O-glycans direct selectin ligands to lipid rafts on leukocytes

Bojing Shao, Tadayuki Yago, Hendra Setiadi, Ying Wang, Padmaja Mehta-D’souza, Jianxin Fu, Paul R. Crocker, William Rodgers, Lijun Xia, and Rodger P. McEver

*Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104; †Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; and Division of Cell Signaling and Immunology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

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Palmitoylated cysteines typically target transmembrane proteins to domains enriched in cholesterol and sphingolipids (lipid rafts). P-selectin glycoprotein ligand-1 (PSGL-1), CD43, and CD44 are O-glycosylated proteins on leukocytes that associate with lipid rafts. During inflammation, they transduce signals by engaging selectins as leukocytes roll in venules, and they move to the raft-enriched uropods of polarized cells upon chemokine stimulation. It is not known how these glycoproteins associate with lipid rafts or whether this association is required for signaling or for translocation to uropods. Here, we found that loss of core 1-derived O-glycans in murine C1galt1−/− neutrophils blocked raft targeting of PSGL-1, CD43, and CD44, but not of other glycosylated proteins, as measured by resistance to solubilization in nonionic detergent and by copatching with a raft-resident sphingolipid on intact cells. Neuraminidase removal of sialic acids from wild-type neutrophils also blocked raft targeting. C1galt1−/− neutrophils or neuraminidase-treated neutrophils failed to activate tyrosine kinases when plated on immobilized anti-PSGL-1 or anti-CD44 F(ab)2. Furthermore, C1galt1−/− neutrophils incubated with anti-PSGL-1 F(ab)2 did not generate microparticles. In marked contrast, PSGL-1, CD43, and CD44 moved normally to the uropods of chemokine-stimulated C1galt1+/− neutrophils. These data define a role for core 1-derived O-glycans and terminal sialic acids in targeting glycoprotein ligands for selectins to lipid rafts of leukocytes. Preassociation of these glycoproteins with rafts is required for signaling but not for movement to uropods.

Significance

Leukocytes partition certain proteins into cholesterol- and sphingolipid-rich membrane regions (lipid rafts) that function as signaling platforms. Inflammatory stimuli cause leukocytes to elongate and form lamellipodia and uropods at opposite ends that facilitate migration. Many raft-associated proteins move to uropods. Proteins are typically thought to use their transmembrane and cytoplasmic domains to associate with rafts. Here, we found that some leukocyte adhesion proteins used carbohydrate modification (glycosylation) of their extracellular domains to associate with lipid rafts. These proteins required preassociation with rafts to transduce signals, but unexpectedly, not to move to uropods. These data define a mechanism for localizing proteins to critical membrane regions of leukocytes.

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To whom correspondence should be addressed. Email: roderg mcever@omrf.org.

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PSGL-1 or CD44 from activating SFKs and generating microparticles. However, O-glycans were not required to redistribute PSGL-1, CD43, or CD44 to the uropods of polarized leukocytes.

Results

PSGL-1 Does Not Require Its Transmembrane Domain to Associate with Lipid Rafts. Deleting the cytoplasmic domain of PSGL-1 does not prevent its partitioning into detergent-resistant membranes (DRMs, lipid rafts) (19). We asked whether PSGL-1 requires its transmembrane domain to associate with rafts. We generated PSGL-1 chimeras that substituted the transmembrane domain of PSGL-1 with the transmembrane domain of glycoporphin A or of CD45, which do not partition into rafts (18, 32, 33) (Fig. S1A). Wild-type (WT) PSGL-1 and PSGL-1 chimeras were expressed in transfected Chinese hamster ovary cells at similar densities (Fig. S1B). The cells were also transfected with vectors that express glycosyltransferases required to construct selectin ligands (34). We lysed the cells in cold 1% Triton X-100 and fractionated the extracts by ultracentrifugation in an Opti-Prep gradient. Western blotting revealed that significant portions of WT PSGL-1 and both PSGL-1 chimeras were in lighter-density DRMs that colocalized with the raft-resident protein flotillin 1. In contrast, the nonraft proteins transferrin receptor and moesin were found only in higher-density fractions (Fig. S1C). Thus, PSGL-1 does not require its cytoplasmic or transmembrane domain to associate with rafts.

