O-glycans direct selectin ligands to lipid rafts on leukocytes

Bojing Shao[a], Tadayuki Yago[a], Hendra Setiadi[a], Ying Wang[a,b], Padmaja Mehta-D'souza[a], Jianxin Fu[a], Paul R. Crocker[c], William Rodgers[b], Lijun Xia[a,b], and Rodger P. McEver[a,b,1]

[a]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104; [b]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104; and [c]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.

cell adhesion | cell signaling | inflammation | uropod | neutrophil

Lipid rafts are ordered membrane domains that assemble cholesterol, sphingolipids, and selected proteins (1). They were first defined by resistance to solubilization in cold nonionic detergents, which maintains raft proteins in the lighter fractions of density gradients (2). On intact cells, lateral crosslinking with antibodies or other probes copatches lipid and protein constituents of rafts (3). High-resolution imaging confirms that rafts are small, dispersed structures that can be oligomerized (1). Importantly, rafts serve as signaling platforms, notably on immune cells (4).

How proteins partition to rafts is incompletely understood (5). Hydrophobic residues in some transmembrane domains may interact with sphingolipids and/or cholesterol. Cysteines modified with saturated fatty acids, usually palmitic acid, direct cytosolic proteins such as Src family kinases (SFKs) to raft inner leaflets. Palmitoylated cysteines in transmembrane and cytoplasmic domains of some membrane proteins also interact with rafts. In polarized epithelial cells, apical transport vesicles are enriched in cholesterol and sphingolipids (1). N- and O-glycans on some apical proteins act as sorting determinants, probably through multiple mechanisms. Glycans may enhance association of some apically destined proteins with rafts (6). Whether glycans direct proteins to rafts of hematopoietic cells is unknown.

At sites of infection or injury, circulating leukocytes adhere to activated endothelial cells and platelets and to adherent leukocytes. The adhesion cascade includes tethering, rolling, deceleration (slow rolling), arrest, intraluminal crawling, and transendothelial migration (7). Selectins mediate rolling, whereas β2 integrins mediate slow rolling, arrest, and crawling. Selectins are lectins that form rapidly reversible, force-regulated bonds with glycosylated ligands under flow (8). Leukocytes express L-selectin, activated platelets express P-selectin, and activated endothelial cells express P- and E-selectin. The dominant leukocyte ligand for P- and L-selectin is P-selectin glycoprotein ligand-1 (PSGL-1). Major leukocyte ligands for E-selectin include PSGL-1, CD44, and CD43, although other ligands contribute to adhesion (9). PSGL-1 and CD43 are mucins with multiple O-glycans attached to serines and threonines. Although not a mucin, CD44 is modified with both N- and O-glycans (10, 11). The selectins bind, in part, to the sialyl Lewis x (sLe^x) determinant (NeuAcα2–3Galβ1–4[Fucα1–3]GlcNAcβ1-R), which caps some N-glycans and mucin-type O-glycans (8, 12). CD44 uses N-glycans to interact with E-selectin (13, 14), whereas PSGL-1 uses mucin-type, core 1-derived O-glycans to interact with all three selectins (14–16). The enzyme core 1 β1–3-galactosyltransferase forms the core 1 backbone (Galβ1–3GalNAcα1-Ser/Thr) to which more distal determinants such as sLe^x are added (17). Neutrophils from mice lacking core 1 β1–3-galactosyltransferase in endothelial and hematopoietic cells (EHC *C1galt1*$^{-/-}$) have markedly impaired rolling on P- or E-selectin (14).

PSGL-1 (18, 19), CD44 (20), and CD43 (21) associate with lipid rafts on leukocytes, but how they do so is unclear. In knockin mice, PSGL-1 lacking the cytoplasmic domain still associates with leukocyte rafts (19). In transfected nonhematopoietic cells,

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 to 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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[1]To whom correspondence should be addressed. Email: rodger-mcever@omrf.org.

