

# E-Selectin Ligands as Mechanosensitive Receptors on Neutrophils in Health and Disease

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**Abstract**—Application of mechanical force to bonds between selectins and their ligands is a requirement for these adhesion receptors to optimally perform functions that include leukocyte tethering and activation of stable adhesion. Although all three selectins are reported to signal from the outside-in subsequent to ligand binding, E-selectin is unique in its capacity to bind multiple sialyl Lewis x presenting ligands and mediate slow rolling on the order of a micron per second. A diverse set of ligands are recognized by E-selectin in the mouse, including ESL-1, CD44 (HCELL), and PSGL-1 which are critical in transition from slow rolling to arrest and for efficient transendothelial migration. The molecular recognition process is different in humans as L-selectin is a major ligand, which along with glycolipids constitute more than half of the E-selectin receptors on human polymorphonuclear neutrophils (PMN). In addition, E-selectin is most efficient at raising the affinity and avidity of CD18 integrins that supports PMN deceleration and trafficking to sites of acute inflammation. The mechanism is only partially understood but known to involve a rise in cytosolic calcium and tyrosine phosphorylation that activates p38 MAP kinase and Syk kinase, both of which transduce signals from clustered E-selectin ligands. In this review we highlight the molecular recognition and mechanical requirements of this process to reveal how E-selectin confers selectivity and efficiency of signaling for extravasation at sites of inflammation and the mechanism of action of a new glycomimetic antagonist targeted to the lectin domain that has shown efficacy in blocking neutrophil activation and adhesion on inflamed endothelium.

**Keywords**—E-selectin, L-selectin, Neutrophils, PSGL-1, Cell signaling.

## INTRODUCTION

PMN roll slowly on E-selectin in inflamed mouse microvasculature, which is crucial for optimal immune

functions of cell arrest and transmigration to sites of injury. To support this function, E-selectin ligands (ESLs) on PMN transduce signals in response to tensile forces exerted on them during rolling under the shear forces of blood flow. What is unique about E-selectin, as compared to L-selectin and P-selectin, is the redundancy of ligands through which it tethers PMN and executes a slow rolling immunosurveillance at velocities on the order of one micron per second. Another remarkable feature is that PMN rolling on E-selectin and subsequent ESL clustering transduce signals that elicit calcium flux, activate kinases, and rapidly upshift affinity of  $\beta_2$ -integrins that supports PMN deceleration and arrest. These aspects of E-selectin function make it a fascinating receptor system to study in vascular mechanobiology and an intriguing receptor to target for remediation of inflammatory diseases. Sickle cell disease has emerged as one in which E-selectin plays a central role in PMN mediated vasculopathy that characterizes the painful episodes that individuals suffer. In this review we will focus on the biophysical mechanisms by which ESLs are bound and mechanotransduce signaling of integrin activation and how this process is being targeted for therapeutic advantage.

## E-SELECTIN LIGANDS RECOGNIZED ON PMN

All three selectins are transmembrane proteins of similar structure with three extracellular domains: an amino-terminal lectin domain, followed by an epidermal growth factor domain, and short consensus repeat units.<sup>8,36,43,74,82</sup> The glycobiochemist Ajit Varki proposed a litmus test for designating a glycosylated ESL with the predominant stipulation that they are located at the right place at the right time to provide adequate affinity or avidity to mediate specific biological recognition

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under physiological conditions.<sup>88</sup> Here, we revise it to focus on the PMN and add a functional criteria: (1) it is expressed on the PMN membrane at the proper time of myeloid differentiation; (2) its removal or blockade diminishes the capacity for PMN capture and rolling under shear stress; and (3) its binding selectivity and affinity are consistent with ligand clustering and signal transduction that elicits calcium release and the onset of integrin activation.<sup>57</sup> Common strategies to assess these criteria are to remove specific ligands on PMN, or genetically modify or remove ESLs in mouse models and then measure the alteration in capacity of E-selectin to bind to the surface and support subsequent recruitment. Such studies have demonstrated that the C-type lectin domain of E-, P-, and L-selectin all interact in a calcium-dependent manner with the  $\alpha 2,3$  sialylated and  $\alpha 1,3$  or  $\alpha 1,4$  fucosylated tetrasaccharide sialyl Lewis x (sLe<sup>x</sup>), which decorate the terminal branches of N- and O-glycans of cell-surface glycoconjugates.<sup>21,26,33,49,61,64,83,91,97</sup> E-selectin is the most permissive in recognition of a wide variety of fucosylated and sialylated glycolipids and glycoproteins that can function as high affinity ligands. In contrast, P- and L-selectin preferentially recognize sugars that are expressed with sulfated tyrosine residues on ligands.<sup>5,9,34,46,48,66,70</sup> On PMN, E-selectin engages P-selectin glycoprotein ligand (PSGL)-1,<sup>54,55,58,78</sup> L-selectin (on human and not mouse),<sup>63,99</sup> ESL-1 (on mouse and not human),<sup>46,47,79,80</sup> CD44 (HCELL),<sup>16,38</sup> and  $\alpha M\beta 2$  integrin (CD11b/CD18, Mac-1).<sup>13,41,71,96</sup> In addition, E-selectin binds CD43 on lymphocytes, but it remains controversial whether it is recognized on PMN.<sup>11,24,51,52,93</sup> These major ESLs are briefly reviewed.