PSGL-1, CD43, and CD44 Require Core 1-Derived O-Glycans to Associate with Lipid Rafts. We next considered whether PSGL-1 uses its extracellular domain to associate with rafts. Some epithelial cell proteins use N- or O-glycans for transport into raft-enriched apical domains (6). Therefore, we asked whether the multiple O-glycans on the extracellular domain of PSGL-1 contribute to raft targeting. Leukocytes from EHC C1galt1−/− mice attach GalNAc to serines and threonines but lack core 1-derived O-glycans, including core 1, extended core 1, and core 2 structures (14). They express normal surface levels of PSGL-1, CD43, CD44, and other glycoproteins (14). The cholesterol probe filipin (35) bound...
similarly to plasma membranes of WT and C1galt1−/− neutrophils (Fig. S24). Filipin binding was specific for cholesterol, because it was eliminated by treating neutrophils with methyl-β-cyclodextrin, a cholesterol chelator, but not with α-cyclodextrin, an inactive analog (Fig. S29).

As in transfected Chinese hamster ovary cells, a significant portion of PSGL-1 in detergent extracts of WT neutrophils was in lighter-density DRMs that colocalized with flotillin 1 (Fig. 1A). The O-glycosylated proteins CD43 and CD44 from WT neutrophils were also enriched in raft fractions. However, virtually all PSGL-1, CD43, and CD44 in extracts from C1galt1−/− neutrophils were in higher-density, nonraft fractions (Fig. 1A). In contrast, the N-glycosylated protein siglec-E was enriched in lower-density fractions of both genotypes, and the N-glycosylated protein L-selectin was enriched in higher-density fractions of both genotypes (Fig. 1A).

To identify proteins in lipid rafts of intact cells, we used confocal microscopy to visualize copatching of proteins with rafts by crosslinking cholera toxin B (CTxB) bound to the raft-enriched ganglioside GM1. Before crosslinking (without incubation at 37 °C to cause patching), antibodies to CTxB, PSGL-1, CD43, CD44, and siglec-E homogeneously stained the plasma membranes of both WT and C1galt1−/− neutrophils (Fig. 1B). After crosslinking CTxB at 37 °C, lipid rafts clustered in discrete aggregates on neutrophils of both genotypes (Fig. 1B). Siglec-E, but not the nonraft protein CD45, copatched with CTxB on both WT and C1galt1−/− neutrophils. PSGL-1, CD43, and CD44 also copatched with CTxB on WT neutrophils. In sharp contrast, they remained homogeneously distributed on C1galt1−/− neutrophils (Fig. 1B). Thus, both detergent resistance and copatching assays demonstrate that PSGL-1, CD43, and CD44 require core 1-derived O-glycans to associate with lipid rafts.

**PSGL-1, CD43, and CD44 Require Sialic Acids to Associate with Lipid Rafts.** Sialic acids cap most N- and O-glycans on mammalian cells, including neutrophils (36). We asked whether sialic acids contribute to raft targeting of PSGL-1, CD43, and CD44. For this purpose, we treated WT neutrophils with neuraminidase (sialidase). This treatment effectively removed sialic acids, as measured by increased binding of the lectin, peanut agglutinin, to neutrophil surfaces (Fig. S34), and by altered mobility of PSGL-1, CD43, and CD44 during SDS/PAGE (Fig. S3 B–D). Neuraminidase treatment markedly reduced the amount of each protein in lighter-density DRMs (Fig. 2A). Neuraminidase did not alter basal homogeneous staining of PSGL-1, CD43, CD44, and siglec-E (Fig. 2B), but it substantially decreased copatching of PSGL-1, CD43, and CD44 with the raft marker CTxB (Fig. 2B). However, it did not alter the distribution of siglec-E (Fig. 2A) or CD45 (Fig. 2B). These data demonstrate that PSGL-1, CD43, and CD44 require sialic acids, most likely on O-glycans, to associate with lipid rafts.