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detergent resistance of CD44 is reversed by mutating cysteines in the transmembrane and cytoplasmic domains and an ezrin/radixin/moesin (ERM)-binding site in the cytoplasmic domain (22). Whether similar mechanisms operate in primary leukocytes is unknown. Determining how these proteins target to rafts is relevant because of their important signaling functions in leukocytes. Selectin binding to PSGL-1 or CD44 on neutrophils triggers phosphorylation of SFKs and downstream mediators that convert β2 integrins to an extended, intermediate-affinity state, slowing rolling and contributing to arrest (23–25). Disrupting lipid rafts by depleting or sequestering cholesterol blocks signaling (23). Binding of P-selectin to PSGL-1 on myeloid cells causes shedding of microparticles with proinflammatory and procoagulant properties (26). The microparticles are enriched in raft-associated proteins such as PSGL-1 and tissue factor, but not in nonraft proteins such as CD45 (18). Disrupting rafts by chelating or sequestering cholesterol blocks microparticle generation (18). However, it is not known whether PSGL-1 or other selectin ligands must preassociate with rafts to trigger integrin activation or microparticle shedding.

Leukocytes stimulated with chemokines or bacterial peptides polarize to form leading-edge lamellipodia and trailing-edge uropods. PSGL-1, CD43, and CD44 redistribute to the uropods (27–29). Studies in transfected cells suggest that PSGL-1 moves to uropods through interactions of its cytoplasmic domain with ERM proteins (30). In knockin mice, however, PSGL-1 lacking the cytoplasmic domain relocates normally to uropods of polarized neutrophils (19). It has also been proposed that PSGL-1 moves to uropods by interacting with flotillin, a raft-resident protein (31). Notably, disrupting lipid rafts by chelating cholesterol blocks uropod formation (21). It is not known whether PSGL-1 or other proteins must associate with rafts before moving to uropods.

Here, we found that loss of core 1-derived O-glycans in leukocytes from EHC *C1galt1*$^{-/-}$ mice blocked raft targeting of PSGL-1, CD43, and CD44, but not of other glycosylated proteins. Treating leukocytes with neuraminidase to remove terminal sialic acids had similar effects. Failure to partition into rafts prevented 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 glycophorin A or of CD45, which do not partition into rafts (18, 32, 33) (Fig. S1*A*). Wild-type (WT) PSGL-1 and PSGL-1 chimeras were expressed in transfected Chinese hamster ovary cells at similar densities (Fig. S1*B*). 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 OptiPrep 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. S1*C*). 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