Human L-selectin is an 80–100 kDa glycoprotein that when glycosylated properly is bound by E-selectin<sup>37,40,63,99</sup> and P-selectin.<sup>63</sup> L-selectin is expressed on the surface of almost all circulating leukocytes, but only that on human PMN and not lymphocytes is specifically bound by E-selectin.<sup>63</sup> Since the lectin binding domain from either mouse or human E-selectin can recognize human L-selectin, it appears to be the glycosylation pattern that confers this specificity.<sup>63,99</sup> L-selectin on human PMN is decorated with sLe<sup>x</sup> that supports interaction with E-selectin,<sup>63</sup> but most lymphocyte L-selectin is not recognized by sLe<sup>x</sup>-reactive mAbs.<sup>63</sup> E-selectin binding L-selectin requires sialic acid but is not dependent on N-glycans and this may explain the inability of most lymphocytes to tether and roll.<sup>99</sup> Treating human PMN with anti-L-selectin mAbs partially inhibits the adhesion of the PMN to E-selectin in shear flow assays.<sup>2,40,44,56,63,76,99</sup> Thus, L-selectin supports sLe<sup>x</sup> dependent slow rolling of human PMN on E-selectin substrates.<sup>28,99</sup> L-selectin on both human and mouse PMN can bind to PSGL-1

on an adjacent neutrophil to support homotypic adhesion and this process enables a PMN rolling on an inflamed vessel wall to capture a PMN flowing in the free stream.<sup>20,81</sup> This prompted us to examine the respective efficiencies for PMN homotypic adhesion vs. capture *via* E-selectin expressed on a cell line (300.19 cells).<sup>53</sup> At comparable selectin site densities, PMN capture by 300.19 cells expressing E-selectin was fourfold more efficient than L-selectin. In contrast, L-selectin was observed to support more robust PMN capture over a greater range of shear rate and shear stress than does E-selectin. These studies revealed that this higher capture efficiency of L-selectin was not mediated by a longer bond lifetime during cell collision since doublet formation *via* E-selectin formed bonds that lasted twice as long. Considering the primary role of E-selectin in PMN rolling and L-selectin in secondary capture of PMN from the blood stream, the long lifetime of E-selectin bonds and the rapid bond formation and shear resistance of L-selectin appear to be well-adapted to these distinct functions in PMN recruitment.

PSGL-1 is a disulfide-linked mucin-like homodimeric glycoprotein consisting of two 120 kDa transmembrane subunits with amino-terminal binding regions that protrude above most of the cell surface glycocalyx.<sup>9,58</sup> It is expressed on all leukocytes and is bound by all three selectins.<sup>5,9,27,46,55,69,78</sup> The amino acid sequences of the human and murine transmembrane and cytoplasmic domains are highly conserved, but the extracellular domains share little sequence similarity.<sup>10,94</sup> PSGL-1 has few N-glycans and many more O-glycans distributed into clusters along the extracellular domain.<sup>58</sup> E-selectin binding to PSGL-1 requires sialylated and fucosylated core-2 O-glycans.<sup>9</sup> Unlike P-selectin, which can only bind the amino-terminal region of PSGL-1, E-selectin binds to the amino-terminal region<sup>27,55</sup> and one or more additional sites on PSGL-1.<sup>27,56</sup> E- and P-selectin have essentially normal binding to PSGL-1 expressed by PMN from mice deficient in fucosyltransferase (Fuc-T) IV, but PSGL-1 expressed on Fuc-TVII-deficient PMN is not bound by E- or P-selectin.<sup>32</sup> E-selectin-mediated rolling *via* PSGL-1 is mostly dependent on core-2 O-glycans since sLe<sup>x</sup> is shown to mediate more than 75% of rolling.<sup>32</sup> However, recent studies also suggest that PMN use both core 2 and extended core 1 O-glycans for capture and rolling, which extend the variety of O-glycan modified proteins that function as essential ESLs.<sup>93</sup>

The CD44 protein family is a polymorphic group of transmembrane glycoproteins (80–200 kDa) present on most vertebrate cells.<sup>65</sup> Some of this heterogeneity stems from cell-specific posttranslational modifications,<sup>65</sup> which are required for endowing E-selectin

binding ability, as not all of these CD44 expressing cell types bind E-selectin.<sup>38</sup> The sialofucosylated glycoform of CD44 known as hematopoietic cell E-/L-selectin ligand (HCELL) originally described on human hematopoietic stem cells is the most potent E-selectin and L-selectin ligand expressed on human cells.<sup>35,68</sup> CD44 from peripheral blood PMN of both mice and humans is bound by E-selectin if properly glycosylated (HCELL).<sup>29,38</sup> There is no binding to CD44 from PMN of mice deficient in  $\alpha$ 1,3 fucosyltransferase IV and VII (Fuc-TIV/VII<sup>-/-</sup>).<sup>38</sup> CD44 (HCELL) is decorated with both N- and O-linked glycans,<sup>65</sup> but E-selectin binding is mediated only by the N-glycans.<sup>38,46,93</sup> Unlike PSGL-1, L-selectin, and ESL-1, which are localized to the microvilli on the neutrophil surface, CD44 is expressed on the cell body.<sup>63,90</sup>