**PSGL-1 and CD44 Require Core 1-Derived O-Glycans and Sialic Acids to Initiate Signaling.** Selectin binding to PSGL-1 and CD44 on neutrophils induces tyrosine phosphorylation of SFKs and downstream kinases, including p38 MAPK, which convert β2 integrins to an extended, intermediate-affinity conformation that mediates slow rolling (9, 23, 24, 37). Disrupting lipid rafts by depleting or sequestering cholesterol blocks signaling (23). We asked whether PSGL-1 and CD44 must preassociate with lipid rafts to initiate signaling. We used mAbs to PSGL-1 or CD44 as selectin surrogates. WT neutrophils plated on F(ab′)2 fragments of anti-PSGL-1 or anti-CD44 mAb, but not isotype-control F(ab′)2, phosphorylated tyrosines on SFKs, and p38 MAPK (Fig. 3A). In marked contrast, C1galt1−/− neutrophils plated on anti–PSGL-1 or anti–CD44 F(ab′)2 did not activate SFKs or p38 MAPK. Furthermore, neuraminidase-treated WT neutrophils plated on anti–PSGL-1 or anti–CD44 F(ab′)2 did not activate SFKs or p38 MAPK (Fig. 3B). These results indicate that selectin-triggered signaling in neutrophils requires O-glycan- and sialic acid-dependent association of PSGL-1 and CD44 with lipid rafts.

**PSGL-1 Requires Core 1-Derived O-Glycans to Trigger SFK-Dependent Generation of Microparticles.** Neutrophils stimulated with LPS or the Ca2+ ionophore A23187 or by P-selectin binding to PSGL-1 generate microparticles enriched in lipid raft-associated proteins (18, 26). We labeled the membranes of WT or C1galt1−/− neutrophils with a fluorescent dye and measured agonist-induced release of fluorescent microparticles. The Ca2+ ionophore A23187, but not vehicle control, generated equivalent numbers of microparticles from WT and C1galt1−/− neutrophils (Fig. 4A). By contrast, F(ab′)2 fragments of anti–PSGL-1 mAb, but not of anti–CD45

![Fig. 2.](https://example.com/fig2.png) **Fig. 2.** PSGL-1, CD43, and CD44 require sialic acids to associate with lipid rafts. WT neutrophils were incubated with buffer or neuraminidase (sialidase). (A) The cells were lysed, fractionated on OptiPrep gradients, and analyzed by Western blotting with antibodies to the indicated protein as in Fig. 1. (B) CTxB-bound rafts and antibodies to the indicated protein were visualized by confocal microscopy as in Fig. 1. Results are representative of at least three experiments. (Scale bar, 5 μm.)
PSGL-1 and CD43 are extended mucins with O-glycans attached to many serines and threonines (9, 15, 39). Clustered, sialylated O-glycan “patches” on these proteins are possible raft-targeting signals. However, the less clustered O-glycans on CD44 also mediated raft targeting, whereas the O-glycans on CD45 (40) did not. Thus, the structural features of the signal require further definition. Raft association could involve interactions of glycan determinants on PSGL-1, CD43, and CD44 with a raft-resident lectin. Candidates are siglecs and the structurally related paired Ig-like type 2 receptors (PILRs), which bind terminal sialic acids in particular contexts (41, 42). Siglec-E, the siglec CD33, and PILRα are expressed on murine myeloid cells. However, all three lectins have cytoplasmic immunoreceptor tyrosine-based inhibitory motifs that negatively regulate inflammation (43, 44), whereas raft association of PSGL-1, CD43, and CD44 promotes proinflammatory signaling. CD33 and PILRα prefer sialic acid linked α2–6 to N-acetylgalactosamine (45, 46), not the sialic acid linked α2–3 to galactose that caps core 1-derived O-glycans. Alternatively, desialylation or truncation of O-glycans could indirectly affect the conformation of targeting signals on the protein backbone. However, a single N-acetylgalactosamine attached to serines and threonines, as occurs on C1gal1−/− leukocytes, is sufficient to extend the polypeptide backbone of mucins such as PSGL-1 and CD43 (47, 48).

In epithelial cells, similarly complex signals target glycoproteins to apical membrane domains where they are enriched in cholesterol and sphingolipids (1). Both N- and O-glycans have been implicated in apical targeting (6). Glycosylation of some proteins enhances raft association as well as apical targeting (49), whereas glycosylation of other proteins mediates apical targeting independently of rafts (50).

