Topographic requirements and dynamics of signaling via L-selectin on neutrophils

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Green, Chad E., David N. Pearson, Nadine B. Christensen, and Scott I. Simon. Topographic requirements and dynamics of signaling via L-selectin on neutrophils. Am J Physiol Cell Physiol 284: C705–C717, 2003. First published November 13, 2002; 10.1152/ajpcell.00331.2002.—Cross-linking of L-selectin on leukocytes signals phosphorylation of mitogen-activated protein kinases (MAPKs) leading to activation of CD18 function and enhanced transmigration on inflamed endothelium. We examined how alterations in the topography of L-selectin correlate with the dynamics of CD18 activation and phosphorylation of MAPK. Simultaneous ligation of humanized antibodies DREG55 and DREG200 provided a strategy for regulating the extent of cross-linking. Triggering of CD11b/CD18 upregulation and adhesion required clustering of L-selectin on microvillus-sized patches of ~0.2 μm². Immunofluorescence revealed that L-selectin was colocalized with high-affinity CD18. Anti-L-selectin-coated protein A microspheres indicated that a single site of contact to a 5.5-μm bead, or multiple contacts to 0.94- or 0.3-μm beads, elicited maximum neutrophil activation. Adhesion signaled via L-selectin coincided with the kinetics of MAPK phosphorylation and was inhibited by blocking p38 or p42/44 activity. These data demonstrate the capacity of L-selectin to transduce signals effecting rapid (~1 s) neutrophil adhesion that is regulated by the size and frequency of receptor clustering.

NEUTROPHILS CONSTITUTE the first line of defense against invading microorganisms and are the major cellular component of an acute inflammatory reaction (5, 44). They circulate in a passive state and become tethered to the vessel wall after the expression of the selectin family of cell surface glycoproteins (18). All three selectins share a similar mosaic structure consisting of three extracellular domains, an NH₂-terminal lectin domain followed by an epidermal growth factor domain, and complement regulatory repeat elements (19). L-selectin (CD62L) is constitutively expressed on leukocytes and supports their capture on activated endothelium as well as in homotypic neutrophil aggregation (37). All three selectins bind with relative high affinity to the sialyl Lewisα (sLeα) tetrasaccharide and related structures expressed on a variety of glycoprotein surface receptors (18). The best-characterized selectin ligand is P-selectin glycoprotein ligand (PSGL)-1, a heavily glycosylated receptor expressed as a homodimer on leukocytes (24). L-selectin ligands that are expressed in the vasculature are less well characterized (30). One well-defined L-selectin ligand is GlyCAM-1, a heavily glycosylated polysaccharide shown to activate lymphocytes through L-selectin by presenting sulfated sLeα ligands in a multivalent manner (11, 15). Other potential L-selectin counterstructures include E-selectin (17, 47), CD34, sulfatides, and heparin sulfate proteoglycans (14, 26, 27).

Capture of neutrophils at vascular sites of inflammation by selectins provides a dynamic contact zone that is thought to facilitate ligation of G protein-linked chemotactic receptors including interleukin (IL)-8 and platelet-activating factor (PAF) (29, 46). This in turn leads directly to the rapid activation of β₂-integrins (CD11/CD18) that function to decelerate rolling neutrophils and facilitate the transition to cell arrest by forming stable bonds with intercellular adhesion molecules (ICAMs) also upregulated on the endothelium (39). This multistep pattern of molecular recognition and intracellular signaling broadly defines the transition from selectin-dependent cell capture to shear-resistant firm adhesion mediated by activated integrins (36, 46).

Recent published data suggest that selectins function as both adhesion molecules and transmembrane signaling receptors as leukocytes become tethered and roll on inflamed endothelium (35). Clustering L-selectin on the surface of neutrophils with antibody or sulfated polysaccharide mimetics is synergistic with chemotactic stimuli in activating β₂-integrins (42). Recent studies also suggest that transmembrane signaling via L-selectin enhances the microvascular sequestration of neutrophils at sites of inflammation by inducing rapid cell shape change and reduction in cell deformability that correlate with F-actin assembly and enhanced adhesion via β₂-integrins and colocalization of L-selectin with CD18 (33, 34). A contribution of L-selectin to neutrophil recruitment in the microcirculation of mice, independent of its function in mediating primary capture to inflamed endothelium, was recently reported. Intravital microscopy revealed that neutrophil-
MATERIALS AND METHODS

Isolation of neutrophils. Venous blood was collected from healthy adult donors into a sterile syringe containing 10 U/ml heparin. The neutrophil-containing bands were isolated from the gradient with an intravenous catheter and washed once with CaCl2-free HEPES buffer (HHB; in mM: 30 HEPES pH 7.4, 110 NaCl, 10 KCl, 1 MgCl2, 10 glucose). Cells were resuspended and kept at 4°C. Mac-1-dependent adhesion was quantified with a bead adhesion assay as previously described (33). Briefly, 2-μm carboxylated fluorescent latex beads (Molecular Probes, Eugene, OR) were washed three times with HHB buffer and incubated in HHB with 0.25% HAS and 1.5 mM CaCl2 at 20°C for 60 min with periodic sonication. Cell suspensions were prepared at a 40-to-1 ratio of albumin-coated latex beads (ACLBs) to cells. Neutrophils (5 x 10^6) were sheared with 2 x 10^11 ACLAbs in a final volume of 0.25 ml of HHB, 0.1% HAS, and 1.5 mM CaCl2 with a 5-mm magnetic stir bar as previously described (33).

