the number of infiltrating neutrophils was also reduced by 50% compared with WT mice (Fig. 5). The fact that the number of DKO neutrophils recruited into the skin was comparable to that of PSGL-1−/− neutrophils also suggests that the CD43 deficiency does not affect neutrophil migration into the inflamed skin, even in the absence of PSGL-1.

Leukocyte rolling in venules with TNF-α-induced inflammation

The above results suggested that CD43 does not play a significant role as an E-selectin ligand in vivo or that the role of CD43 as an E-selectin ligand is masked by its role as an antiadhesive molecule. To more directly examine the role of CD43 as an E-selectin ligand in vivo, we studied the rolling behavior of leukocytes of all four genotypes in the postcapillary venules of the TNF-α-treated cremaster muscle by intravital microscopy. TNF-α induces the expression of E-selectin and enhances the expression of P-selectin, and most rolling leukocytes in this model are neutrophils (25). WT, CD43−/−, PSGL-1−/−, and DKO mice were treated with TNF-α, and rolling and adhesion on the cremaster muscle venules were evaluated 2.5 h later. We compared leukocyte rolling in 30–44 venules of each genotype. The microvessel and hemodynamic parameters were closely matched across the four genotypes (Table I). Consistent with previous results (4, 5, 7), the leukocyte rolling flux fraction was reduced to 3.7% in PSGL-1−/− mice compared with 11.5% in WT mice (Fig. 6A). In contrast, the leukocyte rolling flux fraction in CD43−/− mice was increased significantly to 14.0%, compared with WT mice (Fig. 6A). Although the rolling flux fraction in DKO mice (3.3%) was not significantly different from that in PSGL-1−/− mice (Fig. 6A), leukocyte rolling velocities in DKO mice (17.2 ± 0.7 μm/s) were significantly higher than in PSGL-1−/− mice (14.9 ± 0.5 μm/s) (Fig. 6, B, E, and F). In contrast, rolling velocities in CD43−/− mice (16.1 ± 0.5 μm/s) were similar to those in WT mice (16.1 ± 0.6 μm/s) (Fig. 6B–D). These results suggest that CD43 controls rolling velocities in the absence of PSGL-1.

Table 1. Hemodynamic and microvascular parameters of cremaster muscle venules in TNF-α-induced inflammation

<table>
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<tr>
<th>Mouse</th>
<th>Genotype</th>
<th>Mice (n)</th>
<th>Venules (n)</th>
<th>Diameter (μm)</th>
<th>Centerline Velocity (μm/s)</th>
<th>Wall Shear Rate (s−1)</th>
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<td>WT</td>
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<tr>
<td>CD43−/−</td>
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<td>2.0±0.1</td>
<td>604±17</td>
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<tr>
<td>DKO</td>
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<td>1.8±0.1</td>
<td>626±17</td>
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</tbody>
</table>

* Diameter, centerline velocity, and wall shear rate are presented as the means ± SEM of all the venules investigated.

FIGURE 5. Neutrophil migration in croton oil-induced cutaneous inflammation. Absolute neutrophil counts in the inflamed ear were determined 4 h after the application of croton oil. Data are means ± SEM from five to six mice. *, p < 0.05.

FIGURE 6. Leukocyte rolling and adhesion in cremaster muscle venules after TNF-α-induced inflammation. A–F. Leukocyte rolling flux fractions (A) and rolling velocities (B) were determined by intravital microscopy of the cremaster muscle venules 2.5 h after the injection of TNF-α into the scrotal sac. Values are means ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Velocity histograms of rolling leukocytes in WT (C), CD43−/− (D), PSGL-1−/− (E), and DKO (F) mice are also shown. G. Number of adherent leukocytes per square millimeter of venular surface area. Values are means ± SEM. *, p < 0.05; ***, p < 0.001.
Since the rolling velocity influences adhesion, we also examined leukocyte adhesion in this model. The number of firmly adherent leukocytes per square millimeter of venular surface area in the TNF-α-treated venules of PSGL-1−/− mice was reduced by 30% compared with WT mice (Fig. 6G). In contrast, CD43−/− mice showed a slight increase in leukocyte adhesion, although this increase did not reach statistical significance (Fig. 6G). In the DKO mice, the number of adherent leukocytes was also reduced to PSGL-1−/− levels (Fig. 6G). Thus, slightly higher leukocyte rolling velocities in DKO mice compared with PSGL-1−/− mice did not result in a further decrease in leukocyte adhesion.