### FUNCTION OF E-SELECTIN LIGANDS

The membrane organization of ESLs, typically on the tips of microvilli, is critical for their dynamic recognition under shear flow. For instance, disruption of lipid rafts abrogates PMN rolling on E-selectin without altering membrane expression levels of PSGL-1, L-selectin, or sLe<sup>x</sup>.<sup>1</sup> An antibody against the epitope in the amino-terminal region where PSGL-1 is bound by P-selectin significantly inhibits attachment of human PMN to E-selectin expressing Chinese hamster ovary (CHO) cells under shear flow, but has no effect on adhesion under static conditions.<sup>56</sup> The absence of PSGL-1 alone does not increase leukocyte rolling velocity on E-selectin *in vitro* or under inflammatory conditions *in vivo*,<sup>29,78</sup> but when both PSGL-1 and ESL-1 are absent rolling velocity is increased further than the absence of ESL-1 alone.<sup>29</sup> PSGL-1 is sufficient to observe slow rolling of murine and human PMN on E-selectin and ICAM-1 co-immobilized in a shear flow chamber.<sup>42,95</sup> Likewise, PSGL-1, supports slow rolling of PMN on L-cells expressing E-selectin and ICAM-1, which is significantly increased at a low shear stress of 0.1 dyn/cm<sup>2</sup>, but not at 2 dyn/cm<sup>2</sup>.<sup>76</sup> In contrast, PSGL-1 does not significantly contribute to the arrest of leukocytes that are already rolling in TNF- $\alpha$  stimulated venules *in vivo*.<sup>29</sup> PSGL-1 is also partly responsible for the capacity of murine PMN to extravasate into thioglycollate-induced peritonitis.<sup>29,95</sup> These functions are related to PSGL-1's capacity to transduce signals, since engagement was shown to increase tyrosine phosphorylation of its cytoplasmic domain and activate MAP kinases in human PMN.<sup>31</sup> The cytoplasmic domain of PSGL-1 is linked to the cytoskeleton by ERM (ezrin-moesin-radixin) family proteins,<sup>4</sup> which mediate the association of PSGL-1 with spleen tyrosine kinase (Syk) in an

ITAM-dependent manner.<sup>87</sup> Engagement of PSGL-1 induces tyrosine phosphorylation of Syk in the human myeloid cell line U937.<sup>87</sup> Similarly, E-selectin-induced LFA-1-mediated slow rolling of both murine<sup>95</sup> and human PMN<sup>42</sup> is PSGL-1 and Syk-dependent. Following activation-induced shape change and polarization of human PMN, PSGL-1 becomes clustered at the uropod and co-localizes with moesin on stimulated human PMN.<sup>4</sup> This process is associated with weakened adhesion to P-selectin that may facilitate integrin control of adhesion and transendothelial migration.<sup>10,17,28,48</sup> L-selectin is also localized in lipid rafts<sup>1,62</sup> and clustered on the tips of microvilli or membrane ruffles.<sup>9,19,44,63</sup> The epitope of L-selectin that mediates PMN tethering to E-selectin under flow conditions is in the amino-terminal lectin domain and not the short consensus repeats.<sup>44</sup> Thus, the accessibility of ESLs is likely critical considering that capture *via* E-selectin on inflamed endothelium at high shear rates ( $\sim 100 \text{ s}^{-1}$ ) must occur within the duration of collision, perhaps as brief as  $\sim 10 \text{ ms}$ .

