CD44 is a physiological E-selectin ligand on neutrophils

Yoshio Katayama, Andrés Hidalgo, Jungshan Chang, Anna Peired, and Paul S. Frenette

The selectin family of adhesion molecules and their glycoconjugated ligands are essential for blood polymorphonuclear neutrophil (PMN) extravasation into inflammatory and infectious sites. However, E-selectin ligands on PMNs are not well characterized. We show here that CD44 immunopurified from G-CSF–differentiated 32D cells or from peripheral blood PMNs binds specifically to E-selectin. In contrast, CD44 extracted from bone marrow stromal or brain endothelial cell lines does not interact with E-selectin, suggesting cell-specific posttranslational modifications of CD44. PMN–derived CD44 binding activity is mediated by sialylated, α(1,3) fucosylated, N-linked glycans. CD44 enables slow leukocyte rolling on E-selectin expressed on inflamed endothelium in vivo and cooperates with P-selectin glycoprotein ligand–1 to recruit neutrophils into thioglycollate-induced peritonitis and staphylococcal enterotoxin A–injected skin pouch. CD44 extracted from human PMNs also binds to E-selectin. Moreover, we demonstrate that CD44 is hypofucosylated in PMNs from a patient with leukocyte adhesion deficiency type II, suggesting that it contributes to the syndrome. These findings thus suggest broader roles for CD44 in the innate immune response and uncover a potential new target for diseases in which selectins play a prominent role.
of the cells with sialidase. Protein extracts from the same cells were incubated with immunomagnetic beads coated with anti-CD44. Immobilized CD44 bound to soluble E-selectin in a manner similar to intact cells; binding was eliminated with EDTA or by sialidase treatment before lysis (Fig. 1 C). No binding was observed when beads were coated with an isotype-matched antibody binding H9251 M H9252 integrin, or with control rat IgG (unpublished data). Immunoblot analyses revealed that CD44 was the sole protein purified from anti-CD44–coated beads (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20042014/DC1). These results thus suggest that CD44 derived from mature myeloid cells interacts specifically with E-selectin. Further, we evaluated E-selectin binding specificity using CD44 extracted from a bone marrow stromal cell line (MS-5) and a brain endothelial cell line (bEnd.3), which express high levels of CD44 but do not bind to E-selectin. CD44 immunopurified from endothelial and stromal cells did not interact with E-selectin (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20042014/DC1), underscoring differential posttranslangual modifications of CD44 between hematopoietic and nonhematopoietic cells.

To investigate the contribution of O-linked and N-linked carbohydrates in the formation of ESLs on myeloid cells, we treated G-CSF–differentiated 32D cells with O-sialylglycoprotein endopeptidase (OSGE) to remove O-glycans. OSGE affected neither ESLs on the cell surface, nor E-selectin binding to immunopurified CD44 (Fig. 1 D), whereas P-selectin ligands were completely cleaved by OSGE (unpublished data). To assess the contribution of N-linked carbohydrates, ESLs were removed with sialidase and differentiated 32D cells were either treated with OSGE, sialidase, or vehicle before immunopurification of CD44. In the last three bars, ESLs were removed by sialidase and cells were allowed to recover for 36 h in the presence or absence of tunicamycin (15 μg/ml) to inhibit N-glycosylation. Data are average geometric mean values from at least three independent experiments. **, P ≤ 0.003. (E) PMN-derived CD44 is an ESL. PMNs were isolated from (i, iv, and vii) BM and (ii, v, and viii) blood of control mice, or (iii, vi, and ix) blood from FucT IV/VII mice. Upper panels (i–iii) show CD44 expression on PMNs (gated on Gr-1hi). Middle panels (iv–vi) depict E-selectin binding on PMNs (Gr-1hi). Lower panels (vii–ix) show binding of E-selectin on immobilized CD44 extracted from purified PMNs (purity >95%). Gray-filled histograms represent isotype-matched control (CD44 staining) or EDTA treatment (E-selectin binding). (F and G) ESLs on wild-type and CD44–/– PMNs. Blood leukocytes were stained with E-selectin–IgM to determine ESL densities. (F) Histograms of E-selectin binding on Gr-1hi PMNs. (G) Geometric mean fluorescence of E-selectin binding on blood Gr-1hi PMNs. n = 12 mice per group. *, P = 0.02.
from mature myeloid cell binds to E-selectin through N-linked, but not O-linked, glycans.