HuDREG55 and -200 antibodies were used to activate human neutrophils by systematically increasing the valency of binding and cross-linking L-selectin. Treatment with either HuDREG55 or HuDREG200 alone results in a combination of monovalent and bivalent antibody binding to L-selectin, as each arm of the whole IgG is capable of binding a single target epitope per receptor. When HuDREG55 and HuDREG200 are added in combination, the potential for both antibodies to bind the same L-selectin molecule results in multivalent MAb binding and a moderate level of L-selectin clustering. Maximal clustering of L-selectin is achieved by adding secondary polyclonal anti-human F(ab')2 fragments to neutrophils bound with single HuDREG IgG or both HuDREG55 and HuDREG200 in combination. In each case, the secondary anti-human F(ab')2 fragments cross-link individual HuDREG antibodies, creating L-selectin clusters, as identified by highly fluorescent regions on the neutrophil membrane.
To stimulate neutrophil adhesion, anti-L-selectin antibodies were first bound to the neutrophil at room temperature for 10 min. Cells were then centrifuged to remove unbound ligand. L-selectin cross-linking was initiated by the addition of goat anti-human F(ab')2 antibodies (10 μg/ml) as described above followed by incubation at 37°C. Human IgG (10 μg/ml; Ancell, Bayport, MN) alone and goat anti-human F(ab')2 alone were used as antibody controls. Cell-bead collisions were induced by shearing the cell suspension in a test tube at a rotation rate of ~300 rpm corresponding to a shear rate of 100 s⁻¹ and an estimated average shear stress of ~<1.0 dyn/cm². The mixing assembly of magnetic stir bar and test tube was placed upstream from the sample inlet of a FACScan flow cytometer to measure the kinetics of neutrophil-bead adhesion in real time. In experiments using chemotactic stimulation, IL-8 or N-formylmethionyl-leucyl-phenylalanine (fMLP) was added at time zero just before application of shear in the mixing tube. In the MAPK inhibition studies, neutrophils were pretreated with SB-202190 (10 μM) or PD-98059 (100 μM) at 37°C for 45 min (concentrations chosen based on titration studies to obtain optimal inhibition). The pretreated cells were then incubated at 100 V for 1 h described for the ACLB assay, but with kinase inhibitor present throughout the kinetic assay. The final concentration of DMSO in the reaction was ≤0.1%.

Neutrophils and beads were differentiated by forward light scatter and fluorescence emission in the green or FL1 wavelength. A live gate was set on a forward vs. side scatter dot plot to limit data acquisition to neutrophils and neutrophil/bead events, excluding unbound beads. Events were acquired continuously at a resolution of 100–200 particles/s at a flow rate of 15 μl/min for up to 10 min after stimulation. Neutrophils devoid of bound beads emitted a background level of green fluorescence. The mean number of beads bound per neutrophil was detected as a quantal increase in green fluorescence and calculated as previously described (43).

Western analysis of p38 and p42/44 MAPK. Neutrophil suspensions (2 × 10⁶ cells) were first incubated with Hu-DREG55 at 2,000 × Kd (80 nM) for 10 min at 24°C to saturate binding sites. At time zero on the x-axis of Fig. 6B, cells were washed and stimulated by addition of secondary goat-anti-human F(ab')2 antibody fragments at 37°C. For comparison, cells were also stimulated with TNF-α (10 ng/ml). A 90-μl aliquot containing 2 × 10⁶ cells was removed at the indicated time points, centrifuged, aspirated, frozen in liquid N₂, and then stored at −70°C. The cell extracts were prepared by thawing the cells on ice in the presence of lysis buffer containing 50 mM HEPES, pH 7.5, 1% (vol/vol) Triton X-100, 2 mM sodium orthovanadate, 10 mM NaF, 1 mM EGTA, 2× protease cocktail inhibitors (Boehringer Mannheim, Indianapolis, IN), and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 1 h with periodic mixing. Biotinylated secondary goat-anti-rabbit IgG at a concentration of 10 μg/ml was added. The sample was incubated at 4°C for 45 min (concentrations based on titration studies to obtain optimal binding) at an estimated average shear stress of ~<1 dyn/cm². After incubation, samples were washed and resuspended in HEPES buffer containing 0.1% HSA and 1.5 mM CaCl₂ and placed on ice. Cell suspensions were labeled on ice for 10 min and then washed and fixed in HEPES buffer containing 1% paraformaldehyde. Labeled cells were then stimulated with 10 μg/ml TNF-α and 10 μg/ml p38/Erk antibodies (10 μg/ml) for 10 min at 37°C. High-affinity CD18 was detected with MAb 327C-Alexa (generously provided by Don Staunton, ICOS, Bothell, WA), a monoclonal antibody that recognizes an epitope exposed on the I-like domain of activated CD18. For two-color imaging of L-selectin and 327C colocalization, a PE-conjugated anti-CD62L antibody (DREG56; Immunotech, Marseille, France) was used in conjunction with nonlabeled HuDREG antibodies. CD11b/CD18 (Mac-1) was detected with a PE-conjugated anti-human CD11b antibody (2LPM19C; Dako). Cell suspensions were labeled on ice for 10 min and then washed and fixed in HEPES buffer containing 1% paraformaldehyde. Labeled cells were imaged with a Nikon TE2000 inverted microscope employing a ×60 oil-immersion Plan-Apo objective with the appropriate band-pass filters for FITC or PE labels. Images were captured with a charge-coupled device (CCD) camera (Dage-MTI, Michigan City, IN) and then analyzed with Image Pro Plus v4.5 software (Media Cybernetics, Silver Spring, MD). We define a cluster as a localized region of the membrane where pixel intensity is at least threefold greater than background fluorescence intensity. With the analysis software, pixel maps are obtained for each cell visualized. Pixel intensity values are unitless and range from 0 to 255. A threshold intensity value based on these values and the image of the cell is chosen and set, which represents the average background intensity over the surface. This value typically ranges from 80 to 120, with clusters achieving off-scale values that are assigned a maximum intensity value of 255 by default. After thresholding, the number of fluorescent clusters, surface area (μm²) per cluster, and percent colocalization (if appropriate) were determined as reported in Table I. Images and values represent averages from 60–90 cell observations per treatment from three separate experiments.

