Selectin catch-bonds mechanotransduce integrin activation and neutrophil arrest on inflamed endothelium under shear flow

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**Key Points**

- PMN rolling on E-selectin form catch-bonds with L-selectin that mechanosignal high affinity β2-integrin bond formation with ICAM-1.
- Rivipansel blocks E-selectin recognition of sLe\(^x\) on L-selectin, thereby antagonizing outside-in signaling of high-affinity β2-integrin.
Abstract

E-selectin extends from the plasma membrane of inflamed endothelium and serves to capture leukocytes from flowing blood via long-lived catch-bonds that support slow leukocyte rolling under shear stress. Its ligands are glycosylated with the tetrasaccharide sialyl Lewis\(^{x}\) (sLe\(^{x}\)), which contributes to bond affinity and specificity. E-selectin mediated rolling transmits signals into neutrophils that triggers activation of high-affinity \(\beta_2\)-integrins necessary for transition to shear resistant adhesion and transendothelial migration. Rivipansel is a glycomimetic drug that inhibits E-selectin–mediated vaso-occlusion induced by integrin dependent sickle-RBC-leukocyte adhesion. How Rivipansel antagonizes ligand recognition by E-selectin and blocks outside-in signaling of integrin mediated neutrophil arrest, while maintaining rolling immune-surveillance is unknown. Here, we demonstrate that sLe\(^{x}\) expressed on human L-selectin is preferentially bound by E-selectin and upon ligation initiates secretion of MRP8/14 that binds TLR4 to elicit the extension of \(\beta_2\)-integrin to an intermediate affinity state. Neutrophil rolling over E-selectin at precise shear stress transmits tension and catch-bond formation with L-selectin via sLe\(^{x}\) resulting in focal clusters that deliver a distinct signal to upshift \(\beta_2\)-integrins to a high-affinity state. Rivipansel effectively blocked formation of selectin catch-bonds revealing a novel mechanotransduction circuit that rapidly converts extended \(\beta_2\)-integrins to high-affinity shear resistant bond clusters with ICAM-1 on inflamed endothelium.
Introduction

Selectins are C-type lectin glycoproteins that initiate leukocyte recruitment at sites of inflammation. Each selectin contains a lectin-EGF domain that is conserved across mammals that recognizes tetrasaccharide sialyl Lewis$^x$ (sLe$^x$) expressed on glycoprotein and glycolipid ligands including P-selectin glycoprotein ligand-1 (PSGL-1), CD44, and ESL-1 on mouse polymorphonuclear (PMN) leukocytes. Human E-selectin recognizes PSGL-1, L-selectin, and sialylated glycosphingolipids. E-selectin binding to its ligands on PMN supports slow rolling that facilitates interrogation of the vasculature during inflammation. E-selectin ligation of L-selectin and PSGL-1 receptors induces their redistribution into membrane clusters that elicits release of cytosolic calcium and activation of Src-family tyrosine kinases. This process, denoted outside-in signaling, activates an upshift in $\beta_2$-integrin binding affinity, which upon bond formation with endothelial intracellular adhesion molecules (ICAMs) leads to PMN arrest on inflamed endothelium. L-selectin expressed on murine PMN is not recognized by E-selectin, they lack the fucosyltransferase (FUT9) to link fucose onto sLe$^x$ presenting O- and N-linked amino acids on L-selectin. In mouse, the mechanism for outside-in signaling during PMN rolling on inflamed venules is attributed to E-selectin engagement of PSGL-1, which associates with L-selectin at the tips of microvilli. It is important to distinguish the mechanism of ESL recognition and receptor clustering between mouse and human, as this may account for the species difference in mechanosignaling via selectins.

Following colocalization of PSGL-1/L-selectin by E-selectin, a signaling complex forms by interaction with Src-family tyrosine kinases on the cytosolic side of membrane rafts. This in turn phosphorylates immunoreceptor tyrosine-based activation motif (ITAM) leading to extension of the $\beta_2$-integrin LFA-1 (CD11a/CD18) into an intermediate conformation that supports slow rolling on ICAM-1. Subsequent transition of LFA-1 to a high-affinity state that supports PMN arrest on inflamed mouse venules requires chemokine signaling through GPCR ligation. Unlike mouse PMN, human PMN can transition LFA-1 to a high-affinity state via rolling over E-selectin in the absence of GPCR signaling. Human PMN have evolved a means of decorating N-glycans with sLe$^x$ on L-selectin that facilitates clustering and its capacity to signal the conversion of $\beta_2$-integrins to high-affinity. Prenster et. al. demonstrated a distinct pathway for activating $\beta_2$-integrin. They showed mouse PMN rolling on E-selectin results in secretion of myeloid-related protein (MRP)8/14 that signals RAP1-GTPase activation of $\beta_2$-integrin via ligation of toll like receptor-4 (TLR4). However, the events associated with E-selectin mediated outside-in signaling of a shift in $\beta_2$-integrin conformation from intermediate to high-affinity have not been established. We address how E-selectin recognition of PSGL-1 and L-selectin under tensile force leads to distinct bond mechanics which support mechanotransduction of signals that activate $\beta_2$-integrin.