We next assessed E-selectin binding on primary mouse PMNs. BM and peripheral blood (PB) PMNs (Gr-1^hi) express high levels of CD44 but expression of ESLs is heterogeneous on BM PMNs whereas PB PMNs uniformly express high levels of ESLs (Fig. 1 E). E-Selectin binding to CD44 displayed a pattern similar to that of intact cells in that binding was lower on CD44 extracted from BM PMNs than on PB PMNs (Fig. 1 E), suggesting that the density of CD44 molecules that are appropriately decorated with E-selectin binding carbohydrates is greater on circulating PMNs than on maturing bone marrow PMNs. No binding was observed when beads were coated with anti-αM integrin or rat IgG (Fig. S2), or when CD44 was extracted from blood PMNs deficient in α(1,3) fucosyltransferase IV and VII (FucT IV/VII^-/-; Fig. 1 E; reference 14). These results further validate the binding specificity of the assay and indicate that CD44 is a physiological target of leukocyte FucTs.

Fluid-phase binding of E-selectin to peripheral blood PMNs, an assay largely PSGL-1–dependent (10), was significa
cantly reduced in CD44^-/- PMNs compared with wild-type control PMNs (Fig. 1, F and G). Taken together, these data clearly indicate that CD44 from PB neutrophils binds to E-selectin.

E-selectin closely cooperates with P-selectin in promoting PMN–endothelial interactions and PMN extravasation; mice lacking both endothelial selectins have much more severe defects than either singly deficient animals (15–18). E-Selectin–deficient mice display increased leukocyte rolling velocities, suggesting that it is critical in forming stronger adhesion bonds between endothelial cells and PMNs (19). However, leukocyte rolling velocities were not altered in PSGL-1^-/- mice (10), suggesting that another leukocyte ESL mediates the slow rolling. We intercrossed CD44^-/-/PSGL-1^-/- mice to characterize the function of CD44 in E-selectin–mediated leukocyte-endothelial interactions. Double knockout (DKO) animals were viable, fertile, and displayed a significant increase in circulating leukocytes, including PMNs, monocytes and eosinophils (Table S1, available at http://www.jem.org/cgi/content/full/jem.20042014/DC1). We subjected age-matched male wild-type, CD44^-/-, PSGL-1^-/-, and DKO mice to intravital microscopic examination of TNF-α–treated cremaster muscle to explore the ability of CD44 to interact with E-selectin in vivo. Consistent with previous studies (9, 10) and with the notion that P-selectin mediates most rolling activity in inflamed venules, the rolling fraction was significantly reduced in PSGL-1^-/- mice (Fig. 2 A and Table S2). Residual rolling activity in PSGL-1^-/- mice was further reduced by

![Figure 2](image-url)

**Figure 2.** Intravital microscopy of TNF-α–stimulated cremaster muscle venules. Leukocyte behavior in cremasteric venules was recorded between 150 and 210 min after TNF-α administration for off-line analyses. (A) Rolling flux fraction in wild-type (WT, n = 63 venules), WT treated with hyaluronidase (WT+Hase, n = 51), CD44^-/- (n = 79), PSGL-1^-/- (n = 50), and DKO (n = 59) mice. (B–D) The velocity of 2,610 rolling leukocytes from the five groups of mice was measured over 2 s (also see Fig. S4). (B) Mean rolling velocities. (C) Cumulative histograms of rolling velocities. (D) Cumulative histograms of transit times calculated per 100 μm of venule segment. Five-group comparisons were analyzed by one-way ANOVA with Bonferroni/Dunn post hoc test. ***, P < 0.001 (1% risk level) compared with WT group or between indicated two groups.
kocytes were much shorter in CD44−/− mice than wild-type mice (Fig. 2, B and C and Fig. S4). Thus, these data indicate that PSGL-1 can also contribute to this activity when CD44 is absent. To investigate whether CD44 binding to E-selectin can mediate the extravasation of PMNs into inflammatory sites, we injected mice with thioglycollate, a chemical that induces a severe peritoneal inflammation. In this model, PMN recruitment 8 h after thioglycollate injection in PSGL-1−/− mice was significantly reduced (by 44%, P = 0.005) in DKO mice (Fig. 3 A). Because thioglycollate may not reproduce physiological inflammation, we also evaluated PMN extravasation elicited by the staphylococcal entero-toxin A (SEA) in a preformed air pouch model (20). In preliminary experiments using P- and E-selectin−deficient mice, we ascertained that SEA-mediated PMN recruitment was selectin dependent (unpublished data). We then instilled SEA in dorsal skin pouches of mice from the four genotypes to assess the contribution of CD44 and PSGL-1 in this model. We found a severe reduction in the extravasation of PMNs 6 h after SEA injection in DKO mice compared with wild-type controls (77% reduction, P = 0.006), whereas the numbers of extravasated PMNs were not significantly altered in either singly−deficient mice (Fig. 3 B). To exclude further the possibility that the reduced recruitment of PMNs observed in DKO mice was due to CD44 binding to HA, we repeated the thioglycollate-induced extravasation experiments in PSGL-1−/− mice treated with hyaluronidase (20 U i.v.) or vehicle. Hyaluronidase treatment did not significantly alter PMN recruitment (Fig. 3 C). Although we cannot completely rule out the possibility that extravascular HA may have remained available for PMN migration, the results from these two models strongly suggest that CD44 is an ESL that cooperates with PSGL-1 in PMN extravasation into inflamed sites.