Electron microscopy of neutrophils and beads. After the preparation of Lam1.3-coated beads bound to neutrophils, neutrophils were postfixed by suspending in a 2% glutaraldehyde solution overnight at 4°C. Samples were then resuspended in agarose and fixed in 2% buffered OsO₄ and dehydrated in steps with ethyl alcohol. Spurrs resin was used as...
the embedding medium. A transmission electron microscope (H-600; Hitachi, Tokyo, Japan) was used to image the interface at the neutrophil-bead margin at ~×5,000 magnification.

Determination of neutrophil/microsphere contact area. Neutrophils were incubated with maximal dose anti-L-selectin-coated microspheres. Cells with attached microspheres were observed under bright-field phase-contrast microscopy with a ×60 oil-immersion lens, as depicted in Fig. 4B. The contact area was then determined by measuring the length of the cell-microsphere interface from 25 digitized images with reference to a calibrated reticule slide. The contact area was calculated with the formula

\[ SA = \frac{\pi}{2} D \left( D - \sqrt{D^2 - L^2} \right) \]

where D is the microsphere diameter and L is the length of the cell-microsphere contact. This calculation of surface area (SA) is based on the assumption of a circular and continuous area of contact. Furthermore, it is assumed that the area of microsphere contact is based on the presumption that L is measured in the central plane (e.g., maximum length of contact). The assumption of continuous membrane contact between the microsphere and neutrophil was confirmed by transmission electron microscopy (TEM) analysis of >25 cells in which >90% revealed close apposition (Fig. 4B). For the 5.5-μm beads, L was 1.64 μm with an SE of 0.06. For the 0.94-μm beads, L was 0.5 μm with an SE of 0.03. To estimate the measurement error in contact area, the mean value \( L_{avg} \) was computed at the upper and lower values of the SE. Given these values, SA is estimated to be 2.15 ± 0.16 μm² for the 5.5-μm beads and 0.21 ± 0.03 μm² for the 0.94-μm beads.

Statistical analysis. Analysis of data was performed with GraphPad Prism version 3.0 software (GraphPad Software, San Diego, CA). All data are reported as means ± SE. Nonparametric grouping of data was analyzed by ANOVA and secondary analysis for significance with a Tukey or Newman-Keuls multiple-comparison test. Gaussian-distributed mean values were analyzed by Student’s t-test. Group comparisons were deemed significant at P < 0.05.

RESULTS

Binding kinetics of anti-L-selectin HuDREG antibodies on neutrophils. We first characterized the binding of humanized monoclonal anti-L-selectin (HuDREGs) antibodies to L-selectin on the surface of human neutrophils. HuDREG55 and HuDREG200 recognize distinct epitopes within the lectin domain of L-selectin and can bind simultaneously without displacement or steric repulsion. A sigmoidal curve fit the binding of antibody as a function of concentration to neutrophils (Fig. 1). Fluorescent conjugated HuDREG MAbs bound with a \( K_d \) of 0.04 and 0.05 nM for HuDREG55 and HuDREG200, respectively, as computed with nonlinear regression analysis of the dose response.

Mac-1 upregulation in response to increased clustering of L-selectin. We next examined the correlation between clustering of L-selectin by HuDREGs and neutrophil activation as quantitated by upregulation of surface Mac-1 (Fig. 2). Neutrophils were incubated with increasing concentrations of either a single HuDREG or HuDREGs added in combination up to 2,000 \( \times K_d \). Although this dose of HuDREG corresponds to ~500-fold above that required for ~99% receptor occupancy, it is in the range typically used to effectively block L-selectin-dependent capture and rolling on activated endothelium or in a soluble sLeX binding assay (2, 4). Treatment with a single HuDREG even at 2,000 \( \times K_d \) did not elicit significant membrane upregulation of Mac-1 above the baseline detected in response to addition of a humanized IgG isotype control (Fig. 2A). In contrast, a dose-dependent increase in Mac-1 expression was achieved by saturation of L-selectin with a single HuDREG (e.g., HuDREG55 at 1,000 \( \times K_d \)) and titration of the second over a 100-fold range from 10 up to 1,000 \( \times K_d \). Treatment with combined HuDREG55 and HuDREG200 at 1,000 \( \times K_d \)
Maximum activation at 1,000 × $K_d$ of combined HuDREGs achieved activation comparable to that obtained with the chemotactic stimuli IL-8 or formyl peptide (Fig. 2B). Together, the data reveal a threshold in L-selectin ligation necessary to trigger neutrophil activation and amplification as a function of the extent of antibody cross-linking.