The species difference between human and mouse ligand recognition by E-selectin has significant ramifications for efficient targeting of glycomimetics to intervene in inflammatory PMN recruitment. Rivipansel is a selectin antagonist in clinical trials for the treatment of vascular occlusive crisis (VOC) associated with
sickle cell disease.\textsuperscript{25} It mimics the \textit{sLeX} tetrasaccharide structure and contains an extended sulfate domain that is recognized by all three selectins.\textsuperscript{26-28} GSnP-6 is another pan-selectin glycomimetic that structurally resembles the N-terminal domain of PSGL-1 and binds with highest affinity to P-selectin.\textsuperscript{29} Given that Rivipansel functions as an \textit{sLeX} mimetic and GSnP-6 as a PSGL-1 mimetic, we employed them to discriminate the relative importance of E-selectin recognition of \textit{sLeX} on L-selectin versus PSGL-1 during PMN recruitment.

To enable efficient recruitment E-selectin is capable of forming catch-bonds with its ligands on PMN. E-selectin in complex with \textit{sLeX} under force is hypothesized to adopt a high-affinity extended conformation during catch-bond formation, which prolongs bond lifetime and transfers force that pulls membrane tethers during PMN slow rolling.\textsuperscript{30-35} Above a threshold in shear force, catch-bonds transition to slip-bonds characterized by a shorter bond lifetime and higher rolling velocities under flow.\textsuperscript{36} We previously reported that E-selectin binding to \textit{sLeX} on L-selectin and PSGL-1 on a rolling PMN drives co-localization of these receptors on membrane tethers at the trailing edge, which is associated with activation of \(\beta_2\)-integrin mediated cell arrest.\textsuperscript{6,7} In this report, we employ real-time immunofluorescence TIRF imaging of PMN to examine the molecular dynamics of selectin-ligand receptor engagement during the transition from cell tethering and rolling to arrest on recombinant E-selectin and ICAM-1 in a microfluidic channel. We demonstrate that Rivipansel blocks E-selectin recognition of \textit{sLeX} on L-selectin and antagonizes formation of catch-bonds necessary for focal membrane clustering and outside-in signaling. These data highlight a species difference in \textit{sLeX} ligand recognition by E-selectin that accounts for its capacity to mechanotransduce via L-selectin the activation of high-affinity \(\beta_2\)-integrin dependent arrest in human and not mouse PMN.
Materials and Methods

Study Subjects

Rivipansel is a selectin antagonist, that mimics the sLe\(^x\) tetrasaccharide structure, currently in clinical trials for the treatment of VOC in SCD patients. Phase I/II clinical trials were conducted on adults with sickle cell disease.\(^{25,28}\) Rivipansel was intravenously infused (20mg/kg) followed by 10mg/kg dose 10 hours later. Blood samples were collected and PMN adhesion to recombinant E-selectin/ICAM-1 (E/I) substrates assembled on glass coverslips beneath PDMS microfluidic channels\(^{21}\) was measured.

Total Internal Reflection Fluorescent Microscopy (TIRF) and Quantitative Dynamic Footprinting (qDF)

Protein A/G coated coverslips were coated with E-selectin and/or ICAM-1 (1\(\mu\)g/mL) as previously described and assembled on microfluidic devices.\(^{21}\) TIRF and qDF\(^{37,38}\) were employed to record fluorescently tagged antibodies targeting L-selectin (DREG55) and PSGL-1 (PL2) cluster area and frequency during rolling of isolated PMN over E- or P-selectin in the presence or absence of glycomimetic inhibition. DREG55 did not alter L-selectin’s recognition by E-selectin or \(\beta_2\)-integrin activation (Supp. Fig. 2A,B).\(^{39}\) High-affinity CD18 clusters were quantified using TIRF on PMN that rolled to arrest in the presence of mAb24.

PMN Bead Collision Assay

Protein G coated beads (diameter=1\(\mu\)m) were derivitized with E-selectin-IgG as per manufacturer’s instructions. Beads were then treated as described by Edmondson et. al.\(^{40}\) PMN were treated with blocking antibodies to PSGL-1 (KPL-1) and Mac-1 (M1/70\(^{41}\)) prior to perfusion. Adhesive interactions were identified as collisions that showed a pause in PMN motion for one frame with velocities below the hydrodynamic velocity.

