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# Avidity enhancement of L-selectin bonds by flow: shear-promoted rotation of leukocytes turn labile bonds into functional tethers

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L-selectin is a key lectin essential for leukocyte capture and rolling on vessel walls. Functional adhesion of L-selectin requires a minimal threshold of hydrodynamic shear. Using high temporal resolution videomicroscopy, we now report that L-selectin engages its ligands through exceptionally labile adhesive bonds (tethers) even below this shear threshold. These tethers share a lifetime of 4 ms on distinct physiological ligands, two orders of magnitude shorter than the lifetime of the P-selectin–PSGL-1 bond. Below threshold shear, tether duration is not shortened by elevated shear stresses. However, above the shear threshold, selectin tethers undergo 14-fold stabilization by shear-driven leukocyte transport. Notably, the cytoplasmic tail of L-selectin contributes to this stabilization only above the shear threshold. These properties are not shared by P-selectin– or VLA-4–mediated tethers. L-selectin tethers appear adapted to undergo rapid avidity enhancement by cellular transport, a specialized mechanism not used by any other known adhesion receptor.

## Introduction

Leukocyte capture and subsequent rolling, mediated by L-selectin on both endothelial and leukocyte-expressed glycoprotein ligands, require critical thresholds of shear stresses to occur (Finger et al., 1996; Alon et al., 1997; Lawrence et al., 1997; Chen and Springer, 1999; Greenberg et al., 2000). In contrast, rolling adhesion mediated by other selectins, such as P- and E-selectin, exhibits weak or no dependence on shear threshold (Finger et al., 1996; Alon et al., 1997; Lawrence et al., 1997). Furthermore, rolling or capturing mediated by α4 integrins are markedly destabilized by shear (Alon et al., 1995b; Berlin et al., 1995; de Chateau et al., 2001). The dependence of L-selectin adhesions on shear threshold appears to be an intrinsic property of the selectin molecule, as it is shared by cell-free immobilized L-selectin interacting with cell- or bead-presented ligands (Alon et al., 1998; Dwir et al., 2000; Greenberg et al., 2000). However, enhanced L-selectin interactions with adhesive ligands reduce the dependence of L-selectin adhesions on a shear threshold (Puri et al., 1998; Dwir et al., 2000).

Transient leukocyte tethers to low density ligands are the smallest adhesive events observable under shear flow (Alon et al., 1995a). Analyses on such quantal L-selectin tethers performed using regular videomicroscopy, i.e., at a 20–30-ms resolution, revealed that below critical shear values, L-selectin fails to form functional tethers (Alon et al., 1997). This result sharply contrasted the ability of P- and E-selectins, as well as α4 integrins, to form functional tethers at any subphysiological shear stress tested or at stasis (Alon et al., 1997; de Chateau et al., 2001). This collapse of L-selectin tethering was hence postulated to reflect a unique mechanical property of the L-selectin bond (Puri et al., 1998; Dwir et al., 2000). The ability of L-selectin to form functional tethers was attributed to a critical force required to press the L-selectin–expressing cell onto the substrate and to overcome a repulsive barrier for binding to the selectin ligand (Chen and Springer, 1999). To elucidate the molecular basis of this shear dependence of quantal L-selectin tethers, the building units of L-selectin–mediated adhesions, we analyzed L-selectin–mediated interactions with low densities of native endothelial and leukocyte-derived ligands at a much higher temporal resolution than previously used to study the kinetics of quantal L-selectin tethers (Alon et al., 1997). Notably, at these shear stresses, both lymphocyte-based and cell-free L-selectin were found to form specific but exceptionally labile adhesive tethers,

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with lifetimes 30-fold shorter than that estimated from L-selectin tether analysis performed at low time resolution (Alon et al., 1997). Strikingly, these tethers appeared insensitive to increased shear stresses at a range of subthreshold shear rates. Above the threshold shear rate, stabilized tethers formed with up to 14-fold longer duration. This is an exceptional stabilization mechanism by which enhanced shear rate up-regulates L-selectin function.

## Results

### Labile force-insensitive L-selectin–ligand tethers undergo dramatic stabilization above a threshold shear

The dynamic properties of transient L-selectin tethers to low density ligands provide key insights into the unique mechanokinetic properties of L-selectin bonds (Alon et al., 1997; Chen and Springer, 1999). To gain further insights into L-selectin function at shear stresses close to the shear threshold required for functional L-selectin–mediated adhesions, we analyzed L-selectin interactions with immobilized PNAd, the major L-selectin glycoprotein ligands expressed on lymph node high endothelial venules (Berg et al., 1991). Displacements of L-selectin–expressing lymphocytes over low density PNAd, immobilized at a density too low to support rolling interactions (Fig. 1 A), were recorded at either a 20- or 2-ms temporal resolution (Fig. 1 A). Below a critical shear stress of 0.3 dyn/cm$^2$, essentially no tethers were detected at the 20-ms resolution, suggesting loss of L-selectin function, in agreement with previous experiments conducted at a 30-ms video resolution (Alon et al., 1997). However, lymphocyte motions over identical PNAd recorded at a 2-ms resolution revealed a significant number of specific L-selectin–dependent interactions (Fig. 1, AII, BI, and BII; Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200303134/DC1) that could be fully suppressed by lectin domain blockage (Fig. 1, AII and B; unpublished data). The duration of these interactions varied from 4 to 20 ms, and their dissociation fit first-order kinetics (Fig. 1 D) with a very high rate constant, $k_{off}$ (Fig. 1 E). Notably, the majority of these tethers were separated by step distances two- to eightfold larger than the lymphocyte diameter, consistent with their inability to support rolling adhesions (Fig. 1 C). Furthermore, these interactions appeared quantal because their $k_{off}$ values were not further increased when L-selectin–mediated interactions were assessed on lower densities of PNAd, although their formation rates were proportional to ligand density (Table I). Thus, improved temporal resolution allowed the identification of exceptionally labile, quantal L-selectin–ligand tethers that form at subthreshold shear stresses, but are undetectable by regular videomicroscopy.

Unexpectedly, although applied forces accelerate rupture rates of L-selectin bonds (Evans et al., 2001), the stability of these labile L-selectin–PNAd tethers did not decrease over a range of low shear stresses (Fig. 1 E). Even more strikingly, the dissociation rate of L-selectin tethers forming at a shear stress of 0.4 dyn/cm$^2$ was 14-fold lower than the corresponding rate measured for L-selectin tethers at 0.3 dyn/cm$^2$ (Fig. 1 E). Thus, at shear stresses approaching physiological shear threshold values (Finger et al., 1996), the duration of L-selectin–specific tethers dramatically increased (Fig. 1, BIII, BIV, C, and D; Videos 3 and 4, available at http://www.jcb.org/cgi/content/full/jcb.200303134/DC1). Shear-dependent stabilization of tethers was accompanied by a persistent increase in the rate of lymphocyte tethering to PNAd (Fig. 1 AII), increased rate of subsequent tethers (Fig. 1 AII, parenthesis) and progressive decrease in their step distance (Fig. 1 C). Above the threshold shear, L-selectin tethers became progressively shorter with exponential increase in their $k_{off}$ in response to increasing shear stresses (Fig. 1, D and E), as reported previously (Alon et al., 1997; Smith et al., 1999; Dwir et al., 2001). Thus, collection of data at a 2-ms temporal resolution identified a new class of L-selectin tethers undetectable by regular videomicroscopy forming at subthreshold shear values.