During neutrophil rolling, selectin engagement of PSGL-1 or CD44 triggers a signaling cascade similar to that used by the T-cell receptor (9). The cascade activates SFKs and downstream kinases and recruits multiple adaptors. Disrupting lipid rafts by depleting or sequestering cholesterol blocks signaling (23). Lipid rafts function as signaling platforms by assembling signaling components such as SFKs. Ligand clustering may merge T-cell receptors in nonraft domains with coreceptors in raft domains to initiate signaling (4). By contrast, we found that PSGL-1 and CD44 must associate with rafts before engaging a selectin surrogate to trigger signaling. These rafts are probably too small to contain a full complement of SFKs or other signaling proteins. During cell adhesion, selectin binding to PSGL-1 or CD44 likely clusters small rafts into larger domains with sufficient kinases, substrates, and adaptors to trigger signaling. PSGL-1 also requires its cytoplasmic domain to signal (19), suggesting that it directly recruits one or more signaling components. Perhaps PSGL-1 and CD44 require preassociation with lipid rafts to initiate signaling.

PSGL-1 and CD43 require core 1-derived O-glycans to redistribute to the uropods of polarized neutrophils. Chemokine-stimulated leukocytes polarize to form leading-edge lamellipodia and trailing-edge uropods (38). We visualized the distribution of membrane proteins on neutrophils after stimulation with CXCL1. The raft-associated proteins PSGL-1, CD43, and CD44, but not the nonraft protein CD45, redistributed to the uropods of WT neutrophils (Fig. 5 A and B). Disrupting lipid rafts with the cholesterol chelator, methyl-β-cyclodextrin, but not with the inactive analog, the cholesterol chelator, methyl-[β-cyclodextrin, but not with the inactive analog, α-cyclodextrin, blocked polarization, confirming previous studies (21) (Fig. 5A). Unexpectedly, PSGL-1, CD43, and CD44 also redistributed to the uropods of C1gal1−/− neutrophils (Fig. 5B). However, CXCL1 did not alter the density distribution of raft and nonraft proteins in detergent extracts from WT or C1gal1−/− neutrophils (Fig. 5C). Thus, PSGL-1, CD43, and CD44 do not require preassociation with lipid rafts to move to the uropods of polarized neutrophils.

**Discussion**

We defined a critical role for core 1-derived O-glycans and terminal sialic acids in targeting glycoprotein ligands for selectins to lipid rafts on leukocytes. We used complementary assays to identify glycoproteins in rafts: resistance to solubilization in nonionic detergent and copatching with a raft-resident sphingolipid on intact cells. Both assays yielded congruent results that strengthen our conclusions. We further demonstrated that these glycoproteins must preassociate with rafts to transduce biologically important signals.

PSGL-1 lacking its cytoplasmic domain still associates with lipid rafts (19). Here we ruled out a requirement for the transmembrane domain of PSGL-1 for raft targeting. This argues against palmitoylation of cysteines in either domain as an essential mechanism for moving PSGL-1 to rafts. Instead, extension of sialylated core 1-derived O-glycans on the extracellular domain of PSGL-1, and of CD44 and CD43, enabled targeting. Global loss of O-glycans or terminal sialic acids did not indirectly impair raft association of all proteins, because flotillin-1 and N-glycosylated siglec-E remained in rafts.

We visualized the distribution of raft and nonraft proteins in detergent extracts of WT or C1gal1−/− neutrophils incubated with buffer or neuraminidase (sialidase) and then incubated on immobilized F(ab)′2 fragments of type control, anti-PSGL-1, or anti-CD44 mAb. Lysates were probed by Western blotting with antibodies to p-SFK, total SFK, phospho-p38 (p-p38), or total p38. Immunoblotting revealed that the cholesterol chelator, methyl-β-cyclodextrin, but not with the inactive analog, α-cyclodextrin, blocked polarization, confirming previous studies (21) (Fig. 5A). Unexpectedly, PSGL-1, CD43, and CD44 also redistributed to the uropods of C1gal1−/− neutrophils (Fig. 5C). Thus, PSGL-1, CD43, and CD44 do not require preassociation with lipid rafts to move to the uropods of polarized neutrophils.

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rafts because, unlike the T-cell receptor, they lack coreceptors that facilitate movement from nonraft to raft domains. Although not yet tested, E-selectin engagement of CD43 on rolling effector T cells (51, 52) may induce signaling by a similar mechanism.