Additional information in supplementary data.
Results

Rivipansel inhibits neutrophil arrest and migration across inflamed endothelium

E-selectin mediates slow rolling of PMN and triggers integrin mediated deceleration as evidenced by blocking E-selectin or ICAM-1 with antibodies that abrogate transition to arrest.\textsuperscript{6,42-45} This prompted us to examine recruitment under shear flow in microfluidic channels (Supp. Fig. 1A) of PMN isolated from healthy subjects and from blood samples obtained from SCD patients participating in Phase II clinical trials.\textsuperscript{25,28} On IL-1β inflamed endothelium, PMN transitioned from rolling to arrest and within minutes a majority (~60%) adopted a polarized shape before transmigrating underneath the monolayer (Fig. 1A,B). We examined the dose dependent effect of Rivipansel on the multistep process of PMN recruitment on stimulated HUVEC. Rivipansel exhibited an IC\textsubscript{50}~26\textmu M for antagonizing the transition to arrest and a slightly lower IC\textsubscript{50}~17\textmu M in blocking transmigration of PMN obtained from healthy subjects (Fig. 1C, Supp. Fig. 1B). It is noteworthy that Rivipansel exerted a greater inhibitory effect on signaling the transition from rolling to arrest than on PMN capture and rolling (Fig. 1C). Blood samples from SCD patients were assessed \textit{ex-vivo} for serum levels of drug along with the capacity for PMN to roll to arrest over an E/I substrate (Fig. 1D, Table 1). PMN arrest efficiency decreased for all patients over 8 hours of Rivipansel infusion, before rising in a manner inversely correlated with drug serum levels (Fig. 1D, Supp. Table 1). We examined the effect of Rivipansel on healthy African American subjects whose blood was left untreated or doped \textit{in vitro} at concentrations measured in SCD patients. PMN arrest efficiency was equivalent between ethnic controls and patients, as was the efficacy of inhibition for rolling to arrest (Supp. Fig 1C). These data demonstrate that PMN recruitment from blood is initiated by stable adhesion signaled through E-selectin and inhibited in a dose dependent manner by Rivipansel.

E-selectin binds to sLe\textsuperscript{x} on L-selectin during transition from rolling to arrest on ICAM-1.

In mouse, P-selectin and E-selectin upregulated on inflamed endothelium bind to PSGL-1 as the predominant sLe\textsuperscript{x} expressing ligand on PMN, and extension of β\textsubscript{2}-integrin activation is mediated through its co-localization with L-selectin.\textsuperscript{12,23,46,47} To determine the relative contributions of known human ESLs in PMN binding and activation via E-selectin, assays were performed in the presence of antibodies to CD44, PSGL-1, and L-selectin. Anti-PSGL-1 resulted in a ~40% decrease compared to an IgG control antibody, while anti-L-selectin elicited a ~25% decrease in E-selectin binding (Supp. Fig. 2A). Anti-CD44 resulted in a ~20% decrease in E-selectin binding, and no additional inhibition was observed in conjunction with anti-L-selectin and/or anti-PSGL-1. To examine the role of these ESLs in outside-in signaling of β\textsubscript{2}-integrin activation, E-selectin-IgG was bound to PMN and crosslinked with the same blocking antibodies (Supp. Fig 2B). Only anti-L-selectin showed a significant decrease high-affinity CD18 activation. This indicates that CD44 plays a marginal role in E-selectin binding and activation of PMN. We next assessed the relative contributions of L-selectin and PSGL-1 to recruitment of human PMN under shear flow over E/I or P/I substrates (Fig. 2A-B). Blocking L-selectin abrogated the transition to arrest on E-selectin, similar to the absence of ICAM-1, or blocking β\textsubscript{2}-
integrin function with antibody\textsuperscript{42,44} (Supp. Fig. 2C). Blocking PSGL-1 did not diminish arrest efficiency, but in conjunction with anti-L-selectin abrogated rolling on E-selectin (Fig. 2A). Shearing PMN on a substrate of P-selectin revealed a similar frequency of cell capture and rolling, but arrest efficiency was at baseline (Fig. 2B, Supp. Fig. 2D). We conclude that PSGL-1 functions as an efficient ligand for PMN capture and rolling, but does not activate the transition to arrest under shear stress on P- or E-selectin. To directly compare the capacity of PMN isolated from mouse versus human blood to roll and activate on E-selectin, a competitive recruitment assay over human or mouse E/l was performed between fluorescent (LysM-EGFP) murine PMN and Dil labeled human PMN (Supp. Fig. 2E-F). PMN from either species captured and rolled with equivalent efficiency on E-selectin, however, arrest efficiency was markedly higher for human PMN rolling on human E-selectin than on mouse (~48% on hE/hI, ~33% on mE/hI). These data indicate that E-selectin recognition of L-selectin on human PMN is necessary and sufficient to activate arrest via β\textsubscript{2}-integrin-ICAM-1 bonds.

To examine the importance of sLex for selectin ligand recognition, PMN were lysed and immunoprecipitated with human E-selectin or P-selectin conjugated beads. Rivipansel abrogated pull down of L-selectin by E- or P-selectin to baseline (Fig. 2C-H), and reduced by ~50% binding of PSGL-1. GSnP-6 which mimics the N-terminal domain of PSGL-1, abrogated pull down of PSGL-1 by either E- or P-selectin. Unexpectedly, GSnP-6 promoted a significant increase in L-selectin pull down by E- and P-selectin perhaps through antagonizing the interaction between L-selectin and PSGL-1. These data indicate that E- and P-selectin binding to L-selectin occurs predominantly via recognition of sLex that is effectively blocked by Rivipansel. PSGL-1 binding is partially inhibited by Rivipansel, but pull down is abrogated by GSnP-6 that efficiently antagonizes binding of sLex along with the sulfotyrosine-amino acid motif important for its recognition.\textsuperscript{48}