### Mucin presentation and cellular environment do not alter the kinetic properties of L-selectin–carbohydrate bonds

PNAd is comprised of largely extended mucin carriers of carbohydrate L-selectin ligands (Hemmerich and Rosen, 2000). Shear stress has been suggested to directly increase mucin recognition by L-selectin by overcoming a repulsive barrier between the selectin and its counter-receptor (Chen and Springer, 1999). Therefore, we studied the microkinetic properties of L-selectin tethers with a nonmucin ligand, a short L-selectin–binding sulfated sLe$^x$-decorated glycopep-

Table I. **Frequency of initial tethering and dissociation rate constants of L-selectin–mediated tethers at different low densities of PNAd**

| Wall shear stress[a], 0.3 dyn/cm$^2$ | | | | Wall shear stress[a], 0.4 dyn/cm$^2$ | | | |
|---|---|---|---|---|---|---|---|
| PNAd density | Frequency of initial tethering[b] | $k_{off}$[c] | $r^2$ | PNAd density | Frequency of initial tethering[b] | $k_{off}$[c] | $r^2$ |
| *ng/ml* | | $s^{-1}$ | | *ng/ml* | | $s^{-1}$ | |
| 10.0 | 18.6 | 253.6 | 0.991 | 10.0 | 28.3 | 17.4 | 0.999 |
| 7.5 | 10.6 | 250.3 | 0.995 | 7.5 | 15.9 | 17.9 | 0.996 |
| 5.0 | 8.3 | 245.8 | 0.994 | 5.0 | 5.3 | 18.3 | 0.997 |
| 4.0 | 5.5 | 247.3 | 0.984 | 4.0 | <1 | ND | - |
| 0 | 1.3 | ND | - | 0 | 0 | - | - |

ND, not determined.
[a]All measurements were performed at a 2-ms resolution, as in Fig. 1.
[b]Frequency is expressed as the percentage of cells tethered at least once across the 0.67-mm field path. At PNAd, 7.5 ng/ml, >90% of cells tethered only once to the substrate.
[c]More than 92% of all tethers disassociated with the indicated $k_{off}$.

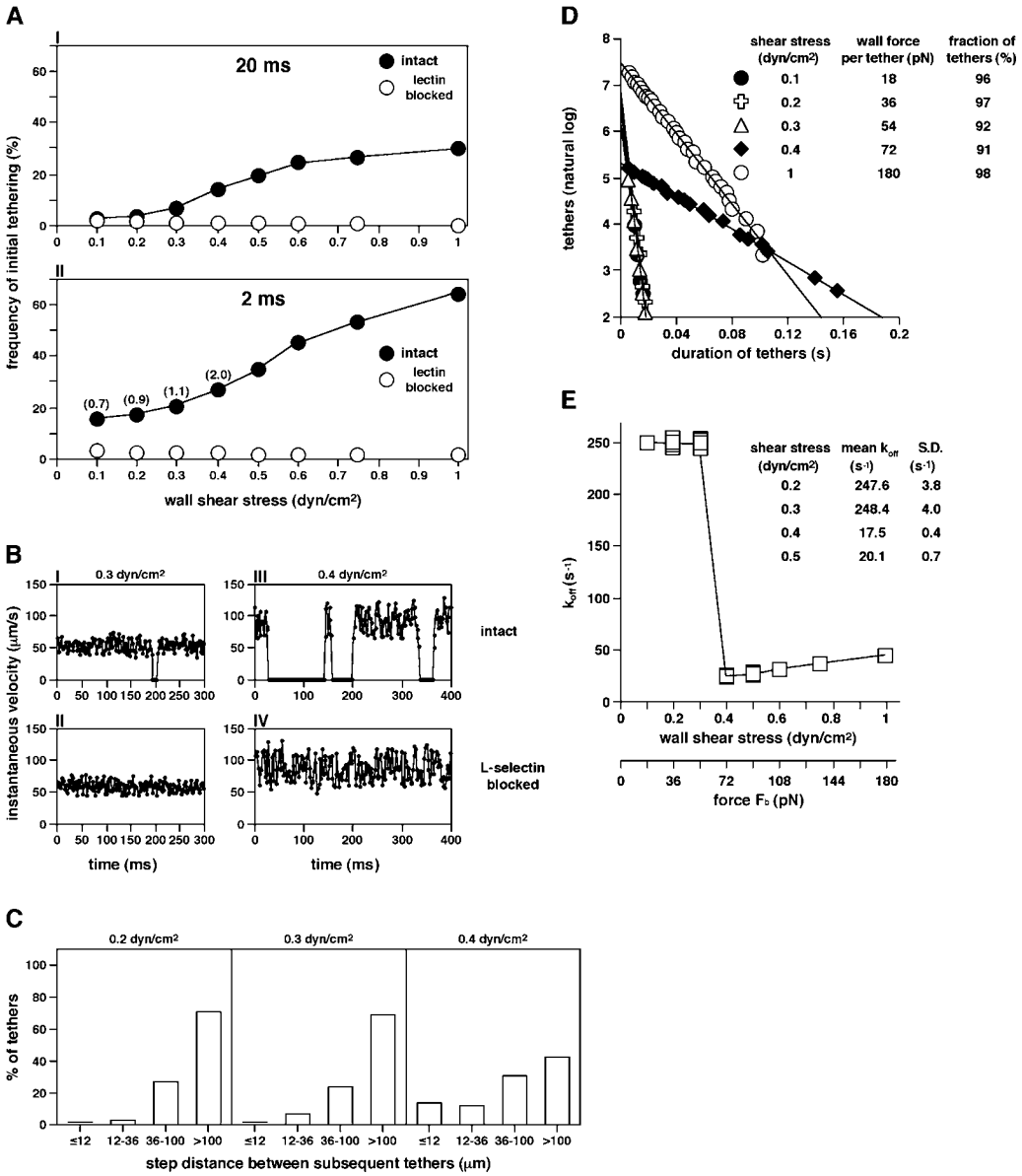


Figure 1. **Transient tethers mediated by L-selectin–expressing lymphocytes interacting with PNAd at low shear stresses undergo dramatic stabilization above critical shear.** (A) Frequency of initial tethering of L-selectin–transfected pre B lymphocytes to substrate containing low density of the L-selectin ligand PNAd, coated at 10 ng/ml. Cell motions were recorded in parallel at either 50 frames/s (20-ms resolution, top) or 500 frames/s (2-ms resolution, bottom). At a standard video resolution, none of the tethered cells established persistent rolling on this substrate at any shear stress tested. The frequency of cells that tethered to the substrate at least once while passing through a 670-μm-long path is shown (filled symbols) at each time resolution. Background tethering in the presence of the L-selectin inhibitory mAb DREG-200 is depicted by open symbols. The mean number of discrete tethers formed by a tethered cell subsequent to the initial tethering point at the indicated stresses is depicted in parentheses. (B) Instantaneous velocities of L-selectin–expressing cells interacting with 10 ng/ml PNAd at shear stresses of 0.3 (left panels) or 0.4 dyn/cm$^2$ (right panels), calculated by analysis of cell motions recorded at a 2-ms resolution. Cells were perfused alone (top panels) or in the presence of DREG-200 (bottom panels). The cell motions analyzed in panels I–IV are shown, respectively, in Videos 1–4 (available at http://www.jcb.org/cgi/content/full/jcb.200303134/DC1). (C) Distribution of mean step distance values at different shear stresses. The step distance, a continuous displacement between successive pauses (BIII) was determined at the indicated wall shear stresses for at least 50 tethered cells. (D) Dissociation kinetics of all L-selectin–mediated tethers recorded at a 2-ms resolution in C. Rate constants of tethers formed at different shear stresses were determined as explained in the Materials and methods section. Data points that fit first-order dissociation curves were connected by straight lines, with slopes equal to $-k_{off}$. Data presented in A–D were determined in a single experiment representative of five. (E) The dissociation rate constants determined in D were plotted as a function of the wall shear stress and the force applied on a singular microvillus. Constants calculated for the indicated stresses from data collected in five independent experiments are shown. Mean ± SD of all experiments are depicted in the inset.