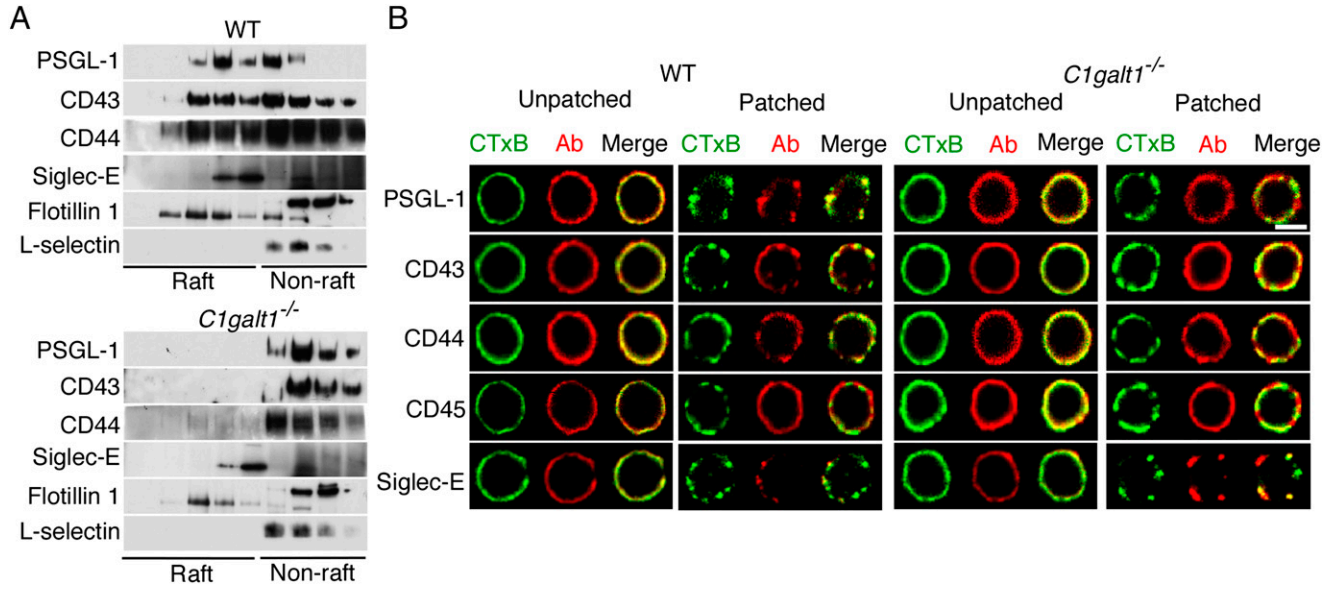

Fig. 1. PSGL-1, CD43, and CD44 require core 1-derived O-glycans to associate with lipid rafts. (*A*) WT or *C1galt1*$^{-/-}$ neutrophils were lysed in cold 1% Triton X-100 and centrifuged in an OptiPrep gradient. Fractions collected from *Top* to *Bottom* (*Left* to *Right*, corresponding to lower to higher density) were analyzed by Western blotting with antibodies to the indicated proteins. (*B*) WT or *C1galt1*$^{-/-}$ neutrophils were incubated with Alexa 488-conjugated CTxB (green) to label GM1-containing lipid rafts. The cells were then incubated with anti-CTxB antibodies at 4 °C as control (unpatched) or at 37 °C to aggregate the rafts (patched). The cells were then fixed and labeled with antibodies to the indicated protein, followed by Alexa 647-conjugated secondary antibody (red). Representative cells were visualized with confocal microscopy to identify CTxB, antibody (Ab), or both CTxB and Ab (merge). Results are representative of at least three experiments. (Scale bar, 5 μm.)

similarly to plasma membranes of WT and *C1galt1*$^{-/-}$ neutrophils (Fig. S2*A*). 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. S2*B*).

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. 1*A*). 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. 1*A*). 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. 1*A*).

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, CD45, and siglec-E homogeneously stained the plasma membranes of both WT and *C1galt1*$^{-/-}$ neutrophils (Fig. 1*B*). After crosslinking CTxB at 37 °C, lipid rafts clustered in discrete aggregates on neutrophils of both genotypes (Fig. 1*B*). 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. 1*B*). 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. S3*A*), 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. 2*A*). Neuraminidase did not alter basal homogeneous staining of PSGL-1, CD43, CD44, CD45, and siglec-E (Fig. 2*B*), but it substantially decreased copatching of PSGL-1, CD43, and CD44 with the raft marker CTxB (Fig. 2*B*). However, it did not alter the distribution of siglec-E (Fig. 2 *A* and *B*) or CD45 (Fig. 2*B*). 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. 3*A*). 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. 3*B*). 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 Ca^{2+} 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 Ca^{2+} ionophore A23187, but not vehicle control, generated equivalent numbers of microparticles from WT and *C1galt1*$^{-/-}$ neutrophils (Fig. 4*A*). By contrast, F(ab′)2 fragments of anti–PSGL-1 mAb, but not of anti-CD45