Because CD44 is expressed on both leukocyte and endothelial cells, we wished to clarify further whether CD44 deficiency on PMNs was sufficient to account for impaired PMN extravasation. We generated chimeric mice by transplantation of a mixture of PSGL-1−/−CD44−/− and PSGL-1−/−CD44+/+ BM-nucleated cells into lethally irradiated mouse. P = 0.24.

Figure 3. Neutrophil extravasation into inflammatory sites. (A) Thioglycollate−induced peritonitis. Extravasated PMNs were determined 8 h after the i.p. injection of thioglycollate (n = 7). (B) SEA-induced inflammation model. Extravasated PMNs were quantified 6 h after instillation of SEA into preformed skin pouches (n = 6). Data are represented by box−and−whisker plots wherein each box represents an interquartile range (central 50%), the median is shown by the horizontal lines, and vertical lines show the full range of data points. Four−group comparisons were analyzed by one−way ANOVA with Bonferroni/Dunn post hoc test. *, P < 0.0083 (5% risk level) compared with WT group. (C) Hyaluronidase treatment does not affect PMN recruitment. PSGL-1−/− mice were injected i.v. with 20 U of hyaluronidase or PBS before i.p. injection of thioglycollate. Each circle represents the value of an individual PSGL-1−/− mouse. P = 0.24.
wild-type recipients. 6 wk after transplantation, >96% Gr-1<sup>hi</sup> leukocytes did not express PSGL-1. Peritoneal inflammation was then induced by thioglycollate for 8 h, and the ratios of PSGL-1−/−CD44<sup>+</sup> PMNs over DKO PMNs in blood and peritoneal exudates were assessed by FACS (Fig. 4 A). In this competitive setting, we found that PSGL-1−/−CD44<sup>+</sup> PMNs were preferentially recruited in the peritoneum (an approximate twofold increase) over those that did not express PSGL-1 and CD44 (Fig. 4 B), suggesting that neutrophil rather than endothelial CD44 plays a critical role for migration into inflammatory sites.

Our data suggest that ESLs may have specialized functions. It is interesting to speculate that this may be controlled by the spatial distribution on the cell surface. For example, PSGL-1 is primarily a tethering molecule localized on the tip of microvilli (21), whereas we show here that CD44, a receptor located on the cell body (22, 23), primarily controls rolling velocity. It is notable that β2 integrins, which can also mediate slow rolling (24), are located on the cell body. Thus, our data are consistent with the notion that adhesion receptors located on microvilli may determine tethering efficiency but not rolling velocity (23, 25). As suggested by the partial defect in leukocyte rolling and recruitment of DKO mice, other functional ESLs exist on PMNs. The various ESLs may exert distinct functions in E-selectin–dependent adhesive and migratory activities. Further studies are needed to ascertain in vivo functions of major candidate ESL glycoproteins, including ESL-1 (expressed on mouse microvilli of myeloid cells; 7) and L-selectin (candidate ESL on human PMNs; 22, 26).