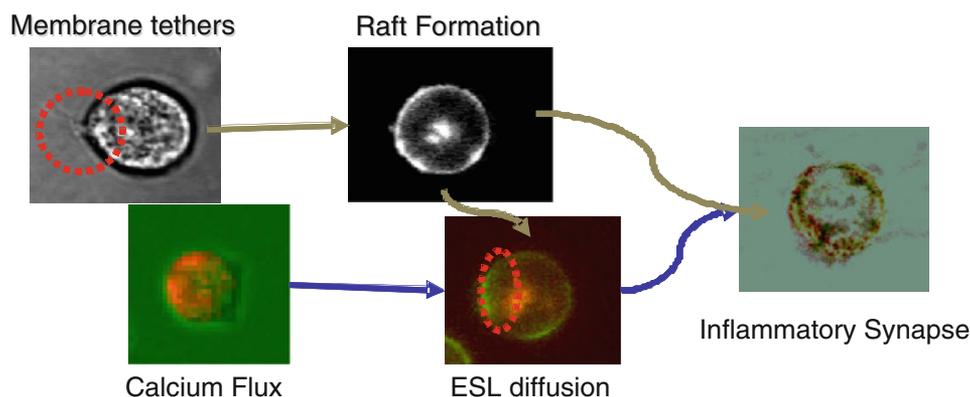
Unlike PSGL-1 and L-selectin, which can be bound by both P- and E-selectin,<sup>5,63</sup> ESL-1 is not bound by P-selectin and is specific to E-selectin.<sup>29,80,99</sup> Immobilized ESL-1, but not murine L-selectin, supports adhesion of murine E-selectin transfected CHO cells in a calcium dependent manner.<sup>80</sup> ESL-1 interacts with E-selectin through presentation of sLe<sup>x</sup> on some of its five N-glycans<sup>46,99</sup> rather than *via* O-glycans like PSGL-1 and L-selectin.<sup>9,99</sup> In contrast to PSGL-1 and L-selectin, which are expressed only on the microvilli, most ESL-1 is found in the Golgi apparatus and a relatively smaller fraction is expressed on the cell surface where it is localized on the microvilli.<sup>1,9,10,19,44,48,63,79</sup> Fucosylation of ESL-1 is essential for E-selectin binding.<sup>80</sup> CHO cells transfected with cDNA for ESL-1 alone or in combination with cDNA for human Fuc-TIII only become competent to bind E-selectin if Fuc-TIII is expressed in these cells.<sup>80</sup> This selective generation of ligand activity correlates with the upregulation of the HECA-452-reactive carbohydrate epitope, which is functionally correlated with ESL activity.<sup>7,16,23,98</sup> Although the absence of ESL-1 alone does not affect leukocyte rolling frequency, it is dramatically reduced when both ESL-1 and PSGL-1 are absent suggesting both are important in mediating the initial leukocyte tethering and rolling *in vivo*.<sup>29</sup> In fact, the ability of soluble E-selectin to bind murine PMN deficient in both ESL-1 and PSGL-1 is completely abrogated suggesting cooperation for high-affinity binding.<sup>29</sup> ESL-1 also cooperates with PSGL-1 and CD44 (HCELL) in controlling rolling velocities and is responsible for steady rolling; without ESL-1 murine leukocytes exhibit a "skipping" behavior.<sup>29</sup> ESL-1 is important in the transition from rolling to

firm arrest and the absence of ESL-1 shows a significant but moderate reduction in the ability of murine neutrophils to extravasate.<sup>29</sup> PSGL-1 is required for E-selectin mediated slow rolling in an autoperfused flow-chamber system,<sup>95</sup> however on E-selectin expressed on inflamed endothelium *in vivo*, CD44 (HCELL) controls rolling velocity to enable slow leukocyte rolling.<sup>29,38</sup> CD44 (HCELL) also plays a major role in the transition of rolling leukocytes to firm arrest *in vivo*<sup>29</sup> and cooperates with PSGL-1 in PMN extravasation into thioglycollate-induced peritonitis and staphylococcal enterotoxin A-injected skin pouch.<sup>38</sup> Less is known regarding the cooperative process by which L-selectin, PSGL-1 and other ESLs support rolling and transduce signals in human PMN, especially *in vivo*.

### TETHERING AND CLUSTERING OF ESLs SIGNAL UNDER SHEAR FLOW

E-selectin engagement under shear flow induces the redistribution and co-localization of PSGL-1 and L-selectin into clusters on the trailing edge of human PMN.<sup>28</sup> This has also been observed in mice treated with TNF- $\alpha$  where a large fraction of rolling leukocytes exhibit L-selectin polarization and co-localization with PSGL-1 in membrane clusters.<sup>29</sup> This E-selectin-dependent redistribution of L-selectin on rolling leukocytes *in vivo* appears to involve CD44 (HCELL), which upon engagement can itself trigger p38 MAPK dependent clustering of L-selectin and PSGL-1.<sup>29</sup> CD44 (HCELL) crosslinking also induces PSGL-1 polarization, which is completely abolished by preincubation of PMN with an inhibitor of p38 MAPK activation.<sup>29</sup> Unlike PSGL-1 and L-selectin,

which cluster on the majority of slow-rolling leukocytes, CD44 (HCELL) remains evenly distributed on rolling cells and in some instances is more densely distributed in areas of contact with the endothelium.<sup>29,47</sup> Our studies suggest that the clustering of ESLs increases the valence of E-selectin recognition and provides a means for slow rolling.<sup>28,72</sup> Clustering of ESLs also provides for amplification of outside-in signaling that contributes to various post-rolling innate immune functions including oxidative burst, degranulation, actin polymerization, and an upshift in CD18 affinity. Dynamic fluorescence video microscopy and total internal reflection fluorescence (TIRF) analysis of rolling and migrating PMN demonstrate that rolling on E-selectin elicits a striking focal co-localization and membrane clustering of L-selectin, PSGL-1, and ADAM-17 that progress to the trailing edge of arresting PMN, which is not apparent on cells activated in suspension (Fig. 1).<sup>72</sup> These receptors diffuse at distinct rates under a shear stress of 2 dyn/cm<sup>2</sup>, thus highlighting a well coordinated dynamic process involving raft formation, calcium transients, and cytoskeletal linkage.<sup>72</sup> Since it is known that PSGL-1 and moesin redistribute toward the cell uropod where activated ERM proteins also assemble, it is likely that the dynamics of the membrane determinants (e.g., lipid rafts) and signaling molecules (e.g., DAG, PLC, PIP<sub>2,3</sub>, PI<sub>3</sub>K, and MAPK) coordinate the precise redistribution of ESLs. Recently it was reported that L-selectin and PSGL-1 ligation by E-selectin activate PI<sub>3</sub>K that in turn catalyzes the phosphorylation of Vav1 and downstream F-actin redistribution.<sup>50</sup> These data suggest that PI<sub>3</sub>K is required for the F-actin-based cytoskeleton changes that occur during neutrophil rolling on E-selectin.