\textit{sLex glycomimetics alter E-selectin bond mechanics}

Slow rolling of PMN on E-selectin involves catch-bond formation with ESLs.\textsuperscript{30,49,50} The efficiency of tether formation and the mechanical properties that dictate the duration to bond rupture is unique for each ESL.\textsuperscript{36,50-52} To assess the influence of glycomimetic dose on the inhibition of PMN rolling and transition to arrest, PMN were perfused over a substrate of E/l. GSnP-6 decreased the frequency of PMN capture on E-selectin, but did not reduce the transition to arrest (Fig. 3A). Rivipansel inhibited arrest by ~60% at a dose of ~6.5μM, which matched its IC\textsubscript{50} in blocking activation of high-affinity β\textsubscript{2}-integrin signaled through crosslinking bound E-selectin-IgG (Supp. Fig. 3). We next examined the influence of sLex recognition on rolling velocity over an E-selectin substrate. PMN rolled at a mean velocity of 3.0μm/sec and variance of 2.83μm\textsuperscript{2}/sec\textsuperscript{2} (Fig. 3B). Treatment with Rivipansel or GSnP-6 elevated mean rolling velocity by 33% and 75%, respectively, and also increased the variance (4.57 and 7.20μm\textsuperscript{2}/sec\textsuperscript{2}, respectively). These data are consistent with glycomimetic disruption of sLex recognition decreasing the number and/or strength of E-selectin bonds and that PSGL-1 predominately mediates rolling velocity, while L-selectin is necessary to activate β\textsubscript{2}-integrin arrest on ICAM-1.
To further assess sLex recognition on the strength and duration of selectin bonds, a PMN-bead collision assay was performed under defined shear stress. E-selectin-IgG coated beads were annealed to the substrate of microfluidic channels and served as targets to capture PMN from the flow field to measure durable membrane tethers and survival until bond rupture (Fig. 3C, Supp. Fig. 4). PMN treated with anti-L-selectin and/or anti-PSGL-1 revealed that either ligand is capable of forming catch-bonds, and in combination abrogated tether formation (Supp. Fig. 4). PMN suspensions were blocked with anti-PSGL-1 to isolate tethering via L-selectin and glycomimetics were infused (Fig. 3D). Tether duration increased with tether force to a threshold of 26pN with vehicle and 17pN, with GSNP-6, before decreasing at higher shear stress (Fig. 3D). Increased tether duration was attributed to E-selectin/L-selectin catch-bond formation, since it was not altered by GSNP-6. In the presence of Rivipansel catch-bond behavior via L-selectin was abrogated as evidenced by decreased tether duration with increased force (Fig. 3D). Catch-bond behavior was also evident in vehicle control and GSNP-6 samples where tether efficiency increased from 20% to 65% up to a shear threshold of 0.75 and 0.50 dynes/cm², respectively (Fig. 3E). Rivipansel treatment abrogated the rise in tether efficiency, which dropped with increased shear stress from a maximum of 20% at 0.25 dynes/cm². We conclude that E-selectin recognition of sLex on L-selectin is necessary for catch-bond formation that promotes PMN capture and slow rolling over a characteristic range of shear stress mediated tensile bond force.

**Rivipansel inhibits L-selectin clustering and mechanosignaling of high-affinity β₂-integrin**

E-selectin recognition of sLex on L-selectin and PSGL-1 elicits receptor co-clustering on rolling PMN, but their relative roles in signaling arrest is unresolved. We employed TIRF imaging to quantify their redistribution during rolling to arrest on E/I substrates. Clusters of ESL assembled within seconds of PMN capture and rolling on E-selectin (Fig. 4A, Supp.Vid.1). Treatment with Rivipansel antagonized the buildup in area of receptor clusters by ~60% compared to vehicle or GSNP-6, and the frequency of contacts was lowered (Fig. 4B, Supp.Vid.2). GSNP-6 effectively antagonized ~70% of the formation of PSGL-1 receptor clusters, but had no effect on L-selectin cluster assembly (Fig. 4C). High-affinity β₂-integrin cluster and bond formation on ICAM-1 was imaged using TIRF as PMN transitioned to arrest (Fig. 4D). Within 10 seconds of arrest, high-affinity β₂-integrin clusters increased in frequency and area. Rivipansel inhibited the area increase by ~50% and the frequency of focal adhesions by ~38% compared to vehicle control or GSNP-6 treatment (Fig. 4E). We conclude that mechanosignaling via sLex dependent clustering of L-selectin rapidly led to the activation and assembly of high-affinity β₂-integrin focal adhesions on ICAM-1.