To investigate whether human neutrophil CD44 was a functional ESL, we purified peripheral blood PMNs from healthy donors and extracted PMN-derived human CD44 from cell lysates for the E-selectin binding assay. To control for binding specificity, we used sialidase-treated PMNs from the same donors and PMNs from a patient with leukocyte adhesion deficiency type II (LADII), characterized by a complete deficit in functional selectin ligands due to G588 nucleotide deletion in the GDP–fucose transporter gene (27). As shown in Fig. 5 A, healthy PMN-derived CD44 bound to soluble E-selectin (empty red histogram), and binding was abrogated by sialidase (green-filled histogram). (B) In contrast, there is no difference in E-selectin binding to immunopurified CD44 between sialidase-treated and sham-treated LADII PMNs. (C) Incubation of LADII PMNs with recombinant FucT VI (20 mU/ml, 40 min at 37°C) and GDP-fucose (1 mM) restores the ability of human CD44 to bind to E-selectin.
In summary, these data clearly indicate that CD44 is a physiological ESL on mature human and mouse myeloid cells. CD44 is widely expressed in multiple cell types but only hematopoietic cells bind to E-selectin, indicating that the affinity for E-selectin is regulated by cell-specific posttranslational modifications. Selective binding to E-selectin (myeloid cells) or HA (activated T cells) endows CD44 with pivotal functions at the nexus of innate and adaptive immunity.

MATERIALS AND METHODS

Cell lines and E-selectin/IgM chimeric protein. The 32D mouse myeloid progenitor cell line (American Type Culture Collection) was cultured in RPMI containing 10% FBS (Hyclone) and 10% Wehi-3B (a murine IL-3–secreting cell line) conditioned medium. Differentiation of 32D cells was induced with 10 ng/ml recombinant human G-CSF (R&D Systems) for 6 d. Mouse BM-derived stromal cell line MS-5 was cultured in αMEM containing 10% FBS, and mouse endothelial cell line bEnd.3 (American Type Culture Collection) was cultured in DMEM containing 10% FBS.

Murine E-selectin/IgM chimera was produced by the transfection of 293T cells with E-selectin/IgM DNA vector using Lipofectamine 2000 (Invitrogen). Saturating concentrations of culture supernatants were used for the fluid-phase selectin binding assay.

Glycan analyses. 32D cells or purified PMNs were treated with 150 mM salidase from Arthrobacter ureafaciens (Roche), or with 20 μg OSC6 (partially purified by membrane dialfiltration and isoelectric point precipitation from Pasteurella haemolytica; Cedarlane) for 1 h at 37°C in HBSS media containing 20 mM Hepes and 0.1% human serum albumin (Baxter). Both treatments completely eliminated P-selectin ligands. Inhibition of N-glycosylation was achieved by culture of salidase-treated 32D cells in the presence or absence of 15 μg/ml tunicamycin (Calbiochem) for 36 h.

Preparation of murine and human neutrophils. Age-matched (6–12 wk) C57BL/6 (NCI, Frederick Cancer Research and Development Center) and FucT IV/VII−/− mice (14; provided by John B. Lowe, University of Michigan Medical School, Ann Arbor, MI) were used as BM and blood donors. In some experiments, lymphopenic NOD/SCID mice were used as blood donor to obtain greater PMN purity. Expression of ESLs and binding of E-selectin to CD44 were similar between C57BL/6- and NOD/SCID-derived PMNs. BM cells were harvested by flushing femora in RPMI using 21-gauge needle and blood was harvested by retroorbital sampling using heparinized capillary tubes. For blood PMNs purification, RBCs were depleted by blood sedimentation in 0.1% methylcellulose for 30 min at room temperature. Cells remaining in the supernatant were washed and resuspended in PBS containing 0.5% BSA and 2 mM EDTA, and PMNs separated by overlaying on a Percoll gradient (65% in HBSS). Contaminating RBCs were lysed with 0.8% NH₄Cl.

Human neutrophils were purified from human venous blood as described previously (27). Human blood samples were obtained in accordance with protocols approved by the Internal Review Board of Mount Sinai.

Flow cytometry and E-selectin binding assay. Cells were stained by incubation with 10 μg/ml of a biotin-labeled anti-CD44 antibody (clone IM7; BD Biosciences) or control antibody followed by incubation with Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories), and washed in PBS before analysis by flow cytometry. For the fluid-phase selectin binding assay, cells or CD44-coated beads were incubated with E-selectin/IgM chimera followed by incubation with Cy5-conjugated anti-human IgM antibody (1:50 dilution; Jackson ImmunoResearch Laboratories) as described previously (28). Control samples were stained in the presence of 5 mM EDTA.

A similar procedure was followed for staining with the P-selectin/IgM chimera. For staining of murine PMNs, 50 μl of blood or 106 BM mononuclear cells were incubated with FITC-conjugated anti-Gr-1 antibody (BD Biosciences) and then labeled with the E-selectin chimera as described above. PMNs were gated on the basis of high Gr-1 expression. All incubations were performed at 4°C for 15 min. Samples were analyzed using a FACSCalibur flow cytometer and the CellQuest software (Becton Dickinson).