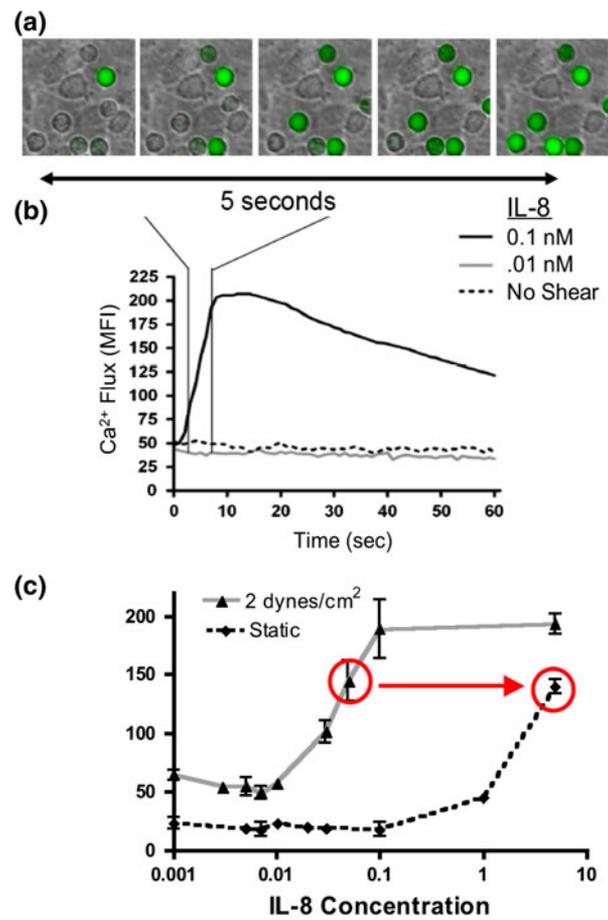


**FIGURE 1.** Dynamic imaging of adhesive and signaling events that support neutrophil recruitment and assembly of the inflammatory synapse. Membrane tethers during rolling on a substrate of E-selectin is observed by phase contrast microscopy as cells flux calcium, detected as a red fluorescence using the intracellular reporter Fluoro-5. E-selectin ligands are clustered in lipid rafts as detected by Dil-C18, a fluorescent indocarbocyanine dye that preferentially partitions into lipid ordered domains (red fluorescence) and antibody to L-selectin (green fluorescence). The inflammatory synapse is imaged with antibodies to L-selectin (green fluorescence) and PSGL-1 (red fluorescence) as previously reported.<sup>28</sup>

A rise in intracellular calcium concentration plays a central role in signaling downstream of chemokine ligation and adhesion function and we and others have detected  $\text{Ca}^{2+}$  flux to be an indicator of the level of PMN activation during recruitment on inflamed endothelium under shear stress.<sup>15,25,45,59,60,77,84,85</sup> A combination of shear stress and E-selectin engagement is a requirement for transition of LFA-1 to an extended conformation and subsequent maximal activation of  $\beta_2$ -integrins in rolling PMN.<sup>42,76</sup> Moreover, it is the superposition of activation *via* ESLs and G-protein coupled receptors that elicit optimal levels of calcium flux on arrested PMN.<sup>73</sup> We have imaged intracellular  $\text{Ca}^{2+}$  flux in real time where PMN were labeled with the dye Fura-2 and perfused at 2  $\text{dyn}/\text{cm}^2$  over L-cell monolayers expressing E-selectin. The kinetics of intracellular calcium release was recorded for PMN rolling to arrest over a dose range of IL-8, added as a bolus to the inlet of the microfluidic channel as depicted in Fig. 2. In the absence of chemokine, or at concentrations of IL-8 below 0.01 nM, rolling PMN elicited a small but significant  $\text{Ca}^{2+}$  flux (i.e., ~50 nM). From this baseline, the level of cortical calcium rapidly increased with IL-8 concentration for PMN rolling to arrest under 2  $\text{dyn}/\text{cm}^2$  of shear stress. For example, stimulation with 0.05 nM IL-8 in suspension yielded ~150 nM of  $\text{Ca}^{2+}$  flux in arrested PMN under shear stress (Fig. 2). To activate this same extent of  $\text{Ca}^{2+}$  flux on PMN stimulated in static suspension required a 100-fold higher concentration of IL-8 (i.e., 5 nM). Thus, ESL clustering under shear stress functions to amplify the sensitivity to chemokine activation and significantly increase the extent of  $\text{Ca}^{2+}$  flux in PMN arrested *via* high affinity  $\beta_2$ -integrins.<sup>73</sup>