**E-selectin engagement with sLex on clustered L-selectin elicits phosphorylation of Lck**

Lck is a Src family protein tyrosine kinase that interacts with the cytoplasmic tail of L-selectin during outside-in signaling following ligation and clustering by E-selectin or sulfatides. We examined Lck activation by quantifying the fraction of phosphorylated to total immunoprecipitated Lck following crosslinking of E- or P-selectin-IgG bound to PMN. Addition of E- or P-selectin-IgG alone did not elicit significant Lck phosphorylation (Fig. 5A). An 8-fold increase in phosphor-Lck was detected in the presence of crosslinked E-
selectin-IgG, but not P-selectin-IgG. Rivipansel inhibited activation by ~70%, while GSNP-6 registered no effect (Fig. 5B). We conclude that E-selectin recognition of sLe\(^x\) presented on L-selectin requires not only ligation but membrane clustering to phosphorylate Lck via a process that is independent of PSGL-1 recognition.

**E-selectin ligation of L-selectin elicits release of MRP8/14, whereas clustering induces high-affinity \(\beta_2\)-integrin**

It was recently reported that murine PMN rolling on E-selectin elicits secretion of MRP8/14 via engagement of PSGL-1, which in turn binds TLR4 that signals the activation of \(\beta_2\)-integrin.\(^{24}\) We assessed whether E-selectin recognition of sLe\(^x\) on L-selectin elicits MRP8/14 release and subsequent activation of \(\beta_2\)-integrin on human PMN (Figure 6A). Addition of E-selectin to PMN in suspension was sufficient to elicit maximum extracellular release of MRP8/14, which was blocked in the presence of Rivipansel (Fig. 6B). We next employed \(\beta_2\)-integrin reporter antibodies to detect the extended and high-affinity conformations following E-selectin ligation and crosslinking.\(^{17,55}\) Ligation of E-selectin-IgG elicited the extension of ~20,000 KIM127 sites, representative of ~25% of total \(\beta_2\)-integrin receptors expressed on untreated PMN (Fig. 6C).\(^{56}\) Crosslinking E-selectin-IgG induced activation of an additional ~25,000 mAb24 bound high-affinity sites, while ~20,000 were maintained in the extended conformation. This level of high-affinity \(\beta_2\)-integrin induction equaled maximal activation via CXCR1/2 ligation by IL-8 (Fig. 6C). Rivipansel abrogated E-selectin mediated activation of high-affinity \(\beta_2\)-integrin and reduced by half the number of intermediate KIM127 sites (Supp. Fig. 3B). To examine the capacity of MRP8/14 to activate PMN in the absence of E-selectin ligation, we added recombinant MRP8 to PMN in suspension. This resulted in the extension of ~35,000 KIM127 sites, which was abrogated by pretreatment with anti-TLR4. MRP8 ligation did not activate high-affinity \(\beta_2\)-integrin, but did induce twice the number of intermediate affinity \(\beta_2\)-integrin as activated by ligation of E-selectin (Fig. 6D). To discriminate the signaling dynamics in conversion from intermediate to high-affinity \(\beta_2\)-integrin during rolling to arrest on E-selectin, PMN were sheared on a substrate of E-selectin with mAb24 or KIM127, while MRP8/14 signaling was blocked in the presence of anti-TLR4. Capture efficiency via E-selectin was equivalent on substrates of mAb24 or KIM127, consistent with the finding that it activates both extended and high affinity \(\beta_2\)-integrin (Supp. Fig. 5A). Direct activation of PMN in suspension via recombinant MRP8 before shearing across a substrate of E-selectin and CD18 reporter antibodies resulted in a ~4-fold higher arrest efficiency on KIM127 compared with mAb24, which was blocked with anti-TLR4 (Supp. Fig. 5B). These data indicate that ligation of E-selectin and/or the extracellular release of MRP8/14 and its ligation of TLR4 activate an upshift of \(\beta_2\)-integrin to an intermediate affinity state. A second distinct signal transduced through E-selectin clustering of L-selectin activates the conversion to high affinity \(\beta_2\)-integrin.
Discussion

Neutrophil surveillance of the microcirculation in most inflamed tissues involves rolling on E-selectin. An immune adaption that humans and not mouse have evolved is the capacity to transition to arrest via outside-in signaling of L-selectin, independent of GPCR mediated inside-out signaling. Here we show that recognition of sLe\(^x\) on L-selectin by E-selectin during PMN rolling initiates shear force resistant catch-bonds that facilitate tether formation and bond clustering. Rivipansel mimics the sLe\(^x\) moiety and targets the lectin domain on E-selectin, abrogating the formation of L-selectin catch-bonds required for receptor clustering and signaling of integrin activation. GSnP-6, a novel PSGL-1 mimetic that blocks its recognition by E-selectin or P-selectin elicited a doubling in PMN rolling velocity, but had no effect on L-selectin outside-in signaling of β\(_2\)-integrin. This differential glycomimetic inhibition of PMN rolling versus signaling reveals a novel mechanosignaling circuit in which E-selectin transmitted tension on L-selectin induces clustering at the adhesive interface that results in high-affinity β\(_2\)-integrin/ICAM-1 bonds. We report that the therapeutic efficacy of Rivipansel in treatment of VOC may involve antagonism of sLe\(^x\) recognition by E-selectin that selectively blocks catch-bond mediated L-selectin mechanosignaling, along with MRP8/14 release and TLR4 signaling of integrin activation.

