Reagents

Murine P-, L-, and E-selectin–IgM Fc chimeras and control CD45-IgM Fc chimera were described previously.[1] Human E-selectin–IgG Fc chimera was expressed and purified as described for human L-selectin–IgG Fc.[2] Human P-selectin was isolated from platelets as described.[3] Human recombinant soluble ICAM-1 and murine recombinant ICAM-1-Fc, CXCL1, and TNF-α were purchased from R&D Systems. Rat anti-murine E-selectin mAb 9A9[4] was a gift from Barry Wolitzky (MitoKor, San Diego, CA). Rat anti-murine P-selectin mAb RB40.34[5] and rat anti-murine PSGL-1 mAb 4RA10[6] were gifts from Dietmar Vestweber (Max Planck Institute of Biomolecular Medicine, Muenster, Germany). Murine anti-human αLβ2 mAb TS1/22 was from Thermo Scientific. Murine anti-human αMβ2 mAb was from Dako. Murine anti-human ICAM-1 mAb LB-2 was from BD Biosciences. The following mAbs to murine proteins were purchased from BD Biosciences: rat anti-$\alpha_L\beta_2$ (M17/4), rat anti-$\alpha_M\beta_2$ (M1/70), rat anti-β2 integrin (GAME46), hamster anti–ICAM-1 (3E2), PE-labeled rat anti–PSGL-1 mAb (2PH1), FITC-labeled rat anti–L-selectin (Mel-14), FITC-labeled rat anti-CD44 (IM7), and Cy5-labeled Y416 in the catalytic domain of all activated SFKs), Syk, phospho-Syk, p38, phospho-p38, and Btk, and goat horseradish peroxidase-conjugated anti-rabbit IgG were obtained from Cell Signaling Technology. Rabbit antibodies to murine Fgr, Hck, and Lyn and goat antibody to murine Btk were from Santa Cruz. Goat anti-human IgG Fc antibody was from Chemicon. Goat anti-human IgM Fc antibody was from Caltag. Murine anti-phosphotyrosine antibody 4G10 was from Millipore. Goat horseradish peroxidase-conjugated anti-mouse IgG and streptavidin were from Thermo Scientific. Signaling inhibitors were purchased from Calbiochem.

Mice

PSGL-1$^{-/-}$ mice were generated as described.[1] C57BL/6J and CD44$^{-/-}$ mice were purchased from The Jackson Laboratory. PSGL-1$^{-/-}$ mice were bred with CD44$^{-/-}$mice to generate double heterozygous mice; these mice were bred to generate PSGL-1$^{-/-}$/CD44$^{-/-}$ mice. SHIP-1$^{-/-}$ mice[7] were a gift from Gerald Krystal (Terry Fox Laboratory, BC Cancer Agency, Vancouver, BC). DAP12$^{-/-}$/FcRγ$^{-/-}$ mice[8] and Fgr$^{-/-}$/Hck$^{-/-}$/Lyn$^{-/-}$mice[9] were a gift from Clifford Lowell (University of California, San Francisco). Fgr$^{-/-}$/Hck$^{-/-}$/Lyn$^{-/-}$mice were bred with C57BL/6J mice to generate Fgr$^{-/-}$, Hck$^{-/-}$, Lyn$^{-/-}$, and Hck$^{-/-}$/Lyn$^{-/-}$mice. LysM-GFP^{+} mice[10] were provided by Klaus Ley (La Jolla Institute for Allergy and Immunology). Btk$^{-/-}$mice,[11] originally from Wasif Khan (Vanderbilt University), were supplied by Carol Webb (Oklahoma Medical Research Foundation). PI3Kγ$^{-/-}$ mice,[12,13] originally from Joseph Penninger (Institute of Molecular Biotechnology of the Austrian Academy of Sciences), were provided by Paul Kubes (University of Calgary). PI3Kδ$^{-/-}$ mice[14] were provided by James Ihle (St. Jude Children's Research Hospital). Chimeric mice were generated by bone marrow transplantation as described previously.[15] WT recipient mice were irradiated lethally (9.5 Gy). After irradiation, a 1:1 mixture injected i.v. into recipients. Experiments were performed 8 weeks after bone marrow transplantation. All mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Cells