#### MECHANICAL FORCES ACTING ON E-SELECTIN LIGANDS FUNCTION IN SIGNALING *VIA* CYTOSOLIC KINASE ACTIVATION

E-selectin bond strength exhibits a weak dependence on the rate of loading (e.g., PMN roll at a constant velocity of ~6  $\mu\text{m}/\text{s}$  over a wide range of fluid shear stress), but these bonds are more durable than those of L- or P-selectin.<sup>44</sup> Shear forces of blood flow acting on membrane tethers with the substrate transmit force to the bonds with ESLs and are a critical determinant in catalyzing kinase phosphorylation and down-stream integrin activation. Thus, the mechanics and kinetics of E-selectin optimize it for recognition of multiple ligands and to function as a dynamic substrate for assembly of cytoskeletal proteins and signaling kinases. An important question is how the forces acting on ESL bonds influence assembly to adaptor molecules in a process summarily referred to as mechanotransduction. We



**FIGURE 2.** PMN rolling on E-selectin and arrested in response to infusion of IL-8 at the indicated concentration. Neutrophils were loaded with Fluo-4 and perfused over monolayers expressing E-selectin at a shear stress of 2  $\text{dyn}/\text{cm}^2$ , then exposed to a dose range of IL-8 following 2 min of shear interaction. (a) Individual neutrophils that have rolled to arrest rapidly increase their intracellular calcium in response to IL-8 (0.1 nM) resulting in an increase in Fluo-4 emission. (b) On average, neutrophils exhibited a rapid increase in Fluo-4 emission indicative of calcium flux in response to IL-8 concentrations of 0.1 nM or higher, but did not significantly increase calcium in unstimulated or at low IL-8 of 0.01 nM. Plot is representative of 4 independent experiments with measurements from at least 60 neutrophils at each labeled concentration of IL-8. (c) Calcium concentration was measured by ratiometric imaging of neutrophils that sedimented onto the monolayer (Static) or rolling on the monolayer under shear stress (2  $\text{dyn}/\text{cm}^2$ ) following exposure to a dose range of IL-8 from 0.001 to 5 nM. The average calcium concentration in all neutrophils in a field of view was measured over time, and the peak value was recorded. Calcium concentration was measured by ratiometric imaging in neutrophils sedimented onto the monolayer (Static) or rolling on the monolayer under shear stress (2  $\text{dyn}/\text{cm}^2$ ) following exposure to a dose range of IL-8 from 0.001 to 5 nM. The average calcium concentration in all neutrophils in a field of view was measured over time, and the peak value was recorded (adapted from Schaff *et al.*<sup>73</sup>).

have begun to dissect how force catalyzes kinase mediated activation using dynamic real time imaging of  $\text{Ca}^{2+}$  flux, upshift in CD18 affinity, and assembly and phosphorylation of kinases with the cyto-domains of

ESLs in human PMN within a region of contact we denote the inflammatory synapse (see Fig. 1).<sup>75</sup> The importance of this process in human disease was recently reported in patients with sepsis-induced acute kidney injury that exhibited elimination of selectin-mediated slow leukocyte rolling that was associated with reduced phosphorylation of spleen tyrosine kinase, Akt, phospholipase C- $\gamma$ 2, and p38 MAPK that correlated with impaired PMN rolling and transmigration.<sup>67</sup> The mechanism by which shear forces actively transport ESLs to sites of active MAPK and Src kinase activation have yet to be elucidated. It should be possible to image the redistribution of L-selectin, PSGL-1, CD44 (HCELL) and other ESLs under well defined conditions of shear stress in order to directly correlate with PMN activation as detected by  $\text{Ca}^{2+}$  flux, assembly of phospho-MAP and Src kinases, and activated CD18. The shortcoming of approaches that rely on imaging phospho-specific antibodies against Src (i.e., Tyr-419) and MAPK (p38MAPK or ERK2) in fixed and permeabilized cells is that the difficulty in identifying those signals elicited by integrins or other membrane receptors engaged. One promising strategy to detect mechanosensing is to disassemble the actin cytoskeleton under defined conditions of receptor ligation. Cuvelier *et al.*<sup>14</sup> adopted such an approach and found that ligation of leukocyte adhesion molecules under flow conditions leads to mechanotransduction in endothelial cells. Treatment of endothelium with latrunculin A prevented ERK2 phosphorylation following ligation of either vascular cell adhesion molecule-1 or E-selectin with antibody coated beads under flow conditions.<sup>14</sup> Rapid phosphorylation of focal adhesion kinase and paxillin as observed for engagement of biomimetic beads resembles that for eosinophil binding to inflamed endothelium.<sup>14</sup> This suggests that focal adhesions are involved in signaling to endothelium, but do not elucidate how leukocyte engagement mechanotransduce such intracellular signals. An intriguing question is whether membrane redistribution of ESLs upon stimulation requires their initial release from cortical actin associated proteins such as talin, vinculin, and  $\alpha$ -actinin under the control of calpain, as compared with other receptors (e.g., CD18, CD45). ESL diffusion into discrete membrane rafts known to be enriched with members of the Ras and Src family also should be analyzed in the context of intracellular signaling under dynamic conditions of shear stress. For example, diffusion of L-selectin/PSGL-1 and CD44 in the membrane can be assessed for PMN incubated with the chelators methyl  $\beta$ -cyclodextrin and filipin to disrupt cholesterol rafts. To directly image selectin-ligands redistributing within rafts, PMN can be labeled with DiI-C18, a fluorescent indocarbocyanine dye that preferentially partitions into lipid ordered domains,