Preparation of immunomagnetic beads for E-selectin binding analyses. Lysates were prepared by incubation of cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, and 1 mM CaCl₂ plus 0.1 M PMSF and protease inhibitor cocktail; Sigma-Aldrich) for 30 min on ice and cell debris were removed by centrifugation at 14,000 rpm for 10 min at 4°C. Anti-rat IgG-coated beads (106 M–Dynabeads; Dynal) were incubated with 2.5 μg anti-CD44 (IM7), anti-αM integrin (M17/10 from American Type Culture Collection, purified from hybridoma supernatants) or rat IgG (Sigma-Aldrich) for 4 h at 4°C under rotation. Beads were then washed twice in cold lysis buffer and incubated overnight with the appropriate cell lysates (2 × 106 cells/106 beads) at 4°C under rotation. Beads were washed once in cold lysis buffer and once in cold RPMI containing 5% FBS and 0.05% NaN₃ before fluid-phase E-selectin binding assay.

In vivo models of leukocyte recruitment. Leukocyte-endothelial interactions were assessed using intravital microscopy of the cremaster muscle as described previously (29). More detailed information is available at http://www.jem.org/cgi/content/full/jem.20042014/DC1. PMN recruitment in the thioglycollate-induced peritonitis model was assessed as described (15, 17). In some experiments, mice were injected i.v. with 20 U of hyaluronidase (Sigma-Aldrich) or control PBS before injection of thioglycollate. SEA-induced inflammation in air pouch was performed as described elsewhere (20). In brief, air pouches were raised on the dorsum by s.c. injection of 2.5 ml sterile air on days 0 and 3 and the pouch was allowed to form until day 6. On day 6, 10 μg of SEA (endotoxin <1 EU/mg; Toxin Technology) in 1 ml PBS was injected in the pouch. Mice were killed 6 h after SEA administration, and the pouches were washed with 5 ml of PBS containing 1% BSA, 0.5 mM EDTA, and 10 U/ml heparin. Total cell numbers in the peritoneal and air pouch exudates were determined by hemocytometer. Differential leukocyte counts were determined from Wright-stained cytospin preparations. All animal experimental procedures were approved by the Animal Care and Use Committee of Mount Sinai.

Generation of PSGL-1/CD44 double-deficient mice. PSGL-1−/− mice were generated by gene targeting (9) and provided by Bruce Furie (Harvard Medical School, Boston, MA). CD44−/− mice were also generated by gene targeting (30) and purchased from The Jackson Laboratory. PSGL-1−/− mice were intercrossed with CD44−/− mice to generate double-heterozygous animals. These doubly heterozygous mice were then bred to yield wild-type control, CD44−/−, PSGL-1−/−, and double-deficient mice. The genotypes were determined by PCR using primers and conditions detailed in supplemental materials. Separate colonies of each genotype were then expanded to yield age- and sex-matched experimental mice with similar mixed background.

Generation of chimeric mice and competitive recruitment assay. Wild-type–recipient mice were lethally irradiated (12 Gy, split dose) and transplanted with 1.5 × 10⁶ PSGL-1−/− BM-nucleated cells and 1.5 × 10⁶ PSGL-1/CD44 double deficient (DKO) BM-nucleated cells. 6 wk after transplantation, engraftment was confirmed by FACS. Leukocytes from thioglycollate-induced exudates were stained with FITC-conjugated anti–Gr-1 antibody (BD Biosciences), PE-conjugated anti-CD44 antibody (KM201; Southern Biotechnologies Associates), and biotin–anti–PSGL-1 antibody (clone 4RA10; gift of Dr. Dietmar Vestweber, The Institute of Cell Biology, University of Münster, Münster, Germany), followed by Cy5-conjugated streptavidin (Jackson ImmunoResearch Laboratories).

Online supplemental material. Online supplemental materials contain detailed information about genotyping of mutant mice, intravital microscopy studies, and statistical analyses. Figs. S1 and S2 further evaluate the
We thank B. Furie for providing PSGL-1 for hemodynamic parameters of intravital microscopy experiments. Online specificity of E-selectin binding to CD44-coated beads. Fig. S3 shows the lymphocyte rolling velocity distributions in TNF-a-treated cremaster muscle venules. Table S1 shows peripheral blood counts and Table S2 details venular hemodynamic parameters of intravital microscopy experiments. Online supplementation material is available at http://www.jem.org/cgi/content/full/jem.20.042014/DC1.