To examine the role of CD43 in a process specifically mediated by E-selectin, the anti-P-selectin mAb RB40.34 was injected into the mice with TNF-α-induced inflammation. We compared leukocyte rolling in 19–34 venules of each genotype. The microvessel and hemodynamic parameters were closely matched across the four genotypes (Table II). The leukocyte rolling flux fraction was reduced to 1.7% in PSGL-1−/− mice and 1.8% in DKO mice compared with 5.8% in WT mice (Fig. 7A). In contrast, the leukocyte rolling flux fraction in CD43−/− mice was slightly increased to 6.7%, compared with WT mice, although the statistical significance between WT and CD43−/− mice was not observed in this E-selectin-mediated model (Fig. 7A). Leukocyte rolling velocities in PSGL-1−/− mice (7.5 ± 0.5 μm/s) were higher than in WT mice (6.7 ± 0.3 μm/s), and those in DKO mice (8.8 ± 0.6 μm/s) were even higher than in PSGL-1−/− mice (Fig. 7, A, C, E, and F). In contrast, rolling velocities in CD43−/− mice (6.0 ± 0.2 μm/s) were significantly lower than in WT mice (Fig. 7B–D). Since leukocyte rolling in TNF-α-stimulated venules blocked with an anti-P-selectin mAb is almost completely mediated by E-selectin, these results suggest that CD43 plays a role as an E-selectin ligand to control rolling velocities in the absence of PSGL-1. Additionally, the increased rolling flux fraction and decreased rolling velocities in CD43−/− mice compared with WT mice suggest that CD43 functions to limit leukocyte rolling, possibly acting as an antiadhesive molecule, in the presence of PSGL-1.

Leukocyte rolling in venules with trauma-induced inflammation

The results of leukocyte rolling in venules with TNF-α-induced inflammation indicated that CD43 functions as an E-selectin ligand to control the rolling velocities and inhibits leukocyte rolling. Thus, we next examined the role of CD43 in leukocyte rolling in venules with trauma-induced inflammation. In this model, leukocyte rolling is largely dependent on P-selectin, so that the contribution of the role of CD43 as an E-selectin ligand does not need to be taken into consideration. Leukocyte rolling was assessed at time points <50 min after the initiation of the surgery in WT and CD43−/− mice. The microvessel and hemodynamic parameters were similar in both WT and CD43−/− mice (Table III). The leukocyte rolling flux fraction was slightly increased to 18.4% in CD43−/− mice compared with 16.3% in WT mice, although this increase did not reach statistical significance (Fig. 8A). Importantly, rolling velocities in CD43−/− mice (40.4 ± 2.1 μm/s) were significantly reduced compared with WT mice (47.8 ± 1.7 μm/s) (Fig. 8B–D). Thus, both in P-selectin-mediated and E-selectin-mediated leukocyte rolling, CD43 functions to increase rolling velocities in the presence of PSGL-1.