tide, derived from the major selectin recognition site on PSGL-1, the key leukocyte-expressed glycoprotein ligand of all three selectins (McEver, 2002). The 19mer peptide was presented on an avidin scaffold via a biotin linked to its non glycosylated terminus (Somers et al., 2000) at a site density fivefold lower than that capable of supporting rolling interactions at physiological shear stresses (unpublished data). Interestingly, both the duration of L-selectin tethers to this

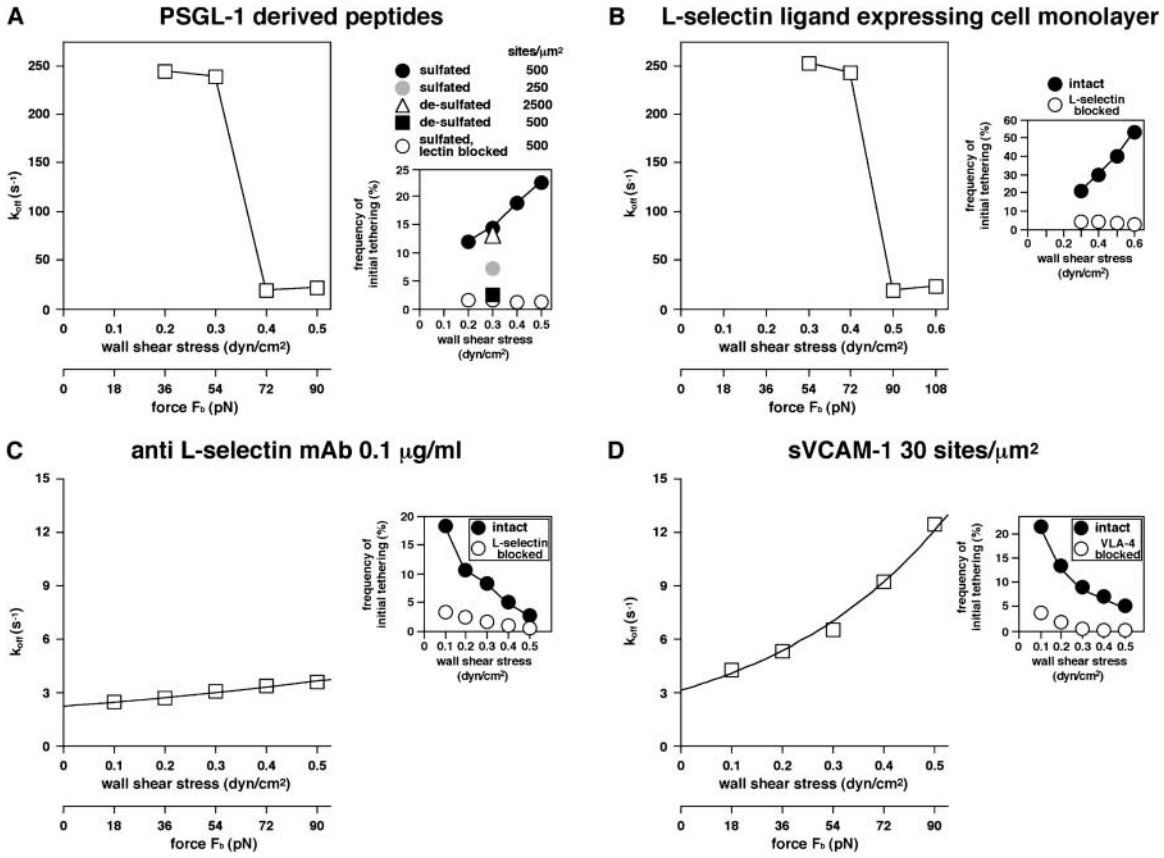


Figure 2. **Shear-promoted stabilization of L-selectin tethers is shared by carbohydrate ligands presented on different scaffolds.** (A) The dissociation rates of L-selectin tethers mediated by L-selectin–expressing pre B lymphocytes perfused over substrate-bound PSGL-1–derived glycopeptides. Duration of tethers was determined by computerized analysis of 2-ms resolution recordings as in Fig. 1, and dissociation rate constants were plotted as in Fig. 1 E. At subthreshold shear stresses, >99% of all tethers dissociated with the indicated first-order $k_{off}$. Avidin-coated substrates were overlaid with a sulfated $sLe^x$-decorated 19mer glycopeptide derived of the N′ terminus of PSGL-1 at 500 sites/$\mu m^2$. Inset: the frequencies of cells initially tethered to either the sulfated PSGL-1–derived glycopeptide or to its desulfated analogue measured at the indicated shear stresses. Background tethering (lectin-blocked) was measured in the presence of 50 μg/ml of the L-selectin blocker fucoidin. (B) Dissociation rate constants for L-selectin bonds mediated by L-selectin–expressing pre B lymphocytes interacting with a monolayer of ECV-304 cells expressing sulfated $sLe^x$-bearing core-2 glycans. Inset: frequency of initial L-selectin–dependent and background lymphocyte tethering to the monolayer. At all indicated shear stresses, >91% of all tethers dissociated with the indicated first-order $k_{off}$. (C) Effect of increased wall shear stress on the dissociation kinetics of L-selectin–mediated tethers of pre B lymphocytes perfused over immobilized anti-L-selectin mAb DREG-200 coated at 0.1 μg/ml. Duration of L-selectin–specific tethers was determined from motion analysis at the 20-ms resolution video recording because all adhesive interactions lasted longer than 40 ms, as verified by high speed videomicroscopy (not depicted). The dissociation rate constants were derived for all L-selectin–specific tethers formed on the DREG-200 and were plotted as a function of the wall shear stress applied on the L-selectin–expressing lymphocytes. Inset: frequency of cells initially tethered to the immobilized mAb at the indicated shear stresses. Background tethers were determined for selectin-expressing lymphocytes preincubated with 1 μg/ml DREG-200. (D) Effect of increased wall shear on the dissociation kinetics of VLA-4–mediated tethers of pre B lymphocytes interacting with low density of sVCAM-1 (30 sites/$\mu m^2$). Tether duration was derived at a 20-ms resolution video recording because all VLA-4–specific adhesive interactions lasted longer than 40 ms, as verified by high speed videomicroscopy. At 0.1 and 0.2 dyn/$cm^2$, 95 and 96% of all tethers, respectively, dissociated with the indicated first-order $k_{off}$. At higher stresses, all tethers dissociated with the indicated koff. Inset: frequency of tethers initiated by VLA-4 (filled symbols), and background tethering measured in the presence of 20 ng/ml of the VLA-4–blocking peptide BIO1211 (open symbols). Data presented in A–D were collected from a single experiment, each representative of three.

nonmucin selectin ligand and their corresponding $k_{off}$, as well as the ability of labile tethers to undergo dramatic stabilization above a threshold shear stress, were indistinguishable from bonds between L-selectin and PNAd (Fig. 2 A). The rate of L-selectin–mediated tethering was proportional to the glycopeptide density and was markedly reduced on the corresponding desulfated PSGL-1–derived glycopeptide (Fig. 2 A, inset). L-selectin–mediated tethering to the sulfated but nonfucosylated isoform of the PSGL-1 peptide was completely eliminated even at coating densities >50,000 sites/$\mu m^2$ (unpublished data). Thus, the kinetics of L-selectin tethers and their tight shear dependence are independent of mucin presentation of the L-selectin ligand. Furthermore, the interactions of L-selectin–expressing lymphocytes with sulfosialylated L-selectin ligands presented on an endothelial monolayer shared similar bond kinetics and stabilization by shear to interactions measured on cell-free PNAd or PSGL-1–derived glycopeptides (Fig. 2 B). Notably, the transition from labile to stable tethers on the monolayer occurred at slightly elevated shear than on PNAd or the PSGL-1 peptide, probably due to the more heterogenous ligand presentation on the monolayer than on the cell-free substrate.