We thank B. Furie for providing PSGL-1 mice, J. Lowe for FucT IV/VII mice, Dietmar Vestweber for the 4RA10 mAb, and C. Cunningham-Rundles (Mount Sinai School of Medicine) for procuring blood samples of the LAD1 patient. We also thank Elaine Chiang for reviewing the manuscript.

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Immunoprecipitation and Western blot analyses. 32D cells differentiated with G-CSF were surface biotinylated by incubation with sulfo-N-hydroxysulfo succinimide–LC biotin (0.5 mg/ml, Pierce Chemical Co.) in PBS buffer for 30 min at 4°C. Lysates of biotinylated and nonbiotinylated cells were prepared and immunoprecipitated with the anti-CD44 antibody (clone IM7) using the anti–rat IgG-coated beads as indicated in the Materials and methods section. After extensive washing of the beads, bound proteins were eluted in SDS sample buffer and separated by SDS-PAGE (7% gel). Proteins were transferred into PVDF membranes (Millipore) and blotted with either peroxidase-conjugated neutravidin (samples from biotinylated cells, Pierce Chemical Co.) or with biotinylated-anti-CD44 antibody (clone IM7, BD Biosciences) followed by peroxidase-conjugated neutravidin (samples from nonbiotinylated cells). Blots were developed with the SuperSignal substrate (Pierce Chemical Co.).

Genotyping of mutant mice. For PSGL-1: PSGL-1 F: 5′/H11032GCT TCC TTG TGC TGC TGA C 3′; PSGL-1 R: 5′/H11032CCT CTG TGG ATG CTG GTT G 3′/H11032; Neo F: 5′/H11032GTC CGG TGC CCT GAA TGA ACT GC 3′/H11032 wherein PSGL-1 wild-type allele was detected by the pair of PSGL-1 F and R, and the mutant allele was detected by the pair of Neo F and PSGL-1 R. The PCR cycle conditions were: initial denaturation step of 3 min at 94°C, followed by 35 cycles of amplification (93°C for 30 s, 59°C for 30 s, 72°C for 1 min) and a final elongation step at 72°C for 7 min. For the CD44 locus, the following primers were used: CD44 F: 5′/H11032GCA GCC CCC AGC CAG TGA 3′/H11032.

Figure S1. Immunomagnetic beads coated with anti-CD44 exclusively immunoprecipitate CD44 isoforms. Cell lysates from biotinylated or nonbiotinylated G-CSF–treated 32D cells were made. CD44 was affinity-purified using IM7-coated beads. The isolated proteins were run in SDS-PAGE and detected with peroxidase-conjugated neutravidin (N-Av; left lane) or with biotinylated IM7 followed by peroxidase-conjugated neutravidin (IM7-biot, right lane). Molecular weight markers are indicated in the left. Two protein species (~200 and ~110 kD) were detected by affinity isolation. Both were recognized by IM7 and correspond to two CD44 isoforms. The high molecular weight isoform was previously reported to contain chondroitin sulfate moieties (5, 6).

Figure S2. Specificity of E-selectin binding to CD44–coated beads. (A) Mouse bone marrow endothelial cells (bEnd.3 cell line) were detached from culture dishes using PBS containing 5 mM EDTA. After extensive washes to remove EDTA, the expression of CD44 and E-selectin ligands was analyzed by flow cytometry as described in Supplemental materials and methods. Despite high levels of CD44 expression (left), E-selectin ligands were neither detectable on the cell surface (middle), nor on CD44 immunopurified from these cells (right). Shaded histograms depict control binding in the presence of 5 mM EDTA or control IgG staining (for CD44 expression). (B) Cell lysates from purified mouse peripheral blood neutrophils were incubated with immunomagnetic beads coated with rat IgG, anti-Mb2 (clone M17/9, IgG2b), or anti-CD44 (clone IM7, IgG2b), and E-selectin binding was analyzed by FACS. E-Selectin ligand activity was detected when CD44 was immunopurified onto beads, but not when anti-IgG was used. Immunopurified Mb2 (mouse MAb) or anti-CD44 (clone IM7) IgG2b, and E-selectin binding was analyzed by FACS. E-Selectin ligand activity was detected when CD44 was immunopurified onto beads, but not when anti-IgG was used.