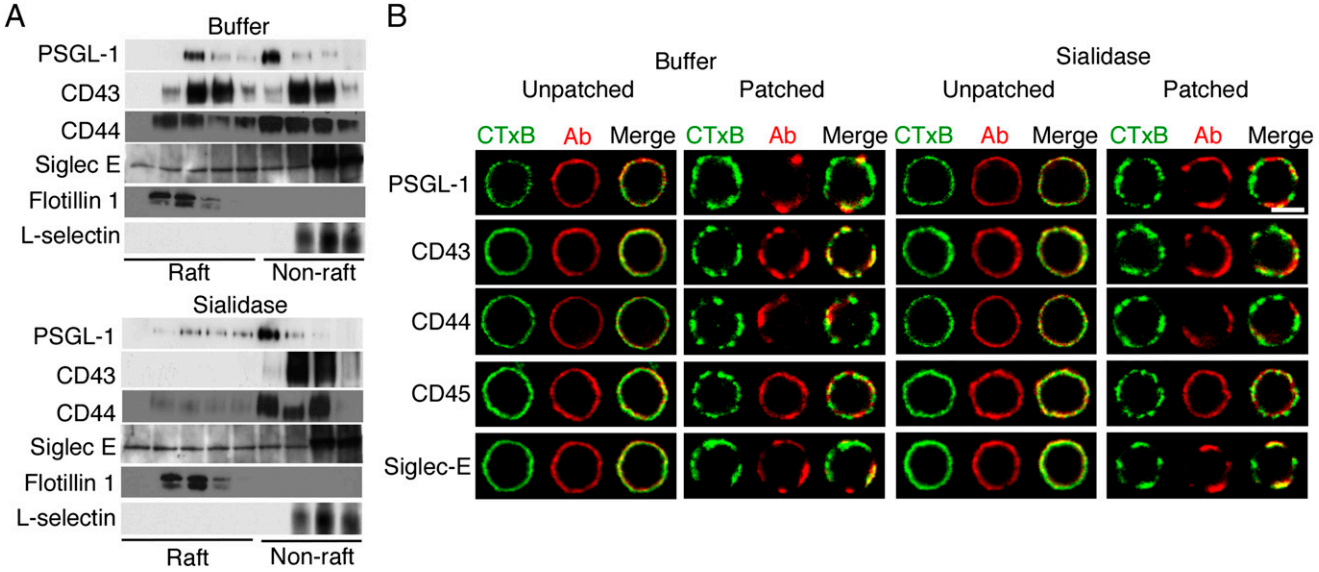

Fig. 2. PSGL-1, CD43, and CD44 require sialic acids to associate with lipid rafts. WT neutrophils were incubated with buffer or neuraminidase (sialidase). (*A*) The cells were lysed, fractionated on OptiPrep gradients, and analyzed by Western blotting with antibodies to the indicated protein as in Fig. 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.)

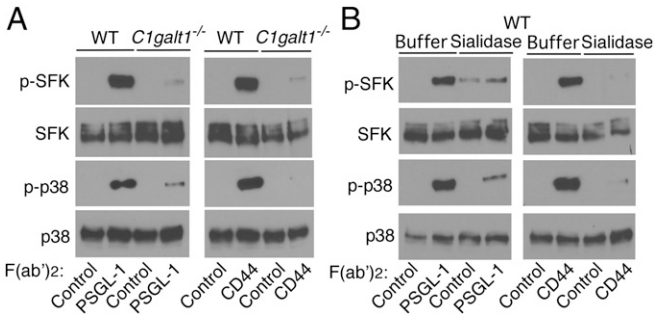

Fig. 3. PSGL-1 and CD44 require core 1-derived O-glycans and sialic acids to initiate signaling. (*A*) WT or *C1galt1*$^{-/-}$ neutrophils were incubated on immobilized F(ab′)$_2$ fragments of isotype control, anti–PSGL-1, or anti-CD44 mAb. Lysates were probed by Western blotting with antibodies to phospho-SFK (p-SFK), total SFK, phospho-p38 (p-p38), or total p38. (*B*) WT neutrophils were incubated with buffer or neuraminidase (sialidase) and then incubated on immobilized F(ab′)$_2$ fragments of isotype control, anti–PSGL-1, or anti-CD44 mAb. Lysates were probed by Western blotting with antibodies to p-SFK, total SFK, p-p38, or total p38. Results are representative of three experiments.