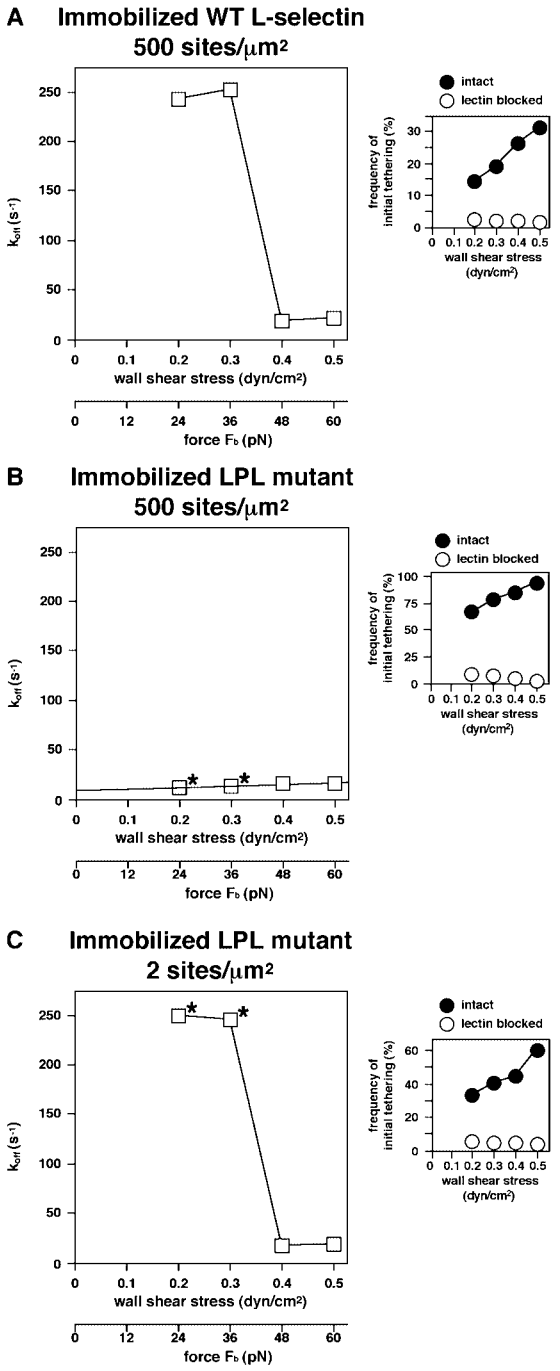


Figure 3. **Enhanced rate of ligand recognition of an EGF domain mutant results in stabilized L-selectin tethers below the shear threshold, but does not alter intrinsic tether lifetime.** Dissociation rate constants of neutrophil tethers mediated by immobilized cell-free wild-type L-selectin (A) or cell-free L-selectin mutated in its EGF domain (LPL) (B) at the indicated shear stresses. Insets: the frequency of neutrophils initially tethered to L-selectin (A, filled symbols) or LPL (B, filled symbols) at the indicated shear stresses. Values depict the percentage of neutrophils tethered at least once while passing at the indicated shear stresses over the immobilized selectin variants. Background tethering (open symbols) was measured in the presence of 50 μg/ml fucoidin. Each L-selectin variant was isolated from pre B lymphocyte transfectants and saturably anchored on substrates (coated at 500 sites/$\mu m^2$) of the tail-specific mAb CA21. The most rapidly dissociating tethers (90% or more) dissociated with the indicated first-order $k_{off}$. (C) Dissociation rate constants and frequency of tethering of neutrophils mediated by highly diluted immobilized LPL. At wall shear stresses indicated by asterisks, a considerable fraction of LPL-specific tethers (23 and 37% at 0.2 and 0.3 dyn/$cm^2$, respectively) had a slower $k_{off}$, similar to values obtained in B, possibly due to multivalent associations. Inset: the frequency of neutrophils initially tethered to immobilized LPL. Tethering frequencies were determined as in previous figures. A representative experiment of three is shown.

Notably, L-selectin tethers to immobilized lectin-binding mAb dissociated at 100-fold slower rates than L-selectin tethers formed on all carbohydrate ligands tested (Fig. 2 C). In sharp contrast to all other tested L-selectin–carbohydrate interactions (Fig. 1, AII; Fig. 2, A and B, insets), the mAb–L-selectin interactions did not undergo any stabilization at elevated shear, and in contrast to L-selectin–carbohydrate interactions (Fig. 1 A), their rate of formation steadily declined as the shear stress was increased from 0.1 to 0.5 dyn/$cm^2$ (Fig. 2 C). In the same lymphocytes, VLA-4 integrin-mediated tethers to low density VCAM-1 also exhibited marked destabilization upon shear increase from 0.1 to 0.5 dyn/$cm^2$, and their rate of formation steadily declined with increasing shear (Fig. 2 D). Interestingly, the lifetime of these quantal VLA-4–VCAM-1 tethers at low shear stresses was 40–50 fold longer than L-selectin–carbohydrate tethers (compare Fig. 2 D with Fig. 1 E). Thus, both VLA-4–VCAM-1 and L-selectin–mAb bonds support long-lived protein–protein interactions that are persistently destabilized by elevated shear stresses, contrasting the effect of L-selectin–carbohydrate bonds.

## Enhanced recognition of ligand by L-selectin rescues its adhesion dependence on a threshold shear

Adhesive interactions between cell-free immobilized L-selectin and L-selectin ligands expressed on flowing neutrophils exhibited nearly identical kinetics and shear-dependent stabilization properties as lymphocyte-based L-selectin interacting with cell-free PNAd or PSGL-1 glycopeptides (compare Fig. 3 A with Fig. 1 E and Fig. 2 A). Therefore, we used cell-free L-selectin systems to further address whether specific alterations of the selectin ectodomain may modulate its intrinsic bonding properties at low shear stresses. Ligand recognition by an L-selectin mutant, in which the native EGF domain was substituted with that of P-selectin (Kansas et al., 1994), was next analyzed at a 2-ms resolution. The mutant (termed LPL) retains the selectin specificity and lectin structure of native L-selectin, but supports functional tethers at a 100-fold higher efficiency than wild-type L-selectin at physiological shear stresses (Dwir et al., 2000, 2002). When immobilized at identical densities, cell-free LPL not only supported much higher frequencies of tethers than cell-free L-selectin at any shear stress and density tested (Fig. 3, A and B, insets; unpublished data), but also mediated stable tethers with $k_{off}$ 20–30-fold lower than L-selectin tethers even at shear stresses below 0.4 dyn/$cm^2$ (Fig. 3, A and B). Consistent with its higher ligand recognition properties, LPL coated at 2 sites/$\mu m^2$ could support comparable levels of neutrophil tethers as L-selectin immobilized at 500 sites/$\mu m^2$ (Fig. 3 C), a density too low to support physiological neutrophil rolling (Dwir et al., 2000, 2002). Under these limiting conditions, the majority of LPL-mediated interactions dissociated at subthreshold shear stresses with similar