Figure S3. Specificity of E-selectin binding to CD44–coated beads. (A) Mouse bone marrow endothelial cells (bEnd.3 cell line) were detached from culture dishes using PBS containing 5 mM EDTA. After extensive washes to remove EDTA, the expression of CD44 and E-selectin ligands was analyzed by flow cytometry as described in Supplemental materials and methods. Despite high levels of CD44 expression (left), E-selectin ligands were neither detectable on the cell surface (middle), nor on CD44 immunopurified from these cells (right). Shaded histograms depict control binding in the presence of 5 mM EDTA or control IgG staining (for CD44 expression). (B) Cell lysates from purified mouse peripheral blood neutrophils were incubated with immunomagnetic beads coated with rat IgG, anti-Mb2 (clone M17/9, IgG2b), or anti-CD44 (clone IM7, IgG2b), and E-selectin binding was analyzed by FACS. E-Selectin ligand activity was detected when CD44 was immunopurified onto beads, but not when anti-IgG was used. Immunopurified Mb2 (mouse MAb) or anti-CD44 (clone IM7) IgG2b, and E-selectin binding was analyzed by FACS. E-Selectin ligand activity was detected when CD44 was immunopurified onto beads, but not when anti-IgG was used.
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CAG3 CD44 R, 5G AAG GGC TGC GGG CAT CCA AGA GTA 3 wherein CD44 wild-type allele was detected by the pair of CD44 F and R, and knockout allele was detected by the pair of Neo F and CD44 R. The PCR cycle conditions for CD44 were: an initial denaturation step of 3 min at 94ºC, followed by 35 cycles of amplification (93ºC for 30 s, 61ºC for 30 s, 72ºC for 2 min) and a final elongation step at 72ºC for 7 min. Null mutations were confirmed by FACS analyses.

Intravital microscopy

2 h before the cremaster muscle surgery, mice (10–14-wk-old males) were injected intrascrotically with 0.5 g mouse TNF-α (R&D Systems) in 250 l PBS. To exclude a role for endothelial hyaluronic acid, a group of wild-type mice was injected through tail vein with 10 U (in 200 l PBS) of hyaluronidase (Sigma-Aldrich), as described elsewhere (1), immediately before TNF-α injection. Mice were anesthetized with an intraperitoneal injection of 2% -chloralose and 10% urethane in PBS (6 ml/kg). A polyethylene tube (PE160; Becton Dickinson) was inserted into trachea to facilitate spontaneous respiration. The cremaster muscle was prepared for intravital microscopy as described previously (2). All microscopic observations were visualized using a custom-designed intravital microscope (MM-40; Nikon), using 60 Nikon water immersion objective (NA 1.0). 15 min after surgery, venules between 25 and 35 mm were visualized using a CCD camera (Hamamatsu) and recorded for ~120s on a Sony SVHS video recorder (SVO-9500) for off-line analyses. Recordings were made between 150 and 210 min after administration of TNF-α. Wall shear rate (y) were calculated based on Poiseuille’s Law for a Newtonian fluid = (πy8 (Vmean /Dv)), where Dv is the diameter of the venule and Vmean is estimated from the center-line red blood cell velocities (Vrbc), using the empirical correlation Vmean = Vrbc/1.6 (3). Vrbc was measured for each venule in real time using an optical Doppler velocimeter (Texas A&M, College Station, TX). Each rolling leukocyte passing a line perpendicular to the vessel axis was counted, and leukocyte rolling flux was expressed as leukocytes per minute. Rolling flux fraction was calculated by dividing leukocyte rolling flux by the total leukocyte flux, which was estimated as (WBC) x Vmean x π x (d/2)², where WBC is the white blood cell count and d is the venular diameter (4). An automated cell counter was used to determine WBC counts from blood samples obtained from retroorbital bleeding at the end of the experiment. Leukocyte rolling velocities were measured for 10 random leukocytes per venule by measuring the distance traveled during 2 s.

Figure S3. Treatment with tunicamycin does not affect O-glycosylation. G-CSF–differentiated 32D cells were treated with sialidase to remove selectin ligands, and further cultured in the presence or absence of tunicamycin (15 μg/ml) for 48 h. (A) Binding of P- and E-selectin chimeras before treatment with sialidase. (B) Binding of P- and E-selectin chimeras after culture in the absence (Nil) or presence of tunicamycin. (C) Cell lysates from the same cultures were incubated with immunomagnetic beads coated with anti-CD44 (clone IM7), and E-selectin binding was analyzed by flow cytometry as described in Supplemental materials and methods. Indicated in each panel is the mean fluorescence intensity (MFI) of P- or E-selectin binding. Tunicamycin treatment greatly reduced (by 85%) the density of surface E-selectin ligands and the ability of affinity-isolated CD44 to bind to E-selectin (by 88%). However, tunicamycin did not affect the density of P-selectin ligands which require O-glycosylation.