**Topography of L-selectin after binding and cross-linking with anti-L-selectin.** We next visualized the membrane distribution of L-selectin on neutrophils after treatment with fluorescein-conjugated HuDREGs in the presence and absence of secondary cross-linker by fluorescence microscopy. Treatment with HuDREG55 revealed a uniform circumferential distribution of L-selectin punctuated with small submicrometer domains of fluorescence clusters (Fig. 3a; Table 1). Subsequent cross-linking of HuDREG55 with secondary polyclonal antibody resulted in a fourfold increase in the area, but not the frequency, of L-selectin clusters (Fig. 3b; Table 1). An identical result was observed for HuDREG200 (data not shown). Incubation of neutrophils with combined HuDREGs elicited a redistribution of L-selectin into clusters that were on average threefold greater than single HuDREG (Fig. 3c; Table 1). Cross-linking combined HuDREGs with secondary polyclonal antibody resulted in a doubling of the frequency and area of L-selectin clusters (Fig. 3d; Table 1). As a control, neutrophils were treated with anti-CD45, which exhibited a uniform fluorescence distribution with no significant clustering (Fig. 3e). Cross-linking of CD45 with goat anti-mouse polyclonal antibody triggered redistribution into patches of fluorescence that were significantly larger than cross-linked L-selectin and less uniformly distributed on the neutrophil surface (Fig. 3f; Table 1). In contrast to L-selectin, clustered CD45 did not result in cellular activation as determined by CD11b/CD18 upregulation (33).

**Anti-L-selectin-coated beads activate neutrophils.** Analysis of L-selectin surface topography confirmed a correlation between the frequency and extent of L-selectin clustering and triggering of neutrophil activation. We further examined this by clustering L-selectin on the neutrophil surface by contact with protein A microspheres (diameter 0.3, 0.9, or 5.5 μm) coated over a range of anti-L-selectin concentrations. The number of antibodies bound per bead was determined for the 5.5- and 0.9-μm beads by comparison with a calibration set of beads containing defined numbers of antibody binding sites (Fig. 4a). Neutrophil suspensions were shear mixed with antibody-coated beads, and the average number bound per neutrophil was measured by phase-contrast microscopy, while the extent of Mac-1 upregulation was detected by flow cytometry. A dose-dependent increase in upregulation was elicited by the binding of beads, regardless of bead diameter. Stimulation increased most rapidly over the lower range of anti-L-selectin coating concentrations (0–100 nM) for the 0.9-μm and 5.5-μm beads compared with the 0.3-μm beads (Fig. 4b). This is the range in which MAb bound to beads increased most rapidly with coating.

**Fig. 2. Upregulation of CD11b/CD18 stimulated by anti-L-selectin.** Neutrophils (1 × 10⁶/ml) were incubated for 10 min at 37°C with the indicated concentration of HuDREG55 and/or HuDREG200. Antibody concentrations are given as fold increase relative to the $K_d$ for HuDREG binding (see Fig. 1). After removal of excess HuDREG by centrifugation, neutrophils were incubated without (A) or with (B) secondary polyclonal antibody (cross-linker at 10 µg/ml) for 10 min at 37°C to initiate cell activation. Neutrophils were then assayed for CD11b/CD18 upregulation by flow cytometry with the anti-Mac-1 antibody 2LPM19C-phycocerythrin (PE). Human IgG alone and cross-linker alone were used as controls for A and B, respectively. Bars indicate fold increase in CD11b/CD18 expression over neutrophils incubated in buffer alone (control) at 37°C. Statistical significance between samples as shown was determined from 3 separate experiments.

**Fig. 3. cluster L-selectin on neutrophils.** A) Neutrophils were suspended in buffer alone (control) or with the indicated concentration of HuDREG55 and/or HuDREG200 for 10 min at 37°C. After removal of excess HuDREG by centrifugation, neutrophils were incubated with combined HuDREGs elicited a redistribution of L-selectin into clusters that were on average threefold greater than single HuDREG (Fig. 3c; Table 1). Cross-linking combined HuDREGs with secondary polyclonal antibody resulted in a doubling of the frequency and area of L-selectin clusters (Fig. 3d; Table 1). As a control, neutrophils were treated with anti-CD45, which exhibited a uniform fluorescence distribution with no significant clustering (Fig. 3e). Cross-linking of CD45 with goat anti-mouse polyclonal antibody triggered redistribution into patches of fluorescence that were significantly larger than cross-linked L-selectin and less uniformly distributed on the neutrophil surface (Fig. 3f; Table 1). In contrast to L-selectin, clustered CD45 did not result in cellular activation as determined by CD11b/CD18 upregulation (33).
concentration. At the highest anti-L-selectin coating concentration (~350 nM), Mac-1 upregulation was not statistically different between each bead diameter. At this concentration, neutrophils bound on average a single 5.5-μm bead, whereas they bound eight of the 0.9-μm beads and many more 0.3-μm beads (estimated to be ~10 beads per cell) (Fig. 4C). Beads (0.9 and 5.5 μm) coated with anti-CD43 did not significantly upregulate CD18 beyond baseline at the maximum coating concentration (data not shown).