$k_{off}$ values to bonds mediated by L-selectin. Thus, the labile nature of the selectin–carbohydrate bond cannot be corrected by an EGF domain substitution that alters the lectin domain reactivity under shear flow. However, native L-selectin, when encountering sufficiently high density ligand, readily converted its labile tethers into stable tethers even at subthreshold shear stresses. At a subthreshold shear stress, for instance, a 1.5- and 2-fold higher density of ligand not only increased tether formation by L-selectin, but also allowed 20 and 50% of L-selectin bonds, respectively, to undergo 15–20-fold stabilization (Fig. 4, PNAd 15 and 20 ng/ml, respectively). Similarly, lymphocytes expressing high L-selectin levels could also stabilize tethers to low density PNAd even at subthreshold shear stresses (unpublished data). Conversely, reduced PNAd density restricted L-selectin–mediated tether stabilization even at the permissive shear stress of 0.4 dyn/cm$^2$ (Table I). Thus, the ability to stabilize short-lived L-selectin bonds can be partially reconstituted, even below the shear threshold, when L-selectin (or ligand) can locally associate with sufficiently high densities of ligand (or of L-selectin).

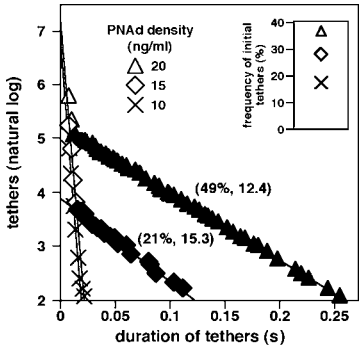


Figure 4. **Slight increase in ligand density stabilizes a fraction of L-selectin tethers even below the shear threshold.** Dissociation kinetics of transiently tethered L-selectin–expressing pre B lymphocytes measured at a subthreshold shear stress of 0.3 dyn/cm$^2$ vary dramatically with PNAd density. Tether duration points that fit a first-order dissociation curve (open and filled triangles) were connected by straight lines, with slopes equal to $-k_{off}$. At all densities, L-selectin tethers fit a straight line with a fast $k_{off}$, $k_1$. More than 98% of tethers formed on 10 ng/ml PNAd dissociated with this $k_1$. At PNAd coated at 15 and 20 ng/ml, fractions of tethers fit a second straight line with a smaller slope, with a second rate constant, $k_2$. The fractions of tethers out of the total tethers that dissociated with $k_2$ and their dissociation rate constants are each indicated in parentheses near the corresponding plots. Inset: frequencies of tethers initiated at 0.3 dyn/cm$^2$ by the L-selectin–expressing lymphocytes interacting with different PNAd densities. Values depict net tethering frequency after subtracting background tethering.

## Shear rate rather than shear stress determines L-selectin tether stabilization

To differentiate between effects of shear force and shear flow rates on L-selectin tether properties, we next altered the viscosity of the perfusion medium by inclusion of the nontoxic dextran Ficoll, which alters the shear stress while maintaining the shear rate under given perfusion conditions (Chen and Springer, 2001). Ficoll at up to 6% wt/vol does not change the medium osmolarity (Chen and Springer, 2001) and was verified to not affect lymphocyte viability, shape, or velocity near the substrate (unpublished data). A 2.6-fold increase in stress experienced by flowing lymphocytes interacting with low density PNAd resulted in a reduction of stable L-selectin–mediated tethers detected by regular videomicroscopy (Fig. 5 A, top, and Fig. 5 B). Notably, at shear rates below a critical value, i.e., 20 and 30 $s^{-1}$ (Fig. 5 B), a 2.6-fold increase in viscosity and shear stress (but not shear rate) caused by the presence of Ficoll did not stabilize L-selectin–mediated tethers (Fig. 5 B), although the shear stresses applied on these tethers, 0.5 and 0.8 dyn/cm$^2$, respectively, were above the threshold shear stresses at which tether stabilization normally took place (Fig. 1 E). Thus, no stable lymphocyte tethers, i.e., with $k_{off}$ <250 $s^{-1}$, occurred at a shear rate of 20 $s^{-1}$ at 2.6 cP (Fig. 5 A, open circle; Fig. 5 B, open squares). In contrast, identical shear stress generated at a shear rate of 50 $s^{-1}$ at 1 cP in the absence of Ficoll promoted significant levels of such stable tethers (Fig. 5 A, filled circle; Fig. 5 B, x symbols). Thus, below a critical shear rate, optimal shear stress was clearly insufficient to generate stable L-selectin tethers. Consequently, at low shear rates, only short-lived L-selectin tethers formed, and a 2.6-fold increase in stress did not further destabilized these tethers (Fig. 5 B, shear rates <40 $s^{-1}$). Thus, although increased shear flow had a proadhesive effect on L-selectin adhesiveness at subphysiological shear rates, increased shear stress at these subphysiological rates lacked any destabilizing effect on L-selectin bonds. However, above a critical shear rate of 30 $s^{-1}$, as soon as stable L-selectin tethers could form, a 2.6-fold increase in stress was accompanied by enhanced tether dissociation (Fig. 5 B, rate of 40 $s^{-1}$). Thus, dissociation of functional L-selectin tethers was governed by the shear stress, and therefore, by the force applied on tethered lymphocytes only above a threshold shear rate.

## Cytoskeletal anchorage of L-selectin stabilizes tethers only above the shear threshold

Constitutive anchorage of L-selectin to the actin cytoskeleton has been reported to facilitate millisecond stabilization of L-selectin tethers (Dwir et al., 2001). Therefore, we next asked whether improper anchorage of L-selectin further destabilizes tethers formed at subthreshold stresses. Although tail-deleted L-selectin supported less lymphocyte tethering to PNAd than intact L-selectin at elevated shear stresses (Fig. 6 A), the L-selectin mutant initiated a comparable number of specific interactions on PNAd at shear stresses <0.4 dyn/cm$^2$. Interestingly, the tail-deleted and intact (wild-type) L-selectin formed essentially identical short-lived tethers at these low shear stresses (Fig. 7 B). Furthermore, tethers mediated by the tail-less L-selectin underwent dramatic stabilization at the shear stress threshold, although stabilized tethers mediated by the tail-less L-selectin were shorter than L-selectin–mediated tethers (Fig. 6 B, stresses ≥0.4 dyn/cm$^2$). In conclusion, lack of cytoskeletal anchorage of L-selectin did not destabilize L-selectin bonds forming at low shear rates and did not abrogate their ability to stabilize tethers, although at reduced magnitude, at or above the threshold shear rate.