CD43 deficiency enhances the ability of BM cells to roll on P- and E-selectin under flow conditions

To directly examine the role of CD43 in selectin-mediated leukocyte rolling under physiological shear flow in vitro, we tested BM cells for their ability to roll on immobilized P- and E-selectin. The percentage of Gr-1high cells in the BM was comparable among the four genotypes, and the P- and E-selectin-IgM binding activities of BM neutrophils from the four genotypes showed a similar pattern to those in Fig. 3B, as determined by flow cytometric analysis (data not shown). When WT cells were infused into capillary tubes coated with P- or E-selectin-IgG chimeras at 1 dyn/cm², they rolled (Fig. 9). The addition of EDTA completely abolished the

Table II. Hemodynamic and microvascular parameters of anti-P-selectin mAb-treated cremaster muscle venules in TNF-α-induced inflammation

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Mice (n)</th>
<th>Venules (n)</th>
<th>Diameter (μm)</th>
<th>Centerline Velocity (mm/s)</th>
<th>Wall Shear Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4</td>
<td>20</td>
<td>29.8 ± 1.1</td>
<td>1.6 ± 0.1</td>
<td>560 ± 23</td>
</tr>
<tr>
<td>PSGL-1−/−</td>
<td>3</td>
<td>23</td>
<td>29.5 ± 0.9</td>
<td>1.5 ± 0.1</td>
<td>547 ± 18</td>
</tr>
<tr>
<td>CD43−/−</td>
<td>4</td>
<td>34</td>
<td>28.8 ± 1.2</td>
<td>1.5 ± 0.1</td>
<td>556 ± 20</td>
</tr>
<tr>
<td>DKO</td>
<td>5</td>
<td>19</td>
<td>31.6 ± 1.4</td>
<td>1.7 ± 0.1</td>
<td>563 ± 23</td>
</tr>
</tbody>
</table>

* Diameter, centerline velocity, and wall shear rate are presented as the means ± SEM of all the venules investigated.

Table III. Hemodynamic and microvascular parameters of cremaster muscle venules in trauma-induced inflammation

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Mice (n)</th>
<th>Venules (n)</th>
<th>Diameter (μm)</th>
<th>Centerline Velocity (mm/s)</th>
<th>Wall Shear Rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>4</td>
<td>38</td>
<td>32.0 ± 1.0</td>
<td>2.4 ± 0.1</td>
<td>784 ± 36</td>
</tr>
<tr>
<td>CD43−/−</td>
<td>3</td>
<td>23</td>
<td>31.9 ± 1.6</td>
<td>2.4 ± 0.1</td>
<td>804 ± 35</td>
</tr>
</tbody>
</table>

* Diameter, centerline velocity, and wall shear rate are presented as the means ± SEM of all the venules investigated.
E-selectin ligand, PSGL-1). Consistent with the role of PSGL-1 as a major B
P-selectin ligand, which also serves as an E-selectin ligand. Additionally, apart from its role as an E-selectin ligand, we showed that CD43 functions as an antiadhesive molecule to limit leukocyte-endothelial interactions.

Our data showed that both PSGL-1 and CD43 are precipitated from BM neutrophils with an E-selectin-IgG chimera, confirming biochemically that PSGL-1 and CD43 are E-selectin ligands on mouse neutrophils. We showed previously that CD43 on mouse Th1 cells functions as an E-selectin ligand (15). We found that the role of CD43 in Th1 cell migration was most apparent in the absence of PSGL-1, and that a CD43 deficiency in the context of a normal PSGL-1 locus did not affect Th1 cell migration detectably (17). Given these observations, we examined the role of CD43 on neutrophils in the absence of PSGL-1. Intravital microscopy showed that a CD43 deficiency increased leukocyte rolling velocities in TNF-α-stimulated venules blocked with an anti-P-selectin mAb, where the rolling was mediated by E-selectin, when PSGL-1 was also absent, suggesting that CD43 functions as an E-selectin ligand to control leukocyte rolling velocities. However, the CD43 deficiency did not affect neutrophil recruitment in thioglycollate- or oyster glycogen-induced peritonitis, nor in croton oil-induced cutaneous inflammation, even in the absence of PSGL-1. These results suggest that the role of CD43 as an E-selectin ligand does not make a significant contribution to the overall efficiency of neutrophil recruitment into inflamed sites.