The best characterized effector response to PSGL-1− or CD44-mediated signaling is conversion of β2 integrins to an extended, intermediate-affinity form that mediates slow rolling on ICAM-1 (9). However, P-selectin binding to PSGL-1 also triggers release of prothrombotic and proinflammatory microparticles (18, 26, 53). We found that PSGL-1 required preassociation with lipid rafts to generate microparticles through an SFK-dependent signaling pathway. Thus, raft-dependent signaling was required to generate raft-enriched microparticles. A downstream event in PSGL-1-induced signaling is activation of phospholipase C (9), which generates intracellular Ca\(^{2+}\) that is probably the proximal inducer of microparticle release. By directly elevating cytosolic Ca\(^{2+}\), the ionophore A23187 bypassed the upstream components of this receptor-mediated signaling cascade.

During polarization of activated leukocytes, membrane domains enriched in cholesterol and sphingolipids, including GM1, coalesce in uropods with a subset of transmembrane glycoproteins that include PSGL-1, CD43, and CD44 (38). Surprisingly, these glycoproteins also moved to uropods of chemokine-stimulated C1galtn−/− neutrophils, even though, before stimulation, they did not copatch with GM1 in lipid rafts, and after stimulation, they remained in higher-density, detergent-soluble “nonraft” fractions. Uropods form through membrane interactions, uropods might sweep PSGL-1, CD43, and CD44 into uropods as rafts coalesce into larger domains that increase binding avidity. These interactions might synergize with binding of the cytoplasmic domains of PSGL-1, CD43, and CD44 to ERMs that link to the cytoskeleton. Because of clustered, high-avidity interactions, uropods might form even if only some cytoplasmic domains bind directly to ERMs. This could explain why PSGL-1 lacking its cytoplasmic domain still moves to uropods of stimulated neutrophils (19).

In addition to selectin ligands, other glycoproteins may use siaylated O-glycans to associate with lipid rafts on hematopoietic cells. Thus, O-glycosylation may influence how membrane domains regulate diverse functions during hemopoiesis, immune responses, and hemostasis.

**Materials and Methods**

All mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation. Details including reagents, mice, cells, isolation of murine neutrophils from bone marrow, detergent-resistant membrane preparation, Western blot, flow cytometry, patching of lipid rafts, neutrophil polarization, activation of SFKs or p38 MAPK by crosslinking PSGL-1 or CD44, and statistical analysis are given in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Reagents. Rat monoclonal antibodies (mAbs) to murine PSGL-1 (clone 4RA10), CD44 (clone KM114), CD45 (clone 30-F11), and L-selectin (clone Mel-14), murine mAb to flotillin-1 (clone 18/3,6,8-sialidase from A. ureafaciens, and fluorescein isothiocyanate (FITC)-labeled goat polyclonal antibody to rat IgG were from BD Biosciences. Goat polyclonal antibody to murine CD43 was from Santa Cruz Biotechnology. Sheep polyclonal antibody to mouse sialic E was prepared as described previously (58). Rabbit mAb to murine Src (clone 36D10), rabbit mAb to murine phospho-Src (Tyr-416, clone D9G4), and rabbit polyclonal antibody to murine phospho-p-38 MAPK (T180/Y182) were from Cell Signaling. Rabbit polyclonal antibody to murine p-38 MAPK (Tyr-416, clone D49G4), and rabbit polyclonal antibody to murine phospho-Src were from Cell Signaling. Rabbit polyclonal antibody to murine L-selectin (clone Mel-14), murine mAb to flotillin-1 (clone 2/transferrin), and L-selectin (clone DREG-56), murine mAb to moesin (clone 38/moesin, crossreacts with human and murine moesin), R-phycocyanin (PE)-labeled rat mAb to murine PSGL-1 (clone 2PH1), and fluorescein isothiocyanate (FITC)-labeled goat polyclonal antibody to rat IgG were from BD Biosciences. Goat polyclonal antibody to murine CD43 was from Santa Cruz Biotechnology. Sheep polyclonal antibody to mouse sialic E was prepared as described previously (58). Rabbit mAb to murine Src (clone 36D10), rabbit mAb to murine phospho-Src (Tyr-416, clone D9G4), and rabbit polyclonal antibody to murine phospho-p-38 MAPK (T180/Y182) were from Cell Signaling. Rabbit polyclonal antibody to murine p-38 MAPK was from BioLegend. Alexa Fluor 647-conjugated goat polyclonal antibody to rat IgG, donkey polyclonal antibody to goat IgG, donkey polyclonal antibody to sheep IgG, and Alexa Fluor 488-conjugated cholera toxin subunit B (CTxB) were from Life Technologies. Goat polyclonal antibody to cholera toxin subunit B was from Calbiochem. Horseradish peroxidase-conjugated anti-mouse IgG, anti-rat IgG, anti-rabbit IgG, anti-goat IgG, and anti-sheep IgG were from Thermo Scientific. Percoll was from GE Healthcare Life Sciences. Filipin III was from Cayman Chemical. α2–3,6,8-Sialidase from Arthrobacter ureafaciens was from New England BioLabs or Sigma, and α2–3,6,8-sialidase from Vibrio cholerae was from Roche LifeSciences. FITC-conjugated peanut agglutinin was from Vector Laboratories. Complete protease inhibitor mixture was from Roche. OptiPrep density gradient, methyl-β-cyclodextrin (mβCD), α-cyclodextrin (αCD), A23187, fibronectin, PKH67 green fluorescent cell linker kit, and poly-L-lysine were from Sigma Aldrich. Recombinant murine CXCL1 was from R&D Systems.