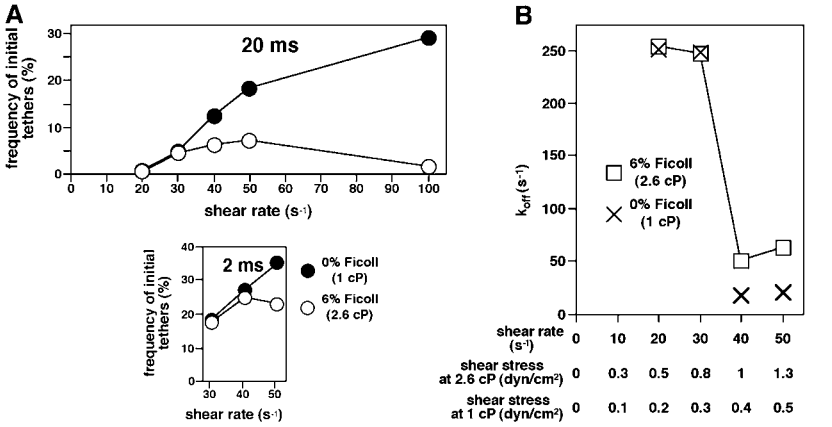


Figure 5. **Shear rate rather than shear stress governs L-selectin tether stabilization, as L-selectin tethers are insensitive to shear stress below a critical shear rate.** (A) Top: frequency of stable tethers mediated by L-selectin at two different medium viscosities, identified by regular video-microscopy at a 20-ms resolution. Pre B lymphocytes were perfused on 10 ng/ml PNAd at the indicated shear rates in binding buffer alone (a viscosity of 1 cP, filled circles) or supplemented with 6% Ficoll, which raised the medium viscosity to 2.6 cP (open circles). Values depict the percentage of lymphocytes tethered at least once while passing over a path of PNAd. Bottom: frequencies of L-selectin–specific tethers initiated at the indicated shear rates on identical fields as in the top panel, but determined at a 2-ms resolution. (B) First-order dissociation rate constants of L-selectin tethers formed on 10 ng/ml PNAd measured at high viscosity (6% Ficoll, 2.6 cP) at a 2-ms resolution, as described in Fig 1. At shear rates of 10 and 20 $s^{-1}$, >98% of L-selectin–mediated tethers dissociated with the indicated first-order $k_{off}$. At higher rates, >94% of the tethers dissociated with the indicated first-order $k_{off}$. For comparison, $k_{off}$ values of L-selectin tethers determined in Fig. 1 E in regular medium (0% Ficoll) are depicted by the x symbols. Experiments in A and B are representative of three.

## Discussion

L-selectin bonds must be kept at low affinity states due to the ubiquitous expression of both L-selectin and its carbohydrate ligands by most circulating leukocytes (Fuhlbrigge et al., 1996). Therefore, L-selectin tethers have evolved to undergo dramatic stabilization rather than accelerated rupture under enhanced shear rates. Consequently, L-selectin is prevented from interacting with its ubiquitous ligands at stasis or very low shear rates both in vitro and in vivo (Finger et al., 1996). The molecular basis for this unique property of L-selectin has been difficult to define. The ability of shear forces to directly strengthen receptor ligand interactions is restricted to very few adhesion receptors (Thomas et al., 2002; Marshall et al., 2003). This reflects the slippage characteristics of almost all adhesive bonds, i.e., their enhanced rupture and dissociation in the presence of tensile forces (Bell, 1978; Dembo et al., 1988; Chang et al., 2000). Therefore, several mechanisms have been suggested for shear-induced activation of L-selectin interactions with its ligands, but experimental evidence for either one has been missing. These include increased probability of encounter between receptors and ligands due to faster transport (Chang and Hammer, 1999), stress-enhanced flattening of tethered cells resulting in a larger number of contacts with ligand (Evans et al., 2001), and increased propensity to overcome a repulsive barrier between selectin and ligand (Chen and Springer, 1999). A recent report also suggested that very low forces applied on singular P-selectin–PSGL-1 bonds prolong their lifetime by about fivefold (Marshall et al., 2003), a unique property potentially shared by other selectin bonds.

However, the present paper suggests a new explanation for the tight dependence of L-selectin adhesiveness on critical shear flow. We demonstrate that at low shear rates, both cell-based and cell-free L-selectin form millisecond-lived tethers with their ligands. Subtle increases in either ligand density (Fig. 4) or in the rate of L-selectin transport over ligand, controlled by the shear rate (Fig. 5, A and B) result, however, in dramatic stabilization of multivalent L-selectin contacts leading to functional adhesive tethers. Thus, the failure of L-selectin to mediate functional adhe-

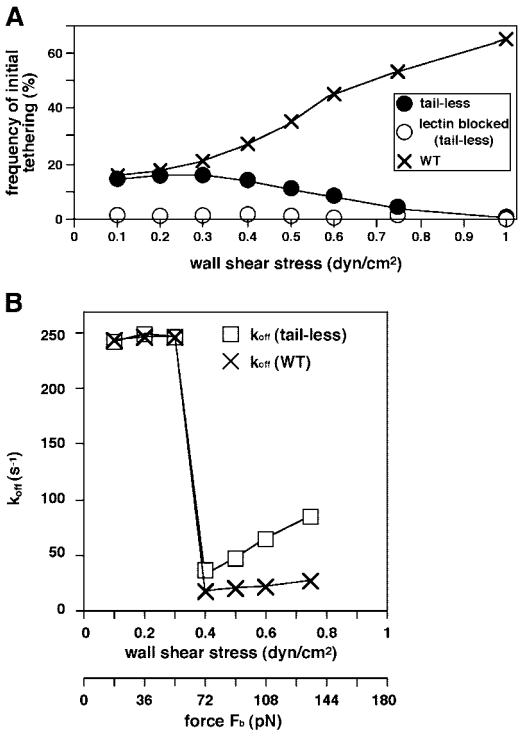


Figure 6. **Cytoplasmic tail deletion of L-selectin does not destabilize L-selectin–ligand tethers formed below the shear threshold.** (A) Frequency of initial lymphocyte tethering mediated by tail-deleted L-selectin to 10 ng/ml PNAd at the indicated shear stresses (filled symbols). Frequency is expressed as in previous figures. For comparison, all tethering values mediated by wild-type (WT) L-selectin and determined in Fig. 1 A II are depicted by the x symbols. Background tethering, measured in the presence of fucoidin, is depicted in the open symbols. (B) Dissociation kinetics of tail-deleted L-selectin–mediated tethers. Rate constants ($k_{off}$) of bonds formed at different shear stresses were plotted as a function of the wall shear stress and the force applied on singular microvilli. At all indicated shear stresses, >95% of all tail-deleted L-selectin–mediated tethers dissociated with the indicated first-order $k_{off}$. For comparison, $k_{off}$ values of bonds mediated by wild-type (WT) L-selectin and determined in Fig. 1 E are depicted by the x symbols. All cellular motions were recorded at a 2-ms resolution and analyzed as in Fig. 1. Data presented were collected in a single experiment representative of three.