These data indicate that a single site of membrane contact to a 5.5-μm bead was sufficient to elicit maximal neutrophil activation. We confirmed by TEM that beads formed a continuous area of contact with the neutrophil over an area of 2.15 ± 0.16 μm² as measured by phase-contrast microscopy. Alternatively, multiple sites of contact to 0.9-μm beads over an area of 0.21 ± 0.03 μm² was also effective at activating Mac-1 upregulation. The specificity of the response was confirmed by coating beads with a control isotype-matched antibody, which did not yield significant upregulation above control levels obtained in presence of beads without bound antibody (data not shown). Together, the data indicate that transmembrane signaling via L-selectin increases with site density of MAb ligation at a single site of contact but also in proportion to an increase in the frequency of bead contacts.

Kinetics of CD11b/CD18 activation and MAPK activity after clustering of L-selectin. A sensitive method for assessing the adhesive function of β2-integrin is real-time detection of the capture of ACLBs by neutrophils under fluid shear with flow cytometry. By using this assay it is possible to correlate the rate at which both constitutively expressed and upregulated CD11b/CD18 participate in adhesion function (31). We previously reported (35) that Mac-1 is activated to bind ACLBs after cross-linking of L-selectin at 2,000 Kd of HuDREG. Here we compare the dose dependence of Mac-1 adhesion in response to binding and cross-linking over a wide concentration range of HuDREG (Fig. 5A). Treatment of cells with HuDREG55 elicited a dose-dependent increase in bead adhesion, but only on addition of secondary cross-linking antibody. As with upregulated expression of CD11b/CD18, a significant increase in bead capture was achieved at antibody concentration.
concentrations corresponding to only 1 × $K_d$ in the presence of cross-linker.

We next compared the dynamics of activation in response to binding of anti-L-selectin or chemoattract-

Fig. 4. Dose response for activation of CD11b/CD18 upregulation by anti-L-selectin coated microspheres. A: antibody binding capacity of 5.5-μm and 0.9-μm protein A-coated beads over the indicated range of antibody coating concentration was determined with Quantum Simply Cellular Beads (QSCBs) as described in MATERIALS AND METHODS. B: protein A-conjugated microspheres of 0.3-, 0.9-, and 5.5-μm diameter were coated with anti-L-selectin (LAM1.3) at various concentrations ranging from 0 to 700 nM. After removal of excess antibody, the coated beads were mixed with neutrophils at a bead-to-neutrophil (PMN) ratio of 20:1 for 10 min at 37°C. CD11b/CD18 upregulation was quantitated by flow cytometry and expressed relative to that obtained with beads coated by a control IgG. Data points represent means ± SE from 2–5 separate experiments, and curves were fit to the data with nonlinear regression analysis. *Significantly higher mean value for the 5.5-μm beads over the 0.3-μm beads ($P < 0.05$). C: no. of anti-L-selectin-coated beads bound to neutrophils as a function of bead diameter. Beads were coated with anti-L-selectin MAb at 700 nM, corresponding to maximum upregulation of CD11b/CD18. The average number of beads (mean ± SE) of each diameter bound per neutrophil was determined with a ×60 phase-contrast microscopy. Representative images showing adherent beads, as well as a transmission electron microscopy (TEM) image of the contact site between a 5.5-μm bead and a neutrophil, are shown for comparison. Beads attached to neutrophils were enumerated on 25–50 cells from 3 separate experiments.

Fig. 5. CD11b/CD18-dependent neutrophil adhesion to albumin-coated latex beads (ACLBs) in response to cross-linking L-selectin. A: neutrophils were stimulated by incubation with the indicated dose of agonist and then washed and resuspended in buffer with (●) or without (○) goat anti-human F(ab')2 cross-linker (10 μg/ml). CD11b/CD18 upregulation was detected by continuously monitoring the capture of 2-μm ACLBs in a sheared cell suspension by flow cytometry. Data points represent the mean ± SE no. of adherent beads/neutrophil after 10 min of shear and stimulation. Each point represents 2–4 separate experiments. *Significant difference ($P < 0.05$) between samples in the presence and absence of cross-linker. B: kinetics of ACLB capture was monitored continuously for 6 min after neutrophil stimulation with the indicated agonist. For L-selectin cross-linking, neutrophil suspensions were preincubated with 2,000 $K_d$ HuDREG55 for 10 min at 20°C. Neutrophils were then stimulated at $t = 0$ by N-formylmethionyl-leucyl-phenylalanine (fMLP; 0.1 μM), interleukin-8 (IL-8; 1 nM), or HuDREG55 cross-linked with goat-anti-human F(ab')2 (10 μg/ml). Stimulated neutrophil-bead suspensions were immediately placed in the shear chamber upstream of the flow cytometer. Samples were recorded continuously, with each data point representing the average number of beads bound to ~150 neutrophils over a 5-s sampling interval. Data points represent means ± SE from 3 independent experiments. C: expansion of adhesion kinetics (see Fig. 6B) over the initial 100 s after application of shear and stimulation. Linear regression analysis was used to compare the rate of bead capture between stimuli. *Statistically greater rate of bead capture after fMLP stimulation ($P < 0.001$).
tants in sheared cell suspensions. Neutrophils and ACLBs in suspension were exposed to stimulus and fluid shear while bead capture was continuously recorded on the flow cytometer (Fig. 5B). Stimulation with IL-8, fMLP, or HuDREG was most rapid over the initial minute of stimulation and resulted in a sustained increase in CD11b/CD18-mediated bead capture that reached a plateau by ~10 min. Stimulation with IL-8 (1 nM) or cross-linked HuDREG55 (2,000 × Kd) elicited similar kinetic rates of capture over the first 100 s (Fig. 5C). In contrast, stimulation with fMLP (1 μM) activated a twofold increase in the rate of capture over IL-8 or HUDREG55.