While L-selectin plays a crucial role on mouse and human PMN for induction of integrin activation, a key difference is in the presentation of sLe\(^x\) to the lectin domain of E-selectin. Clues to the specific glycosylation of ESLs on human PMN come from studies using CRISPR-Cas9 disruption of glycan construction in HL-60 cells. O-glycans contribute to initial capture and rolling of PMN, and when knocked out result in reduced rolling frequency with no effect on transition to arrest. N-glycans are involved in both PMN recruitment and activation of arrest on E-selectin. L-selectin predominantly presents N-glycan linked sLe\(^x\), in contrast to PSGL-1 that is decorated with O-linked sLe\(^x\). GSnP-6 by design is a glycosulfopeptide that effectively blocks E-selectin binding to sLe\(^x\) expressed on PSGL-1, as well as its docking to the EGF domain. In contrast, Rivipansel occludes docking of sLe\(^x\) to the lectin domain independent of amino acid sequence of ESLs. This may explain its specificity in antagonism of L-selectin and dimeric PSGL-1 recognition and its minimal influence in increasing PMN rolling velocity. Antibody to L-selectin blocked signaling as effectively as Rivipansel suggesting that ancillary ESLs, such as glycosphingolipids and PSGL-1, play a role in L-selectin clustering within lipid raft domains enriched in signaling kinases. Binding via E-selectin is the critical event in transmission of mechanical force necessary for outside-in signaling.

Binding of L-selectin by its cognate ligands under shear force results in catch-bond behavior. Unlike human PMN, Mouse L-selectin is not glycosylated with sLe\(^x\) and consequently is not bound by E-selectin. Structural details of how shear resistant bonds form between E-selectin and L-selectin and the importance of sLe\(^x\) docking by its lectin domain provide a basis for catch-bond behavior. X-ray scattering measurements of E-selectin's lectin domain in conjunction with a molecular dynamics model predict that tensile force can induce a 10Å shift in receptor headpiece angle that promotes strong engagement with sLe\(^x\). Our data revealed efficient...
catch-bond formation within a narrow regime of shear between \( \sim 1-2 \) dynes/cm\(^2\), whereby tensile forces on the order of 25pN supported L-selectin bond clusters capable of mechanotransducing outside-in signaling.\(^{61}\) We observed \( \beta_2 \)-integrins convert from a low or intermediate to high affinity state during rolling, as measured by capture on a substrate co-expressing E-selectin-IgG and mAb24 under shear flow. A different result was recently reported by Kuwano et al., who observed that a majority of PMN sheared in microfluidic channels at 6 dynes/cm\(^2\) were not activated to arrest on ICAM-1 and were captured on a substrate of E-selectin and KIM127, but not on mAb24.\(^{17}\) They concluded that signaling via E-selectin clustered PSGL-1 activates only the intermediate and not high affinity conformation of \( \beta_2 \)-integrin. This apparent discrepancy may be explained by the fact that selectins display slip-bond behavior at shear stresses beyond 3 dynes/cm\(^2\), a regime in which tethers form less efficiently and dissociate more rapidly. This highlights a fundamental mechanism by which hydrodynamics regulates PMN adhesive signaling in order to optimize recruitment at appropriate shear stress, while minimizing arrest and vaso-occlusion at very low flow rates.

L-selectin clustering induces effector activities prior to its down-regulation by ectodomain shedding.\(^{52}\) It promotes phospho-Lck activation at the cytoplasmic tail that in turn activates the Ras pathway and an upshift in \( \beta_2 \)-integrin affinity.\(^{54,62}\) Rivipansel inhibited activation of phospho-Lck by antagonizing E-selectin catch-bond engagement necessary for L-selectin clustering and signaling. Our data supports a mechanism in which Rivipansel antagonizes E-selectin interaction with L-selectin more efficiently than with PSGL-1 on human PMN. Our finding of a 4-fold higher dose of Rivipansel required to abrogate PMN rolling to arrest than to block induction of high affinity \( \beta_2 \)-integrin, supports a two-step mechanism of outside-in signaling. Initial ESL ligation was sufficient for MRP8/14 release and of \( \beta_2 \)-integrin extension via TLR4. A distinct signal resulted from crosslinking and focal clustering of L-selectin during cell rolling, which triggered phospho-Lck and activation of high-affinity \( \beta_2 \)-integrin via Rap-1-GTPase. Thus, induction of high affinity CD18 is cooperative but independent of \( \beta_2 \)-integrin extension signaled via MRP8/14/TLR4. It is noteworthy that the superposition of selectin mediated outside-in signaling and ligation of CXCR to induce GPCR signaling can effectively amplify the number of high-affinity \( \beta_2 \)-integrin by \( \sim 100 \)-fold.\(^ {63}\) Thus, PMN recruitment involves temporal cooperativity between selectins and CXC receptors to regulate the number and affinity state of \( \beta_2 \)-integrin.