Murine bone marrow leukocytes were isolated as described previously.[16] Briefly, bone marrow cells were isolated by gently flushing femurs and tibias with 10 ml Hanks' balanced salt solution

(HBSS) without Ca^{2+} or Mg^{2+}. Red blood cells were lysed by adding 5 ml lysis buffer (150 mM NH4Cl, 10 mM NaHCO3, 1 mM EDTA) to 0.5 ml bone marrow cells. The cells were incubated for 20 s, and then 5 ml HBSS was immediately added. After centrifugation at 100 × g for 5 min, the cells were resuspended at 2×10^6/ml in HBSS containing 1.26 mM Ca^{2+}, 0.81 mM Mg^{2+}, and 0.5% human serum albumin. Human neutrophils were isolated as described previously.[17]

Flow chamber assay

For studies with murine leukocytes, a goat antibody against the Fc portion of human IgM (10 μg/ml) was adsorbed on 35-mm polystyrene dishes. For some experiments, 20 μg/ml murine ICAM-1–Fc chimera and 10 μg/ml murine CXCL1 were also adsorbed. Dishes were blocked in 1% human serum albumin, after which murine P-selectin-IgM or E-selectin–IgM was captured on the dish. For studies with human neutrophils, a goat antibody against the Fc portion of human IgG (10 μg/ml) was adsorbed on dishes. For some experiments, 20 μg/ml human ICAM-1 was also adsorbed. After blocking the dish with 1% human serum albumin, human E-selectin–IgG was captured on the dish. Alternatively, P-selectin (1 μg/ml) isolated from human platelets was perfused over P-selectin or E-selectin in dishes mounted in a parallel-plate flow chamber at a wall shear stress of 1 dyne/cm^2. Site densities of immobilized murine adhesion proteins were measured by binding of ^{125}I-labeled anti–P-selectin mAb RB40.34, anti–E-selectin mAb 9A9, or anti–ICAM-1 mAb 3E2. Human neutrophils or murine bone marrow leukocytes were suspended at 10^6/ml in HBSS with 0.5% human serum albumin. In some experiments, dishes were preincubated with 20 μg/ml anti–P-selectin mAb, anti–E-selectin mAb, anti–ICAM-1 mAb, or isotype-matched control mAb, or 20 μg/ml anti-αLβ2 and/or anti-αMβ2 mAb were included in the cell suspension. For some studies, cells were preincubated with 25 μg/ml OSGE (Cedar Lane) for 20 min at 37˚C.[16] In other studies, cells were incubated for 30 min at room temperature with the Syk inhibitor piceatannol (20 μM), the SFK inhibitor PP2 (20 μM) or its inactive analogue PP3 (20 μM), the PI3K inhibitor LY294002 (50 μM), the Tec kinase inhibitor LFM-A13 (50 μM), or the p38 inhibitor SB203580 (50 μM), or an equal volume of DMSO as a vehicle control. To disrupt lipid rafts, cells were preincubated with 10 mM MβCD with or without 15% FBS, 10 μM α-cyclodextrin, or 5 μg/ml filipin III for 30 min at 37˚C.[18] After 5 min, cells were analyzed using a videomicroscopy system coupled to a digital analysis system on a Silicon Graphics workstation. Velocities of rolling cells were measured over a 5-s interval.