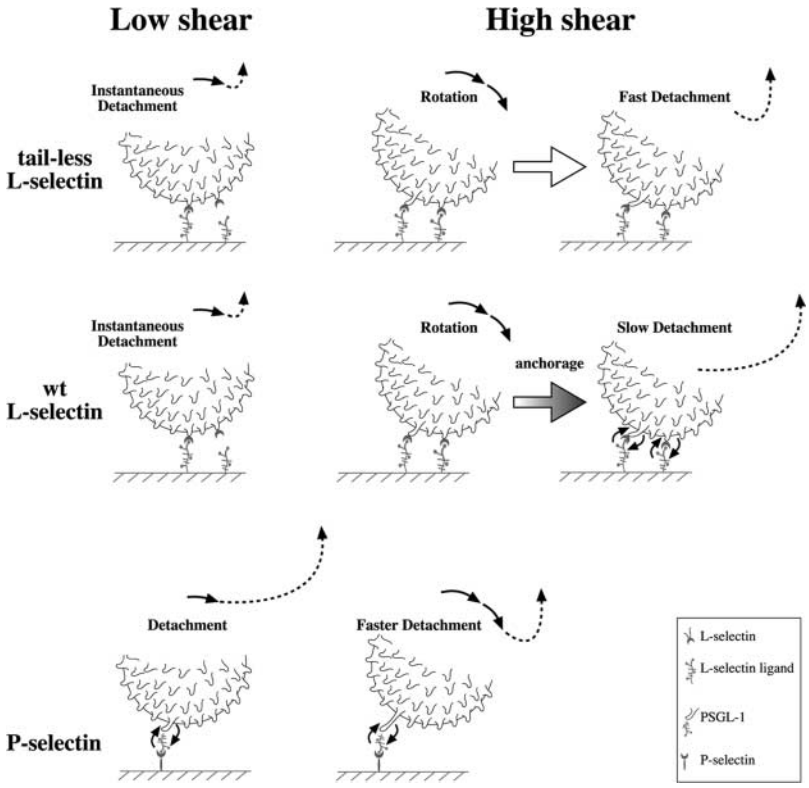


Figure 7. **A postulated two-step mechanism accounting for the role of cellular transport and of cytoskeletal anchoring of L-selectin in stabilization of L-selectin–mediated tethers.** The L-selectin–expressing lymphocyte translates close to the ligand with hydrodynamic velocity dictated by the shear rate. At a low subthreshold shear rate, the initial L-selectin–ligand bond (top left) dissociates within a few ms before a second bond (e.g., on a neighboring microvillus) can form downstream of the first bond. At high threshold shear rate (top middle), the leukocyte held by the first bond may rotate rapidly enough to encounter a second ligand site (top middle) and to form a multivalent tether. This initial stabilization is conditional for a second, cytoplasmic tail–mediated stabilization event (anchorage) to take place and further prolong tether lifetime, possibly via local rebinding (depicted as ↑↓). As force is loaded, the trailing microvillus extends and the cell detaches (top right). In contrast, the high affinity P-selectin–PSGL-1 bond (bottom left) lasts two orders of magnitude longer than the L-selectin–carbohydrate bond, and undergoes local rebinding favored by selectin and ligand dimerization within the first occupied microvillus, often without a need for a secondary contact (bottom left). Consequently, at elevated shear rates and stresses (bottom right), the P-selectin–mediated tether is therefore generally more rapidly disrupted than at low shear rates.

sion below critical shear stresses is not due to improper recognition of ligand, but reflects a failure to stabilize singular contacts via leukocyte transport over ligand-bearing surface. High resolution videomicroscopy (Videos 1–4, available at http://www.jcb.org/cgi/content/full/jcb.200303134/DC1) suggests that an L-selectin–expressing cell can rotate onto or translate over the surface as it is held by the lever arm provided by its first ligand-occupied microvillus. Enhanced shear rate increases the probability of encounter with secondary ligand sites by the initially tethered leukocyte (Fig. 1, A and C; Fig. 7, top panels). The fact that stabilization is tightly regulated by the shear rate, rather than shear force experienced by the tethered leukocyte (Fig. 5 B), indicates that force is not a major positive regulator of L-selectin adhesion, at least not at low shear conditions. Thus, labile tethers formed below the shear threshold correspond to single L-selectin bonds, whereas above the shear threshold, the shear rate provides sufficient cellular transport to stabilize a functional multivalent tether (Fig. 7). In principle, the order of magnitude stabilization in the lifetime of this contact could be provided by mere increase in bond number (Chen and Springer, 1999). However, if multiple L-selectin bonds would simply decay in parallel, the 14-fold increase in tether lifetime observed at the shear threshold would predict a five-orders of magnitude increase in L-selectin bond number (Goldstein and Wofsy, 1996). Because a twofold increase in ligand density resulted in about half of L-selectin interactions undergoing a 20-fold stabilization (Fig. 4), mere increase in microvilli engagements and in bond number per microvillar contact could not have accounted for such dramatic stabilization. Therefore, avidity amplification of L-selectin tethers should involve exceptionally fast and local rebinding events between microvillar L-selectin and clusters of carbohydrate ligands on single scaffolds.

These millisecond-rebinding events are favored by cytoskeletal anchorage of L-selectin, a process dependent on the cytoplasmic tail of L-selectin (Kansas et al., 1994; Dwir et al., 2001). However, the present paper suggests that below the shear threshold, individual L-selectin bonds form, but also break very rapidly before selectin anchorage provides stabilization to the newly formed tether (Fig. 7). Thus, contacts formed below the shear threshold and lasting 4 ms are insufficient for the L-selectin tail to stabilize the nascent selectin-mediated tether (Fig. 6 B). Critical cellular transport over ligand is mandatory for initial stabilization of this tether because once the contact is prolonged to ~30 ms (Fig. 6 B), the cytoplasmic tail of L-selectin can now participate in further tether stabilization (Fig. 6 B). Interestingly, the cytoplasmic tail facilitates both preformed and ligand-induced cytoskeletal anchorage of L-selectin (Pavalko et al., 1995; Evans et al., 1999), and so, stabilization of the nascent L-selectin tether may involve both the primary ligand-occupied L-selectin and its neighbor L-selectin molecules on the same ligand-occupied microvillus (Fig. 7).

Bond lifetime has been predicted to decrease with any increased loading forces (Bell, 1978), and this has been experimentally confirmed with several types of counter-receptors both by single-bond force spectroscopy measurements (Merkel et al., 1999; Evans et al., 2001) and by measurements of bond duration between counter-receptors experiencing increasing shear forces (Alon et al., 1995a; Pierres et al., 1996; Ramachandran et al., 1999). Nevertheless, over a range of low subthreshold shear stresses generated by increasing shear rates at a fixed medium viscosity (Fig. 1 E) or by increasing viscosity at a fixed rate (Fig. 5 B), the $k_{off}$ of single L-selectin

bonds remained practically force insensitive. Thus, L-selectin bonds are adapted to increase their avidity to surface-bound ligands at low physiological shear stresses with little cost in stress-enhanced bond rupture. Such a mechanism could potentially enhance the formation of other multivalent receptor-mediated bonds, including shear-promoted platelet tethering to von Willebrand factor (Doggett et al., 2002). Successful selectin anchoring to the cytoskeleton, driven by ligand occupancy, may also increase the duration of the selectin tether, reducing its sensitivity to rupture by forces. Indeed, the higher the force loading at higher shear stresses, the higher is the contribution of L-selectin anchorage to stabilization of selectin tethers (Dwir et al., 2001).