or isotype-control mAb, generated microparticles from WT but not *C1galt1*$^{-/-}$ neutrophils (Fig. 4*B*). Anti–PSGL-1 F(ab′)2 did not generate microparticles from PSGL-1–deficient neutrophils, confirming its specificity (Fig. 4*B*). Furthermore, anti–PSGL-1 F(ab′)2 did not generate microparticles from SFK-deficient neutrophils (Fig. 4*B*). These data indicate that PSGL-1 requires O-glycan–dependent association with lipid rafts to generate microparticles through an SFK-dependent signaling pathway.

PSGL-1, CD43, and CD44 Do Not 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, α-cyclodextrin, blocked polarization, confirming previous studies (21) (Fig. 5*A*). Unexpectedly, PSGL-1, CD44, and CD43 also redistributed to the uropods of *C1galt1*$^{-/-}$ neutrophils (Fig. 5*B*). However, CXCL1 did not alter the density distribution of raft and nonraft proteins in detergent extracts from WT or *C1galt1*$^{-/-}$ neutrophils (Fig. 5*C*). 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.

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 PIRLα 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 *C1galt1*$^{-/-}$ 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 that 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

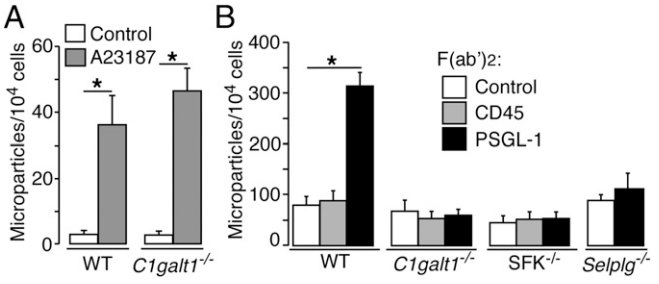

Fig. 4. PSGL-1 requires core 1-derived O-glycans to trigger SFK-dependent generation of microparticles. (*A*) Fluorescent WT or *C1galt1*$^{-/-}$ neutrophils were incubated with vehicle control or with the Ca^{2+} ionophore A23187. The number of microparticles generated was measured by flow cytometry. (*B*) Fluorescent neutrophils of the indicated genotype were incubated with F(ab′)$_2$ fragments of isotype control, anti-CD45, or anti–PSGL-1 mAb. The number of microparticles generated was measured by flow cytometry. The data represent the mean ± SEM of five experiments. *$P < 0.01$.