In summary, we reveal that ligation of L-selectin induces MRP8/14 and TLR4 signaling that primes \( \beta_2 \)-integrin in an extended conformation which supports slow rolling of human PMN. Shear resistant arrest requires signaling through clustered E-selectin/L-selectin bonds that result in Lck phosphorylation and the rapid activation of \( \beta_2 \)-integrin to a high-affinity state capable of shear resistant bond formation with ICAM-1. We demonstrate that catch-bond behavior via sLe\(^a\) recognition on L-selectin is critical for outside-in signaling by E-selectin, thereby revealing a novel aspect of inhibition with a new class of glycomimetic anti-inflammatories. Rivipansel antagonizes outside-in mechanosignaled PMN arrest while leaving intact rolling dependent immunosurveillance within the microcirculation.
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Authorship

V.A.M. performed research, analyzed data, wrote the manuscript, and designed the research. S.C. performed research and analyzed data. T.W. designed the research. E.L.C. contributed vital new reagents. J.L.M. contributed vital reagents and S.I.S. designed the research and edited each draft of the manuscript. All authors contributed to editing the manuscript.

J.L.M. has declared a financial interest in Glycomimetics Inc. whose drug GMI-1070 is presently under license and clinical trials with Pfizer. Although J.L.M. contributed reagents no additional funds were provided for these studies. Further, none of the other authors have declared financial interests.
Figure 1. PMN arrest and transmigration on inflamed endothelium is inhibited by GMI-1070. Isolated human PMNs were perfused over IL-1β stimulated HUVEC monolayers in a microfluidic flow chamber at physiological shear stress of 2 dynes/cm². (A) PMN rolling to arrest and transmigration across HUVEC monolayers are superimposed. PMN are phase bright until migration below endothelial monolayer, then become phase dark (marked by white arrows, scale bar: 10 μm). (B) PMN density, adherent on inflamed HUVEC measured after 2 minutes of shear flow. Arrest and TEM averaged from 5 fields data represents mean +/- SEM for 40 cells (n=3 separate exps). Significant differences are indicated for PMN arrest and TEM compared to stimulation and no shear condition (***, p<0.001; **, p<0.01; *, p<0.05). (C) PMN rolling at 3 min, arrest, and TEM density on HUVEC at 7 min in presence and absence of Rivipansel inhibition are superimposed. Data points represent mean +/- SEM from 5 fields per condition (n=4 separate exps). Rolling (*), arrest ($) and TEM (#) significance (at p < 0.01) compared with untreated reported. (D) PMN arrest efficiency (# PMN captured/count in blood) of whole blood sheared over recombinant E-selectin/ICAM-1 for four patients treated for sickle cell disease before (t=0) and post Rivipansel infusion (left y-axis) and avg. serum concentrations of Rivipansel post-infusion (right y-axis). Each data point is the mean +/- SEM of 5-11 fields of view along the centerline of the channel (***, p<0.001; **, p<0.005; *, p<0.01 denote significance from baseline at t=0).
Figure 2. E-selectin binds sLe\(^\alpha\) on L-selectin and inhibition with Rivipansel. Isolated human PMN were treated with IgG, anti-PSGL-1 antibody (KPL-1), anti-L-selectin (DREG56), the combination, or anti-β\(_2\)-integrin (IB4) antibody and perfused over (A) E/I or (B) P/I substrate in a microfluidic chamber at 2 dynes/cm\(^2\). PMN rolling (# p < 0.05, ## p < 0.001) and arrest ($ p < 0.01$) significance compared to IgG control, subtracting background adhesion. Values depicted are mean +/- SEM for n=3 separate exps. L-selectin and PSGL-1 were immunoprecipitated from isolated human PMN with recombinant E-selectin or P-selectin treated with vehicle, or in presence of Rivipansel (100 μM), or GSnP-6 (120 μM). Western blot protein content of: (C) E-selectin-IgG pulldown and quantitation of the ratio of (D) L-selectin (~69 kDa) or (E) PSGL-1 (~120 kDa + ~210 kDa) to untreated immunoprecipitation of total protein. (F) P-selectin pulldown of (G) L-selectin and (H) PSGL-1 relative to total protein pulldown. Relative density of L-selectin and PSGL-1, respectively was compared between mean +/- SEM as depicted (n=3 separate experiments; * p < 0.05; ** p <0.01; *** p < 0.001).