Woodman et al. (19) reported that neutrophil infiltration in an oyster glycogen-induced peritonitis model was reduced in CD43−/− mice. In contrast, our data showed no defect in neutrophil migration in the same model. The reason for this apparent inconsistency is not known, but possible explanations may include differences in the age, sex, and genetic background of the mice or in the activity of the oyster glycogen used: in their study, the number of neutrophils recruited in the peritoneal cavity was ~8 × 10⁶ in WT mice; in ours, it was ~2 × 10⁶. Note, however, that Carlow and Ziltener (20) obtained results consistent with ours. That is, they found CD43−/− neutrophils to be recruited in comparable numbers to WT neutrophils, using competitive migration assays, in both thioglycollate- and oyster glycogen-induced peritonitis models.

Intravital microscopy showed slightly enhanced leukocyte rolling in cremaster muscle venules of CD43−/− mice compared with WT mice in both the trauma model and TNF-α-stimulated model with P-selectin blockade, where the rolling is mostly P-selectin and E-selectin dependent, respectively. Additionally, leukocyte rolling velocities were decreased in CD43−/− mice in both models. These results are in agreement with those by Woodman et al. (19), which demonstrated significantly enhanced rolling in cremaster muscle venules after chemotactic stimuli in CD43−/− mice compared with WT mice. Additionally, they showed enhanced rolling of CD43−/− leukocytes on immobilized E-selectin under flow conditions in vitro. Similarly, we showed enhanced rolling of CD43−/− leukocytes on both P- and E-selectin in vitro, supporting
the view that CD43 interferes with cell-cell interactions. Such an antiadhesive role of CD43 has been documented for various cell types expressing CD43, and it is thought to be mediated through steric hindrance or charge repulsion (32). However, this antiadhesive role of CD43 was not evident in E-selectin-mediated rolling when PSGL-1 was absent. We hypothesize that CD43 on neutrophils generally attenuates leukocyte rolling on endothelial cells, functioning as an antiadhesive molecule, but when E-selectin is expressed, it also plays a role as an E-selectin ligand. These apparently contrasting functions of CD43 may complicate the interpretation of the effect of CD43 deficiency on neutrophil migration in several models of inflammation, and may explain why the contribution of CD43 to neutrophil migration was not detectable in vivo.

Neutrophils deficient in both PSGL-1 and CD43 still rolled on E-selectin in vivo, indicating the existence of other E-selectin ligands. We showed that ESL-1 is precipitated with E-selectin-IgG from BM neutrophils, suggesting that ESL-1 also serves as a physiological E-selectin ligand in vivo. Additionally, CD44 has been previously reported to mediate the E-selectin-dependent rolling of neutrophils (7). Although CD44 is highly expressed on mouse BM cells, we could not detect the band for CD44 in the E-selectin-IgG precipitate in our hands (data not shown). Hidalgo et al. (9) proposed a model where PSGL-1 mediates the initial leukocyte capture, ESL-1 converts initial tethers to slowly rolling, and CD44 controls rolling velocities and initiates signaling events. Our study showed that CD43 serves as one of the E-selectin ligands to regulate leukocyte rolling velocities, raising the possibility that CD43 functions in later stages of E-selectin-mediated rolling. Whether CD43 interaction with E-selectin initiates signaling events, such as activation of β2 integrins, remains to be determined.

In conclusion, our study shows that CD43 on neutrophils has both antiadhesive and proadhesive functions. CD43 generally serves as an antiadhesive molecule in neutrophil-endothelial interactions, but when E-selectin is expressed on endothelial cells, CD43 plays a proadhesive role as an E-selectin ligand. Our results may reconcile, at least partly, the inconsistencies of the phenotype caused by a CD43 deficiency in various models of inflammation, and they suggest that the balance between proadhesive and antiadhesive functions controls neutrophil migration in various inflammatory diseases.

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Disclosures

The authors have no financial conflicts of interest.

References