Mice. EHC C1galt−/− (59), Selplg−/− mice (60), and Hck/Fgr/Lyn-deficient (SKF−/−) mice (61) were of mixed genetic background (129S1/SvIm and C57BL/6J). Littermate controls were used. All experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Cells. Transfected Chinese hamster ovary (CHO) cells expressing core 2 GlcNAcT-I, FucT-VII, and WT or mutant murine PSGL-1 were generated as previously described (62).

Isolation of Murine Neutrophils from Bone Marrow. Murine bone marrow leukocytes, isolated as described (60), were suspended in 45% (vol/vol) Percoll. Percoll concentrations of 81%, 62%, 55%, and 50% were layered from the bottom sequentially. The cells were laid on the top of the four-layer Percoll gradient. After centrifugation (1,200 × g, 30 min, room temperature, no braking), neutrophils at the interface between 62% and 81% Percoll solutions were collected. The cells were washed twice with Hank’s balanced salt solution (HBSS) without Ca2+ and Mg2+ and then suspended in the indicated buffer for experiments.

Detergent-Resistant Membrane Preparation. Membranes were isolated as described previously (63). Briefly, chilled murine neutrophils or transfected CHO cells were lysed with 1% Triton X-100. After homogenization and centrifugation, the cell lysate was mixed with OptiPrep solution to a final concentration of 40% (vol/vol). This mixture was put at the bottom of a 2.4-mL centrifuge tube. On the top of the mixture, OptiPrep solutions at concentrations of 30%, 20%, and 5% were layered sequentially. After centrifugation at 100,000 × g for 4 h at 4 °C, nine equal fractions were collected from top to bottom of the tube. In some experiments, neutrophils were preincubated with 10 units/mL α2–3,6,8-sialidase from A. ureafaciens at 37 °C for 1 h before lysis.

Western Blot. Western blots were performed as described previously (60).

Flow Cytometry. Flow cytometry was performed as described previously (60). Cholesterol in the plasma membrane of neutrophils was detected by binding of filipin as described (35). Briefly, after fixation with 2% paraformaldehyde, neutrophils were washed, stained with 50 μg/mL filipin III at room temperature for 2 h, and then immediately analyzed by flow cytometry. In some experiments, cells were treated with mβCD or αCD before fixation.

Patching of Lipid Rafts. Patching was performed as described previously (3). Briefly, neutrophils were incubated with Alexa 488-conjugated CTxB in PBS with 0.1% BSA for 30 min on ice. Cells were washed at 4 °C and incubated with polyclonal antibody to CTxB on 30 min on ice. To patch lipid rafts, the cells were incubated at 37 °C for 15 min and then fixed with 2% paraformaldehyde in PBS at room temperature. After fixation, neutrophils were stained with primary antibodies to the indicated membrane proteins and with Alexa 647-conjugated secondary antibodies. Immunofluorescence was detected with a confocal laser-scanning microscope equipped with an argon/krypton laser light (LSM 510, Zeiss). In some experiments, neutrophils were preincubated with 10 units/mL α2–3,6,8-sialidase from A. ureafaciens at 37 °C for 1 h before patching.