Our finding that the lifetime of unstressed singular L-selectin bonds falls in the range of 4 ms should lead to reevaluation of previous affinity measurements of monovalent L-selectin bonds. The $k_{on}$ of selectin ligand association previously estimated from BIAcore analyses to be in the order of $10^5$ $M^{-1}s^{-1}$ (Nicholson et al., 1998) may be in fact much higher, approaching a value of $2.3 \times 10^6$ $M^{-1}s^{-1}$, well within the range of the P-selectin–PSGL-1 association rate ($4.4 \times 10^6$ $M^{-1}s^{-1}$; Mehta et al., 1998). This value may explain the high efficiency by which L-selectin, although the shortest of all selectins, efficiently captures flowing leukocytes to endothelial surfaces and adherent leukocytes. Our new kinetic results also explain why L-selectin partially loses its shear threshold requirement when interacting with multivalent glycoprotein ligands such as GlyCAM-1 (Dwir et al., 1998) or with ligand clusters such as $sLe^x$-bearing glycolipids or polysulfated polymers (Finger et al., 1996). High valency L-selectin ligands in extravascular tissues including the basal aspects of HEV (Hemmerich et al., 2001) may thus stably associate with and signal through lymphocyte L-selectin in shear-free settings. Clearly, L-selectin transport over these multivalent ligands is not required to produce multivalent tethers. Similarly, L-selectin ligands chemically modified to prolong their selectin occupancy do not require shear to generate stable tethers (Puri et al., 1998; Greenberg et al., 2000). In conclusion, the specialized kinetic properties of native L-selectin carbohydrate interactions depicted in this paper may reflect an evolutionary pressure to down-regulate L-selectin interactions with carbohydrate ligands abundantly expressed on circulating leukocytes, and possibly on subsets of blood vessels. These carbohydrates may serve as a pool of emergency ligands that can abruptly promote L-selectin–dependent leukocyte capture to blood vessels and other leukocytes in response to abruptly elevated shear, without de novo ligand synthesis or translocation.

## Materials and methods

### Reagents and antibodies

PNAd, purified from human tonsil lysates (Berg et al., 1991), was a gift from Dr. J.J. Campbell (Children's Hospital, Boston, MA). PSGL-1–derived glycopeptides (sulfated or nonsulfated) were a gift from Dr. R.T. Camphausen (Wyeth/Genetics Institute, Cambridge, MA). Recombinant sVCAM and the VLA-4–specific blocker BIO1211 (Feigelson et al., 2001) were gifts from Dr. R. Lobb (Biogen Inc., Cambridge, MA). The function-blocking or cytoplasmic tail–specific anti-L-selectin mAbs, DREG-200 and CA21, respectively (Kahn et al., 1994), were gifts from Dr. T.K. Kishimoto (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). These mAbs and the anti-PSGL-1 mAb KPL-1, directed against the major P- and L-selectin–binding site (Snapp et al., 1998), were used as purified Ig. Fucoidin, a blocker of the lectin domains of L- and P-selectin (Rosen and Bertozzi, 1994), BSA (fraction V), protein A, $Ca^{2+}$- and $Mg^{2+}$-free HBSS, and Ficoll-Hypaque 1077 were all obtained from Sigma-Aldrich.

### Cells

The tail-deleted analogue L358stop, lacking the 15 carboxyl-terminal cytoplasmic residues, and LPL, L-selectin in which the EGF-like domain of L-selectin has been replaced with the homologous P-selectin domain, were described elsewhere (Kansas et al., 1994; Dwir et al., 2001). These constructs and full-length human L-selectin were stably expressed in 300.19 pre B cells as described previously (Dwir et al., 2000, 2001). The human umbilical vein endothelial cell–derived line, ECV-304 (LS12), stably transfected with FucTVII and *N*-acetylglucosamine 6-*O*-sulfotransferase (Kimura et al., 1999), was a gift from Dr. R. Kannagi (Aichi Cancer Center, Nagoya, Japan). Cells were maintained in RPMI 1640/10% FCS, 2 mM glutamine, and antibiotics. Peripheral blood granulocytes were isolated from anti-coagulated blood after dextran sedimentation and density separation over Ficoll-Hypaque (Dwir et al., 2000).

### Preparation of ligand-coated substrates for flow experiments

PNAd diluted to 5–100 ng/ml in coating medium (PBS supplemented with 20 mM bicarbonate, pH 8.5) were adsorbed onto a polystyrene plate for 15 h at 4°C. DREG-200 and sVCAM-1 diluted in the same coating medium were coated at 37°C for 2 h. All substrates were washed and blocked with PBS supplemented with 2% human serum albumin (PBS/HSA). The ligand density of PNAd was expressed as input-coating concentrations (ng/ml). PSGL-1–derived monobiotinylated glycopeptides were immobilized on avidin-coated substrates as described previously (Dwir et al., 2002). Coating densities were determined by coating equimolar input densities of [$^{14}$C]biotin (Amersham Biosciences). Cell-free L-selectin and LPL mutant were each derived from lysates of the corresponding transfected 300.19 cells, and were captured on plates coated with the selectin tail-specific mAb CA21, as described previously (Dwir et al., 2000).

### Laminar flow assays

Plates coated with adhesive ligands or cell monolayers were assembled in a parallel plate laminar flow chamber, and laminar flow adhesion assays were performed as described previously (Dwir et al., 2000). Cells resuspended in cell-binding medium H/H medium (HBSS/10 mM Hepes, pH 7.4), supplemented with 2 mM $CaCl_2$ at $1–2 \times 10^6$ cells/ml were perfused at RT through the flow chamber at desired flow rates, generated by an automated syringe pump (Harvard Apparatus). Media viscosity was increased from 1 to 2.6 cP by supplementing it with 6% (wt/vol) Ficoll ($M_r$ = 400,000; Sigma-Aldrich) as described previously (Chen and Springer, 2001). Cellular interactions were visualized with a 20× objective (Diaphot 300; Nikon). Cells were videotaped at either a 0.02-s resolution with a CCD camera (model LIS-700; Applitech) or at a 0.002-s resolution with a high speed camera (Kodak Motion Corder Analyzer, FASTCAM-SUPER 500; Kodak). For L-selectin inhibition, leukocytes were perfused in medium supplemented with 2 mM EGTA or preincubated with 1 μg/ml DREG-200 or 50 μg/ml fucoidin. For PSGL-1 inhibition on neutrophils, leukocytes were presuspended with 1 μg/ml KPL-1.

High temporal resolution microkinetics of individual leukocytes was analyzed on video segments recorded at 500 frames/s (2 ms), and cell-tracking analysis was performed with the WSCAN-Array-3 software as described previously (Dwir et al., 2001). Motions of cells perfused at shear rates lower than 30 $s^{-1}$ were manually analyzed from played back segments. Initial tethers were defined as those freely flowing cells moving closest to the lower wall of the flow chamber, which transiently tethered to the adhesive substrate at least once during a 670-μm-long path. Initial tethers or pauses of leukocytes reversibly interacting with the adhesive substrates were defined as displacements of <0.1 μm within three or more consecutive frames. Pauses <4 ms were considered nonspecific, as they could not be eliminated by blocking selectin function. The natural log of the number of pauses with a given duration after pause initiation was plotted against pause duration. A first-order dissociation plot yielded a straight line with the slope equal to $-k_{off}$. The error on each $k_{off}$ value was derived by linear regression analysis. In experiments with L-selectin expressing 300.19 lymphocytes, the force on the bond was calculated to be 180 pN per 1 dyn/cm$^2$ wall shear stress using a diameter of 12 μm and assigning a bond angle of 50°. In neutrophils, the force on the bond was estimated to be 120 pN per 1 dyn/cm$^2$ (Alon et al., 1997).

### Online supplemental material

Videos 1 and 3 are digitized videos recorded with a high speed camera (at 500 frames/s) of a representative L-selectin–transfected pre B lymphocyte tethering to PNAd at shear stresses of 0.3 and 0.4 dyn/cm$^2$, respectively.

Videos 2 and 4 are control experiments depicting an L-selectin–transfected lymphocyte preblocked with the L-selectin mAb DREG-200 and perfused at 0.3 and 0.4 dyn/cm$^2$, respectively, over the same PNAd. The frame number and time (in seconds) elapsed from the beginning of the recording is shown. Also included is a supplementary section describing results and discussion of high speed camera analysis of transient P-selectin–mediated neutrophil tethers determined at low shear stresses. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200303134/DC1.

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