Previous work by our laboratory (35, 40) demonstrated a central role for p38 MAPK in signaling of neutrophil degranulation and adhesion via β2-integrin in response to L-selectin cross-linking. To delineate the relative role of the p38 and p42/44 MAPKs in the dynamics of CD11b/CD18 adhesion triggered via clustered L-selectin, neutrophils were pretreated with the specific p38 inhibitor SB-202190 or the p42/44 inhibitor PD-98059 and the rate of bead capture was assessed (Fig. 6A). To control for the potential toxic effects of the inhibitors on cell function, we pretreated cells and assessed bead capture in response to fMLP stimulation. As expected, the inhibitors did not affect G protein-mediated signaling of adhesion, suggesting that they were nontoxic at the doses applied. In contrast, stimulation by cross-linking anti-L-selectin was blocked to baseline (e.g., ~85% inhibition) with treatment by either the p38 or the p42/44 inhibitor. Combined pretreatment with both inhibitors failed to further decrease the rate of bead capture observed for the individual inhibitors alone (data not shown).

Given the rapid kinetics by which cross-linking L-selectin signaled neutrophil adhesion, we next examined the time course of p38 and p42/44 MAPK phosphorylation after stimulation. Phosphorylation of threonine and tyrosine residues on p38 (Thr<sup>180</sup>/Tyr<sup>182</sup>) and p42/44 (Thr<sup>202</sup>/Tyr<sup>204</sup>) was quantitated by densitometry of Western blots from lysates of neutrophils treated with cross-linked anti-L-selectin, TNF-α, or cross-linked human IgG control (Fig. 6B). Specific rabbit polyclonal antibodies that report on the active, phosphorylated forms of p38 and p42/44 produced bands at the expected molecular weights. As a positive control, neutrophils were stimulated with TNF-α, previously shown to be a potent stimulus of p38 phosphorylation and Mac-1 upregulation. Addition of secondary cross-linker to HuDREGs at time zero elicited a rapid rise in both p38 and p42/44 phosphorylation that peaked at three- to fourfold above the baseline activity by 1 min and stayed high up to 3 min (Fig. 6B). Stimulation with TNF-α elicited a comparable increase in p38 and p42/44 phosphorylation that was sustained beyond 3 min. The inhibitor PD-98059 reduced p42/44 phosphorylation fourfold at 3 min; however, SB-202190, which is known to only inhibit the α- and β-isoforms of p38, did not inhibit total p38 phosphorylation.

Clustered L-selectin colocalizes with activated β2-integrin. We next examined the mechanisms by which clustered L-selectin could signal CD18 adhesion dynamics on par with that elicited through chemotactic stimulation. As shown in the two-color immunofluorescence images of Fig. 7, cross-linking HuDREGs induced clusters of L-selectin that colocalized with 327C. This antibody binds to an epitope exposed on the I-like domain of CD18 and reports on the active ligand-binding conformation (1, 23). In the absence of secondary cross-linking, L-selectin cluster area was small and coincided with minimal CD18 activation and no colocalization between L-selectin and 327C (Fig. 7A). However, on cross-linking, 60 ± 12% of activated CD18

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colocalized with clustered L-selectin (Fig. 7b). Further analysis indicated that there were \(3.0 \pm 0.94\) clusters of 327C per cell with an area of \(0.37 \pm 0.21\) after L-selectin cross-linking. Colocalization was found to be specific to activated CD18, because cross-linking L-selectin did not elicit colocalization of CD11c expressed on neutrophils (Fig. 7c). Moreover, activation of CD18 was specific to L-selectin, because cross-linking CD18 or CD11c did not elicit significant 327C expression (Fig. 7, d and e). We next assessed whether CD18 activation and colocalization with cross-linked L-selectin involved signal transduction via p38 MAPK. Pretreatment of neutrophils with SB-202190 effectively eliminated expression of 327C in response to cross-linked L-selectin (Fig. 7f). By comparison, we confirmed that blocking p38 activity did not inhibit CD18 activation or clustering in response to stimulation with IL-8 (data not shown).