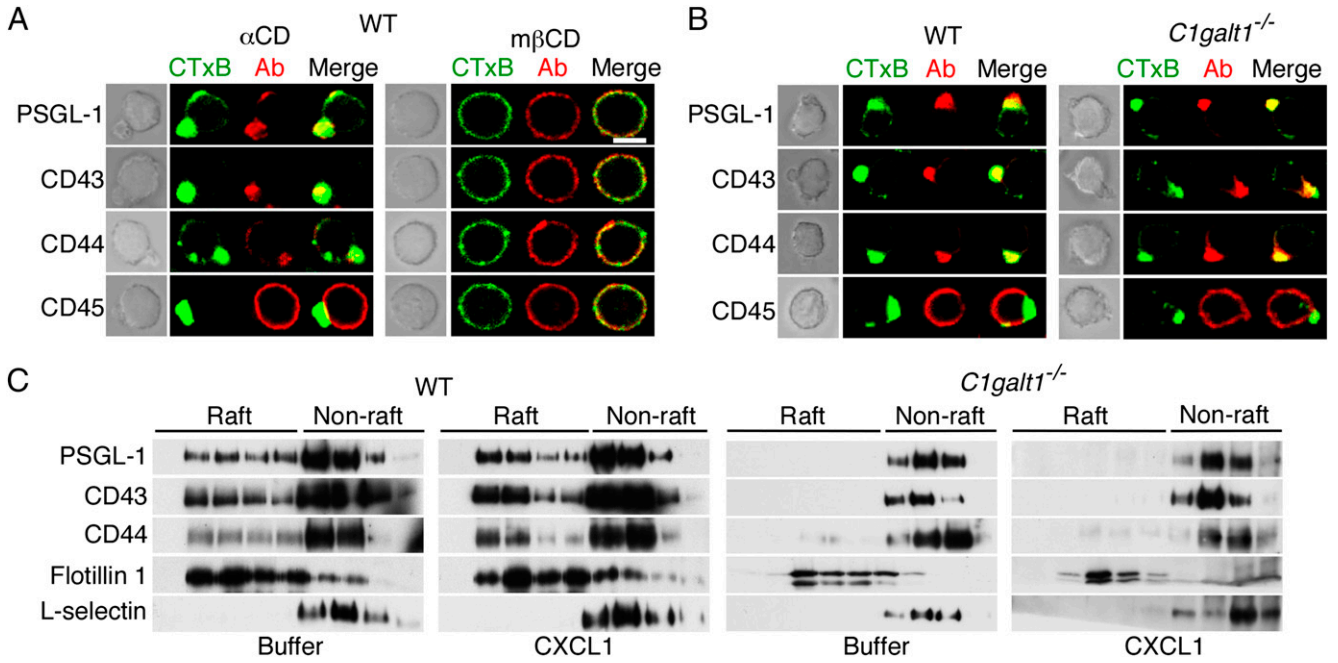

Fig. 5. PSGL-1, CD43, and CD44 do not require core 1-derived O-glycans to redistribute to the uropods of polarized neutrophils. (*A*) WT neutrophils pre-incubated with methyl-β-cyclodextrin (mβCD) or control α-cyclodextrin (αCD) were stimulated with the chemokine CXCL1. After fixation, the cells were labeled with CTxB (green) or antibodies to the indicated protein (red). Representative cells were visualized by phase-contrast microscopy (*Left*) or confocal microscopy (*Right*). (*B*) WT or *C1galt1*$^{-/-}$ neutrophils were stimulated with CXCL1, fixed, labeled, and visualized as in *A*. (*C*) WT or *C1galt1*$^{-/-}$ neutrophils were incubated with buffer or CXCL1. The cells were lysed, fractionated on OptiPrep gradients, and analyzed by Western blotting with antibodies to the indicated protein as in Fig. 1. Results are representative of at least three experiments. (Scale bar, 5 μm.)

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 was 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 *C1galt1*$^{-/-}$ 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 with flotillins 1 and 2 and with the actin cytoskeleton (54, 55), in part through binding of ERM adaptors to the cytoplasmic domains of membrane glycoproteins (56). PSGL-1 associates with flotillins as measured by coimmunoprecipitation in detergent extracts and by a proximity-ligation assay in intact cells (31, 57). However, direct binding of PSGL-1 to flotillins has not been demonstrated. Direct interactions, if they occur, may have low affinity, because flotillins dissociated from PSGL-1, CD43, and CD44 in gradients of *C1galt1*$^{-/-}$ neutrophil extracts. On intact *C1galt1*$^{-/-}$ neutrophils, however, low-affinity interactions with flotillins 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 ERM proteins that link to the cytoskeleton. Because of clustered, high-avidity interactions, uropods might form even if only some cytoplasmic domains bind directly to ERM proteins. 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 sialylated O-glycans to associate with lipid rafts on hematopoietic cells. Thus, O-glycosylation may influence how membrane domains regulate diverse functions during hematopoiesis, 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, neutrophil microparticle preparation, and statistical analysis are given in *SI Materials and Methods*.

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