Figure 3. sLe$^x$ glycomimetics alter rolling to arrest on E-selectin and bond mechanics. (A) PMN rolling to arrest over E/I substrates (<0.4 μm/sec) treated with vehicle (PBS), Rivipansel, or GSnP-6 was measured and normalized to total number of interacting PMN, data is reported as mean +/- SEM (n=3 separate exps; * p < 0.05; *** p < 0.001 compared to vehicle) (B) PMN rolling velocity was quantified over an E-selectin substrate treated with Vehicle, Rivipansel (6.5 μM), or GSnP-6 (12 μM) and binned at intervals of 0.4 μm/sec. Comparison of GMI-1070 showed * significance and GSnP-6 showed *** significance over untreated controls (n=3 separate exps; * p < 0.05 and *** p < 0.001). (C) Schematic depicts dynamic interaction between PMN and recombinant E-selectin coated protein-G beads recorded at 50 fps in the flow channel. Isolated human PMN were treated with an anti-Mac-1 and anti-PSGL-1 blocking antibodies and then perfused through flow chambers. PMN pivot over beads and pull a membrane tether at defined wall shear stress. Adhesive interactions were identified as collisions that had a visible pause in PMN motion for at least one frame along with velocities below the hydrodynamic velocity. (D) Tether duration was compared to step-wise increases in calculated tether forces. (E) Tether efficiency (collisions resulting in adhesion divided by the total collisions observed) as shear stress was ramped in a stepwise manner. Data reported as mean +/- SEM (n=3 separate exps; **, p < 0.01; *, p < 0.05 glycomimetics compared to 9 pN tether force and 0.25 dynes/cm$^2$ wall shear stress).
Figure 4. Glycomimetic antagonism of selectin mediated β₂-integrin activation. (A) PMN rolling on a substrate of E-selectin in presence of vehicle control, Rivipansel (6.5 μM), or GSnP-6 (12 μM) was dynamically imaged using qDF to detect L-selectin (AF488 anti-human DREG55) and PSGL-1 (PE anti-human PL-1) engagement in the plane of adhesive contact. (B) L-selectin (FITC DREG-55) and (C) PSGL-1 (PE PL-2) receptor cluster area and frequency were determined and reported as mean +/- SEM (n=3 separate exps; ** p < 0.01 compared to vehicle). (D) PMN imaged by TIRF were pretreated with a membrane dye (DiI) and HA β₂-integrin reporter antibody (mAb24) and perfused over E/I substrates treated with vehicle, Rivipansel (6.5 μM), or GSnP-6 (12 μM). (E) HA β₂-integrin cluster number (MFI >10 pixels above background over ~.1 μm in area) in plane of contact were quantified using qDF in real time and compared between fixed cells that had rolled to arrest between the three conditions (n=3 separate exps; ** p < 0.01 compared to vehicle).
Figure 5. E-selectin engagement of sLeX on L-selectin requires crosslinking to induce phospho-Lck. (A) E-selectin or P-selectin crosslinked by addition of polyclonal secondary antibody were immunoprecipitated in absence and presence of glycomimetics. Western blot applied to detect Lck along with its tyrosine phosphorylated state. (B) Activation of phospho-Lck was normalized to total protein to quantify the increase following E-selectin or P-selectin crosslinking in presence of Rivipansel (6.5 μM) or GSnP-6 (12 μM). No significant increase quantified for crosslinked P-selectin. Data is presented as mean +/- SEM for crosslinked E-selectin was significant compared to E-selectin alone. (n=3 separate exps; ** p < 0.01; *** p < 0.001).
Figure 6. E-selectin crosslinking activates release of MRP8/14 and TLR4 signaled extension of β2-integrin with upshift to a high-affinity state. (A) Diagram of outside-in mechanosignaling of β2-integrin activation via engagement of E-selectin and subsequent clustering of L-selectin on human PMN. E-selectin binding to L-selectin induces extracellular release of MRP8/14 that then binds TLR4, which activates upshift from low affinity to an extended intermediate affinity state of β2-integrin. Further clustering of E-selectin bound L-selectin activates high-affinity β2-integrin. (B) MRP8/14 release by human PMN treated with E-selectin and crosslinked in absence or presence of Rivipansel (100 μM) or activation with IL-8 (10 nM). Release into supernatant measured by ELISA as mean +/- SEM (n=3 separate exps; **p < 0.01, ***p < 0.001 significance as indicated). (C) Affinity state of β2-integrin detected on PMN in suspension by flow cytometry. β2-integrin receptors bound by KIM127 (extended intermediate affinity) versus mAb24 (high-affinity) is shown for PMN treated with E-selectin with or without cross-linker in absence or presence of Rivipansel (100 μM), TLR4 blocking antibody, or IL-8 (10 nM) (n=3 separate exps; # denotes analysis of extended, * for high-affinity with p<0.01 compared to crosslinked E-selectin). (D) MRP8/14 (0.8 ng/mL) activation and extension of β2-integrin analyzed by flow cytometric detection of KIM127 and mAb24 receptor number in the absence and presence of TLR4 blocking antibody. Data shown as mean +/- SEM (n=3 separate exps; # p<0.05 compared to unstimulated control or TLR4 blocked samples).
References


Figure 3

A. PMN Adhesion (% Total Interaction)

- Treatment (μM): Rivipansel
- GSnP-6

B. PMN Rolling (%)

- Rolling Velocity (μm/sec)
  - Vehicle
  - Rivipansel
  - GSnP-6

C. Flow

- Tether
- Bead
- PMN

- Contact
- Lengthening
- Adhesion
- Detachment

D. Tether Duration (sec)

- Tether Force (pN)

- Vehicle
- Rivipansel
- GSnP-6

E. Tethering Efficiency (f)

- Wall Shear Stress (dynes/cm²)

- Vehicle
- Rivipansel
- GSnP-6
Figure 5

A. E-selectin

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B. % Activated Lck (pTyr Lck/Total Lck)

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Selectin catch-bonds mechanotransduce integrin activation and neutrophil arrest on inflamed endothelium under shear flow

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