**DISCUSSION**

This study demonstrates a direct correlation between the extent of L-selectin clustering and transmembrane signaling of neutrophil adhesion. With a combination of two humanized anti-L-selectin MAb in development as anti-inflammatory agents (7), a threshold in the area of L-selectin clustering required to trigger neutrophil activation was revealed. Cross-linking with two bivalent anti-L-selectin antibodies was necessary and sufficient to induce membrane patching that correlated with sites of CD18 activation and rapid induction of CD11b/CD18-dependent adhesion with kinetics that paralleled the phosphorylation of p38 and p42/44 MAPK. The data establish a hierarchy in the relative number and site density of clustered L-selectin associated with signaling adhesion of neutrophils.

A common in vitro strategy used to simulate ligand-induced receptor clustering and cellular activation via L-selectin is by ligation and cross-linking of MAbs. Previous reports demonstrated that binding of L-selectin with a single anti-L-selectin was insufficient to trigger Mac-1 function even at doses several thousandfold in excess of that required to saturate L-selectin receptors (33). The current study extends these findings by showing that simultaneous binding of HuDREG55 and HuDREG200 exceeded this threshold for neutrophil activation but only at excessive concentra-
tions of MAbs. Evidence correlating the level of cellular activation with the extent of clustering was the finding that secondary cross-linking of HuDREGs with polyclonal antibody decreased the concentration required to trigger activation by 1,000-fold to a level corresponding to just ~50% of L-selectin saturation. This applied to HuDREG bound individually at $1 \times K_d$ or combined at $0.5 \times K_d$.Remarkably, combined HuDREGs amplified Mac-1 activation by 100% over single MAB treatment and, at maximum stimulation, elicited Mac-1 upregulation on par with chemotactic stimulation by IL-8 or fMLP.

On the basis of the immunofluorescence of L-selectin clustering, we speculate that the large excess of MAB necessary to trigger neutrophil activation reflects a requirement for continuous receptor occupancy of HuDREG55 and -200 to their respective epitopes on L-selectin. Given the affinity and valency of HuDREGs, addition of a single HuDREG at saturation results in dimerization by binding of each Fab on the IgG to a single lectin domain of L-selectin (Fig. 8). Bivalent ligation is apparently insufficient to elicit significant receptor clustering or neutrophil activation. Rather, further constraint and aggregation of receptors by simultaneous occupancy of both HuDREGs recognizing distinct epitopes on the lectin domain was necessary and sufficient to trigger activation. Immunofluorescence data confirmed that combined HuDREGs elicited a redistribution of L-selectin from a uniform punctate expression in the presence of single MAB to one of dense clusters of fluorescence. This correlated with a threefold increase in the area of L-selectin clusters and increased neutrophil activation (Table 1). Further patching of L-selectin with secondary cross-linker doubled again the area and frequency of clusters that correlated with potent activation. Patching of receptors in response to the clustering of a primary MAB by a secondary polyclonal antibody is not unique to L-selectin, as shown by the patching of fluorescent anti-CD45. However, the correlation between the extent of patching and amplification in transmembrane signaling appears to be a distinct property of L-selectin on neutrophils.

A second piece of data revealing a direct relationship between the extent of L-selectin engagement and signaling was detection of Mac-1 upregulation in response to the binding of microspheres presenting anti-L-selectin. Figure 4 reveals a direct relationship between anti-L-selectin presented on beads and activation elicited by binding over the range of bead diameters tested. Activation increased most rapidly over the lower range of anti-L-selectin coating concentrations, correlating to the increase in the number of antibody sites presented on the beads. At the highest concentration of anti-L-selectin, maximum activation was equivalent for each bead diameter. We conclude that amplification in signaling via clustered L-selectin is effectively transduced at a single site of contact or through multiple smaller sites of membrane contact.

A single site of membrane contact to a 5.5-μm latex bead over an area of ~2.0 μm² was sufficient to trigger maximum upregulation of Mac-1. We estimate that this contact area could accommodate engagement of up to ~10 microvilli on the surface of a neutrophil over an area that is on the same order of magnitude measured for a leukocyte rolling on microvascular endothelium (20). Alternatively, neutrophils bound to a 0.9-μm bead over an area at ~0.2 μm² could accommodate contact with a single microvillus (32). This area is consistent with the immunofluorescence data that revealed L-selectin in clusters of ~0.2 μm², which was sufficient to trigger activation. Amplification in signaling also correlated with an increase in cluster frequency and area after addition of the secondary polyclonal cross-linker. However, maximum activation correlated with the binding of ~10 of the 0.9-μm beads, compared with a single 5.5-μm bead. Given the site density of anti-L-selectin on beads, we estimate that ligation of as few as ~100 sites at a single area of contact was sufficient to trigger neutrophil activation. The capacity of L-selectin to amplify transmembrane signals in this manner is distinct from receptor-mediated activation of T cells. For example, it was reported that activation via major histocompatibility complex I presented on 4-μm microspheres required a threshold contact area, but amplification in signal was not achieved by increased frequency in binding of smaller-diameter microspheres (25).