and ESL co-localization analyzed using 3-color immunofluorescence as depicted above in Fig. 1.

### E-SELECTIN PLAYS A DOMINANT ROLE IN THE CELLULAR EVENTS OF VASO-OCCCLUSIVE CRISIS IN SICKLE CELL DISEASE

Sickle cell disease (SCD) is among the most common monogenetic hematological disorders in the United States.<sup>86</sup> It is characterized by a single amino acid substitution in the  $\beta$ -chain of hemoglobin, which during hypoxic conditions induces its polymerization and causes the characteristic rigid sickle shape of the red blood cells. SCD sufferers undergo periodic vaso-occlusive events in which PMN adhere to the endothelium and bind and entrap circulating sickle red blood cells eventually leading to blockage of blood flow, ischemic damage to organs and intense pain. While a genetic disorder, the underlying cause of the vaso-occlusive crisis (VOC) is a heightened inflammatory response to stimuli ranging from general stress to bacterial and viral infections. Signs of chronic vascular inflammation are common in sickle cell disease. Numerous markers of inflammation such as TNF- $\alpha$ ,<sup>6,22</sup> C-reactive protein,<sup>3</sup> and interleukins 1 and 8<sup>18</sup> are elevated in sickle cell individuals and result in activation of the endothelium. Clinical studies have also shown that elevation of circulating leukocytes in patients correlates with episodes of VOC, stroke, and acute chest syndrome.<sup>89</sup> PMN activation is also pronounced in SCD and results in increased cell adhesion, while the activation of monocytes results in the formation of platelet-monocyte aggregates.<sup>92</sup>

Mice expressing human sickle hemoglobin in their red blood cells mimic the human disease by undergoing VOC upon inflammatory stimuli. The cellular events that initiate VOC in these mice were directly observed using the technique of intravital microscopy.<sup>86</sup> PMN initiate the process by rolling and achieving stable arrest on the vascular endothelium. Sickie erythrocytes are observed to then bind to the immobilized PMN eventually causing further entrapment and resulting in vaso-occlusion. To explore the possible role of adhesion molecules in VOC, a sickle cell mouse model was created by bone marrow transplantation and crossed with a genetic background lacking E- or P-selectins. Leukocyte recruitment to the vessel wall is defective in these knockout mice, which affords protection from VOC.<sup>86</sup> These studies demonstrate the critical roles of E- and P-selectins, as all of the other adhesion molecules in these sickle cell mice are present yet they do not undergo VOC.

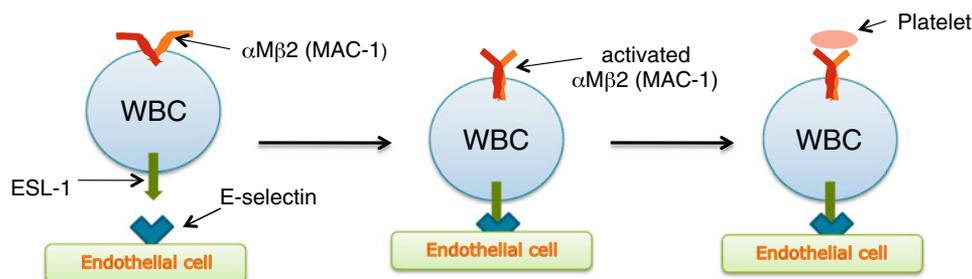
Hidalgo *et al.*<sup>30</sup> explored the relative roles of E-selectin and P-selectin during vaso-occlusive events