SFK, Syk, Btk, and p38 phosphorylation

6-well plates were coated with anti-human IgM Fc antibody and incubated with E-selectin-IgM or P-selectin–IgM. Bone marrow leukocytes suspended in Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} containing 0.5% human serum albumin were incubated in selectin-coated plates on a rotary shaker at 65 rpm for 5 min. The cells were lysed with 1% Triton X-100, 125 mM NaCl, 50 mM Tris pH 8.0, 10 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, pepstatin, 50 μg/ml bestatin, 2 mM PMSF, 0.1% SDS, and 0.5% sodium deoxycholate. Cell lysates were analyzed by Western blotting,[16] using rabbit antibodies against phospho-SFK (Y416), Syk, phospho-Syk, p38, phospho-p38, or Btk. To examine phosphorylation of Fgr, Hck, Lyn, or Btk, lysates were incubated with protein G agarose for 3 h at 4˚C and centrifuged. The precleared lysates were incubated with rabbit antibodies to Fgr, Hck, Lyn, or goat antibody to Btk at 4˚C overnight and then with protein G agarose for 3 h at 4˚C. After washing the beads, bound proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by Western

blotting with antibodies to phosphotyrosine, Btk, and phospho-SFK, or with biotinylated antibodies to Fgr, Hck, and Lyn.

Intravital microscopy

Mice were anesthetized by i.p. injection of 1.25% Avertin (tribromoethanol/amylene hydrate, 0.2 ml/10 g body weight). After tracheal intubation, the right carotid artery was cannulated for systemic administration of mAbs and blood sampling for leukocyte counts. The cremaster muscle was superfused with thermocontrolled (35˚C) bicarbonate-buffered saline 3.5 to 4 h after intrascrotal injection of 500 ng murine TNF-α. Microvessel diameters, hemodynamic parameters, and mean leukocyte rolling velocities were analyzed offline. All neutrophil rolling in cremasteric muscle venules 4 h after TNF-α stimulation was blocked by a combination of mAbs to P- and E-selectin, excluding a contribution of L-selectin to rolling at this time point. In some experiments, 50 µg anti–P-selectin mAb or anti-β2 integrin mAb was injected i.v. Some mice received 4 µg pertussis toxin (PTx) i.v. 2 h before cremaster muscle exteriorization. In other experiments, 10^7 bone marrow leukocytes from WT or gene-targeted mice were labeled at room temperature for 5 min with red (PKH26) or green (PKH67) fluorescence dye (Sigma) and then was incubated with 200 ng/ml PTx at 37˚C for 2 h, and then injected i.v. 2 h after intrascrotal injection of TNF-α.

Thioglycollate-induced peritonitis

Mice were injected i.p. with 1 ml of 4% thioglycollate. Some mice received 4 µg PTx i.v. 2 h before thioglycollate injection. After 4 h, peritoneal cells were collected with 10 ml PBS containing 0.1% BSA and 5 mM EDTA, and the cells were analyzed by flow cytometry. Neutrophils were counted based on scatter properties and high expression of Gr-1.[19]

Competitive neutrophil recruitment assay

Competitive recruitment was performed as described.[20] Bone marrow leukocytes from WT or gene-targeted mice were labeled with red (PKH26) or green (PKH67) fluorescent dye. The labeled cells were resuspended in HBSS at 10^8 cells/ml and mixed at a 1:1 ratio. Some labeled cells and recipient mice were pretreated with PTx. Recipient WT mice were injected with 1 ml of 4% thioglycollate i.p., and the labeled cell mixture (200 µl per mouse) was then injected retroorbitally. After 4 h blood was collected. The mice were then sacrificed, and peritoneal cells were collected with 10 ml PBS containing 0.1% BSA and 5 mM EDTA. Neutrophils were counted in blood and peritoneal exudate as described above. In all experiments, 10^4 to 2×10^5 fluorescent neutrophils were recovered from the peritoneum. The data were plotted as the ratio of PKH26-labeled neutrophils from the indicated population compared to PKH67-labeled WT neutrophils in blood or peritoneal lavage.

Statistics

The unpaired Student *t* test was used to determine P values as indicated in the figures.

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