Figure S4. Leukocyte rolling velocity distributions in TNF-α–treated cremaster muscle venules. The rolling velocity of 2,610 leukocytes was determined over a constant 2-s time window as described in Supplemental materials and methods. All leukocytes rolling at velocities over 40 μm/s are represented in the last bar. These fast rollers are mostly found in CD44+ and DKO mice. n = number of leukocytes evaluated for each genotype.

Figure 3. Treatment with tunicamycin does not affect O-glycosylation. G-CSF–differentiated 32D cells were treated with sialidase to remove selectin ligands, and further cultured in the presence or absence of tunicamycin (15 μg/ml) for 48 h. (A) Binding of P- and E-selectin chimeras before treatment with sialidase. (B) Binding of P- and E-selectin chimeras after culture in the absence (Nil) or presence of tunicamycin. (C) Cell lysates from the same cultures were incubated with immunomagnetic beads coated with anti-CD44 (clone IM7), and E-selectin binding was analyzed by flow cytometry as described in Supplemental materials and methods. Indicated in each panel is the mean fluorescence intensity (MFI) of P- or E-selectin binding. Tunicamycin treatment greatly reduced (by 85%) the density of surface E-selectin ligands and the ability of affinity-isolated CD44 to bind to E-selectin (by 88%). However, tunicamycin did not affect the density of P-selectin ligands which require O-glycosylation.
There was no significant difference among the five groups of mice. WT, wild-type mice; WT + Hase, wild-type mice treated with hyaluronidase; DKO, CD44/PSGL-1 double-knockout mice.

Table SII. Venular hemodynamic parameters in TNFα-induced inflammation.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of mice</th>
<th>Number of vessels</th>
<th>Vessel diameter</th>
<th>Centerline velocity</th>
<th>Wall shear rate</th>
<th>Genotype</th>
<th>Number of mice</th>
<th>Number of vessels</th>
<th>Vessel diameter</th>
<th>Centerline velocity</th>
<th>Wall shear rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6</td>
<td>63</td>
<td>30.0 ± 0.4</td>
<td>1,868 ± 102</td>
<td>662 ± 36</td>
<td>WT + Hase</td>
<td>5</td>
<td>51</td>
<td>30.0 ± 0.4</td>
<td>1,873 ± 127</td>
<td>662 ± 41</td>
</tr>
<tr>
<td>CD44−/−</td>
<td>7</td>
<td>79</td>
<td>30.3 ± 0.4</td>
<td>2,333 ± 117</td>
<td>785 ± 43</td>
<td>PSGL-1−/−</td>
<td>5</td>
<td>50</td>
<td>29.4 ± 0.4</td>
<td>1,780 ± 911</td>
<td>642 ± 33</td>
</tr>
<tr>
<td>DKO</td>
<td>5</td>
<td>59</td>
<td>29.6 ± 0.4</td>
<td>2,256 ± 142</td>
<td>819 ± 56</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

There was no significant difference among the five groups of mice. WT, wild-type mice; WT + Hase, wild-type mice treated with hyaluronidase; DKO, CD44/PSGL-1 double-knockout mice.

Statistical analyses

Data are presented as mean ± SEM except for Fig. S3 where data are represented by box-and-whisker plots. Data were analyzed by one-way ANOVA with Bonferroni/Dunn post hoc test for multiple group comparison using StatView software. For two-group comparisons (Fig. S1 G), P < 0.05 (5% risk level) or P < 0.01 (1% risk level) were considered statistically significant. For three-group comparisons (Fig. 1 D), P < 0.0167 (5% risk level) or P < 0.0033 (1% risk level) were statistically significant. For four-group comparisons (Fig. S3 and Table SII), P < 0.0083 (5% risk level) or P < 0.0017 (1% risk level) were deemed significant. For five-group comparisons (Fig. S2 and Table SII), the threshold for statistical significance was P < 0.005 (5% risk level), P < 0.001 (1% risk level). Paired t-test was used for Fig. S4 B where significance was set at P < 0.01 with 1% risk level.

REFERENCES


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