Sites of clustered L-selectin were fourfold more likely to overlap with expression of CD18 subunits activated to adopt the ligand-binding conformation. The high-affinity CD18 colocalized with L-selectin constituted ~20% of expressed CD18 (~40,000 sites) (33). We estimate that in a contact region of 2.0 μm², on the order
of 600 high-affinity CD18 receptors are associated with L-selectin. We recently reported that neutrophil activation by IL-8 also elicits rapid expression of activated CD18. A similar fraction of membrane CD18 (~15%) was found to adopt a clustered topography, which was the minimum required for efficient neutrophil capture to ICAM-1 in shear flow (23).

Recently, we reported (40) that signal transduction as a result of clustering L-selectin by addition of combined HuDREGs on neutrophils utilizes p38 MAPK to effect shape change, integrin activation, and release of secondary, tertiary, and secretory granules. A second MAPK associated with signaling in neutrophils is p42/44 ERK, which is phosphorylated in response to engagement of either PSGL-1 or L-selectin (13, 40). Here we analyzed the rate of p38 and p42/44 MAPK phosphorylation in response to the more potent stimulus of cross-linking anti-L-selectin with polyclonal antibody. Treatment of neutrophils at 2,000 × K_0 with a single HuDREG alone was insufficient to signal MAPK activity. However, within a minute of addition of the secondary goat anti-human antibody, MAPK activity was boosted greater than tenfold. In neutrophils, it appears that both MAPK pathways are activated by the binding of L-selectin, as demonstrated by the equivalent and near total functional inhibition observed with SB-202190 and PD-98059. We confirmed that blocking p42/44 with PD-98059 indeed abrogated phosphorylation of Thr^{202}/Tyr^{204}, however, SB-202190 specific for p38 did not block its kinase activity. This is not entirely surprising given that p38 is made up of four isoforms of which only α and β are blocked by SB-202190. The conclusion is that the intact p38 phosphorylation activity presumed to be generated by the γ- and δ-subunits is not sufficient for activation of CD18.

There is also evidence suggesting that p38 and p42/44 converge at a common regulatory point on activation. These two MAPKs have been demonstrated to form a molecular complex in HeLa and HEK293 cells on stress activation (45). Formation of a complex involving both kinases on signal transduction would account for the near complete inhibition by either p38 or p42/44 inhibitor after L-selectin clustering in neutrophils. However, we confirmed that the p38 and p42/44 signaling pathways are independent after activation, in that inhibitors to each did not affect phosphorylation of the other kinase. We are currently pursuing the question of whether each kinase is capable of triggering distinct cellular functions that superpose to effect optimal activation of β2-integrin adhesion.

Several mechanisms may provide for multivalent binding and clustering of L-selectin as it recognizes and is bound by distinct vascular ligands. L-selectin is expressed on the tips of microvilli and membrane ruffles in a conformation that constitutively recognizes sLe^x expressed on ligands presented on inflamed endothelium and bound to extracellular matrix (3, 8). sLe^x present on L-selectin is also bound with high affinity by E-selectin and has been shown to be one of only a few leukocyte surface receptors that can be isolated with an E-selectin affinity column (16, 28, 47). Moreover, E-selectin binding to L-selectin forms bonds of sufficient strength to facilitate slow rolling of neutrophils (47). We previously reported (35) that neutrophils are activated during rolling on E-selectin in a parallel plate flow chamber. We are currently pursuing the hypothesis that E-selectin upregulated on inflamed endothelium binds multivalently to L-selectin and other sLe^x-presenting receptors, resulting in the rapid signaling of firm adhesion of neutrophils in shear flow (Pearson et al., unpublished observations).

There is also recent evidence that indicates L-selectin may undergo a conformational change in response to specific epitope binding that exposes a high-avidity site within the lectin domain (21). This in turn triggers association of L-selectin with the cytoskeleton and an increase in the avidity of adhesion to physiological ligands. Signaling via L-selectin has also been induced through dimerization of its cytoskeletal domains in a transfected lymphoblastoid cell line (22). This resulted in increased binding of carbohydrate ligands, an increase in the strength of lymphocyte adhesive interactions with vascular endothelium, and constitutive induction of intercellular aggregation (9). In this regard, we have demonstrated here that ligation of L-selectin by bivalent IgGs at distinct epitopes elicited membrane patching. Thus one mechanism for signal transduction may involve clustering of L-selectin by engagement of vascular counterreceptors that bind multivalently to O-linked sLe^x, that in turn trigger cytoskeletal association and assembly of signal-promoting elements. A second mechanism that can promote clustering of L-selectin as leukocytes interact with vascular ligands is its colocalization with other receptors in the plane of the membrane. We previously reported (34) on the colocalization between L-selectin and CD18 on the surface of neutrophils after anti-L-selectin antibody binding and cross-linking, and others have demonstrated the association of urokinase plasminogen activator receptor with the lectin domain of L-selectin (38). Here we show preferential association of high-affinity CD18 at sites of L-selectin clustering.

The physiological implication is that the extent of L-selectin engagement by extensively sialylated carbohydrate ligands or multivalent recognition and clustering by vascular lectinlike receptors at sites of inflammation provides for amplification in transmembrane signaling of β2-integrin adhesion function. Moreover, this mechanochemical transduction through L-selectin can occur at a single site of membrane contact involving a few microvilli expressing a few hundred receptors, thereby providing a means for the local and rapid recruitment of activated β2-integrin and the efficient conversion from neutrophil rolling to arrest.

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