in a mouse model of SCD. Two critical events that occur during the crisis are the foundation of the occlusions created by the binding of RBC to immobilized leukocytes and reduced blood flow. Blocking E-selectin with antibodies or genetic deletion in knockout mice fully inhibits the adhesion of RBCs to immobilized leukocytes over 4 h of observation during a vaso-occlusive event. In contrast, blocking P-selectin exhibits transient inhibitory effects on the binding of RBCs to leukocytes, which return to control values over this time period. Likewise, blocking E-selectin, but not P-selectin, has a significant effect on increasing blood flow during VOC.<sup>30</sup> The prevalence of E-selectin function over P-selectin is also seen in sickle cell patients. Kato and colleagues at the NIH<sup>39</sup> measured the level of adhesion molecules in the plasma of 160 sickle cell patients over a four year period and found a strong correlation of soluble E-selectin, but not P-selectin, with mortality over that time period. Similarly, in the sickle cell mouse model a genetic knockout of E-selectin, but not P-selectin, correlates with increased survival after induction of VOC.<sup>30</sup> In mice, endothelial E-selectin, rather than P-selectin, engagement of ligand ESL-1 on the PMN surface induces secondary signaling events that generate activated Mac-1 integrin microdomains at the leading edge of adherent PMN in TNF- $\alpha$  stimulated post-capillary venules. These activated Mac-1 clusters participate in heterotypic interactions with circulating RBCs.<sup>30</sup> This suggests that E-selectin is responsible for signaling the adherent neutrophil to capture red blood cells and leukocytes, which is a critical step in the formation of an occlusion. A mechanism by which E-selectin affects the adhesion of either RBCs or platelets to leukocytes, as gleaned from the studies of Frenette, is depicted in Fig. 3. Rolling and adhesion of leukocytes on E-selectin mechanotransduces signals including an upshift of CD11b/CD18 (Mac-1) into a high affinity conformation capable of binding RBCs or platelets. This model is congruent with the findings that elimination of either E-selectin or activated Mac-1 in

knockout mice reduces the formation of RBC or platelet/leukocyte aggregates.<sup>30</sup>

### INHIBITION OF VOC BY SMALL MOLECULE PAN SELECTIN ANTAGONIST GMI-1070

GMI-1070 is a small molecule pan selectin antagonist currently in Phase II clinical trials for VOC in sickle cell patients. It was designed to mimic the bioactive conformation of the functional carbohydrate ligands of the selectins and to incorporate the charged groups required for a pan selectin antagonist. Although GMI-1070 is bound by all three selectins, it demonstrates an order of magnitude greater potency in binding to E-selectin and blocking ligand binding.<sup>12</sup> Chang *et al.*<sup>12</sup> tested the ability of GMI-1070 to reverse ongoing acute VOC in the sickle cell mouse model. The protocol was designed to be a clinically relevant model in which treatment with GMI-1070 was delayed as long as possible to simulate the arrival of a sickle cell patient already in VOC at the clinic. After initiation of VOC in the sickle cell model, mice were allowed to progress in VOC for 110 min. Mice were then treated with GMI-1070 and shortly thereafter control untreated mice began to die. GMI-1070 was observed to reverse measures of VOC as determined by dramatic increases in blood flow and the elimination of the capture of sickle RBCs by arrested leukocytes in the treated cohort of mice. This reversal of ongoing VOC in the treated mice also increased survival. Treatment with GMI-1070 saved half of the cohort and extended survival for the remaining half compared to untreated mice. These data suggest that although both E- and P-selectin have been associated with VOC, the effects of E-selectin clearly predominate, in the mouse where inhibition of E-selectin is sufficient for full inhibitory effects of VOC. Ongoing human studies on blood samples obtained from sickle cell disease subjects participating in Phase I and II clinical studies of the safety and efficacy of GMI-1070 reveal that it is well tolerated by patients following infusion at serum concentrations shown



**FIGURE 3.** Schematic of ESL ligation and signaling of Mac-1 dependent VOC. Following white blood cell tethering by E-selectin a signal is transduced resulting in a conformational upshift in Mac-1 to high affinity that facilitates binding to specific ligands on platelets captured from the blood stream.

effective in the mouse VOC studies. Moreover, *in vitro* assays of blood samples to assay PMN capture on E-selectin and integrin activation show a clear inhibitory benefit of GMI-1070 at low micromolar concentrations.

### SUMMARY

E-selectin is the most promiscuous of selectins in recognition of a variety of sLe<sup>x</sup> expressing glycoprotein and glycolipid ligands on leukocytes. Its role in immune surveillance may be to amplify the sensitivity to activation with chemokine at sites of vascular inflammation and locally activate PMN arrest and migratory function. Key issues to be elucidated regarding mechanotransduction of these functions are how the direction, magnitude, and duration of the hydrodynamic forces acting on each ESL influence their respective capacity to signal in the cytosol. Remaining to be discovered is the distinction between how distinct ESLs signal during rolling on E-selectin as compared to dynamic interaction *via* P-selectin and L-selectin, which are less efficient at mechanotransducing a calcium flux and integrin activation. In addition, how FAK/Src-dependent and ESL-mediated p38 MAPK and ERK activation is central to the organization and localization of active Raf-MEK1-MAPK signaling complexes and other guanine nucleotide exchange factors (i.e., Vav-1) within the PMN-endothelial inflammatory synapse. The role of E-selectin in various inflammatory diseases is coming to light and in particular its function in VOC associated with SCD. The capacity of the glycomimetic antagonist GMI-1070 to block both PMN recruitment by effectively antagonize signaling of CD18 activation and RBC capture, highlights a complex process by which ESL recognition and reorganization are associated with mechanotransduction of PMN activation.

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