Neutrophil Polarization. Neutrophils were plated on fibronectin-coated coverslips in the presence or absence of 20 ng/mL CXCL1 at 37 °C for 30 min (19). The polarized cells were fixed with 2% (wt/vol) paraformaldehyde at room temperature for 10 min. The fixed cells were stained with Alexa 488-conjugated CTxB and primary antibodies to indicated proteins, followed by Alexa 647-conjugated secondary antibodies. Immunofluorescence was detected with a confocal laser-scanning microscope equipped with an argon/krypton laser light (LSM 510, Zeiss).

Activation of SFKs or p38 MAPK by Crosslinking PSGL-1 or CD44. F(ab′)2 fragments of anti-PSGL-1 mAb 4RA10, anti-CD44 mAb IM7, or isotype control rat IgG were generated with an F(ab′)2 preparation kit (Thermo Scientific). Fifty μg/mL F(ab′)2 were coated in 48-well plates overnight at 4 °C. The plates were blocked with 1% human serum albumin for 1 h at room temperature. Bone marrow neutrophils from WT or EHC C1galt−/− mice (5 × 105) in 200 μL HBSS with 0.5% human serum albumin were incubated in F(ab′)2-coated wells for 10 min at room temperature. In some experiments, neutrophils were preincubated with 0.6 units/mL α2–3,6,8-sialidase from A. ureafaciens and 5 μg/mL α2–3,6,8-sialidase from Vibrio cholerae in HBSS with 0.5% human serum albumin) at 37 °C for 1 h before incubation in F(ab′)2-coated wells. The cells were lysed in 1% Triton X-100, 125 mM NaCl, 50 mM Tris, pH 8.0, 10 mM EDTA, 2 mM PMSF, and 1/100 protease and phosphatase inhibitor mixture. The cell lysates were probed by Western blotting under reducing conditions with 

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conditions with antibodies against SFK, phospho-SFK (Y416), p38, or phospho-p38.

**Neutrophil Microparticle Preparation.** Neutrophil microparticles were prepared as described (18, 26), with minor modifications. Briefly, neutrophils isolated from murine bone marrow were labeled with PKH67 fluorescent membrane dye. The cells were incubated with 20 μg/mL F(ab')2 fragments of anti–PSGL-1, anti-CD45, or isotype control mAb in RPMI 1640, 10% (vol/vol) FBS at 37 °C for 12 h. The cell suspension was centrifuged sequentially at 500 × g, 1,500 × g, and 5,000 × g to remove cells and debris. The supernatant was then spun at 20,000 × g for 20 min to pellet microparticles for quantification by flow cytometry. In some assays, neutrophils were stimulated with 20 μM calcium ionophore at 37 °C for 15 min before the microparticles were isolated and quantified.

**Statistics.** Data are expressed as mean ± SEM. Comparisons used the Student’s t test (unpaired and two tailed). P < 0.05 was considered to be significant.

Fig. S1. PSGL-1 does not require its transmembrane domain to associate with lipid rafts. (A) Schematic of wild-type (WT) murine PSGL-1 or of PSGL-1 constructs containing the transmembrane domain (TMD) of glycophorin A (GpA) or CD45. (B) Flow cytometry of transfected cells expressing the indicated PSGL-1 construct labeled with PE-conjugated anti–PSGL-1 mAb. (C) Transfected cells were lysed in cold 1% Triton X-100 and centrifuged in an OptiPrep gradient. Fractions collected from Top to Bottom (Left to Right, corresponding to lower to higher density) were analyzed by Western blotting with antibodies to the indicated proteins. Results are representative of at least three experiments.
Fig. S2. The cholesterol probe filipin binds similarly to WT and C1galt1−/− neutrophils. (A) WT or C1galt1−/− neutrophils or (B) WT neutrophils pretreated with the cholesterol chelator methyl-β-cyclodextrin (MβCD) or its inactive analog α-cyclodextrin (αCD) were incubated with DMSO vehicle or filipin. Flow cytometry was used to measure filipin binding by UV light emission. Results are representative of three experiments.

Fig. S3. Neuraminidase effectively removes sialic acids from glycans on neutrophils. (A) WT neutrophils were treated with control buffer or neuraminidase (sialidase) and then stained with FITC-conjugated peanut agglutinin (PNA). (B–D) WT neutrophils treated with or without sialidase were lysed and analyzed by Western blotting with mAbs against PSGL-1, CD43, or CD44 under nonreducing conditions. Results are representative of three experiments.