Interactions through L-selectin between Leukocytes and Adherent Leukocytes Nucleate Rolling Adhesions on Selectins and VCAM-1 in Shear Flow

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Abstract. We demonstrate an additional step and a positive feedback loop in leukocyte accumulation on inflamed endothelium. Leukocytes in shear flow bind to adherent leukocytes through L-selectin/ligand interactions and subsequently bind downstream and roll on inflamed endothelium, purified E-selectin, P-selectin, L-selectin, VCAM-1, or peripheral node addressin. Thus, adherent leukocytes nucleate formation of strings of rolling cells and synergistically enhance leukocyte accumulation. Neutrophils, monocytes, and activated T cell lines, but not peripheral blood T lymphocytes, tether to each other through L-selectin. L-selectin is not involved in direct binding to either E- or P-selectin and is not a major counterreceptor of endothelial selectins. Leukocyte–leukocyte tethers are more tolerant to high shear than direct tethers to endothelial selectins and, like other L-selectin–mediated interactions, require a shear threshold. Synergism between leukocyte–leukocyte and leukocyte–endothelial interactions introduces novel regulatory mechanisms in recruitment of leukocytes in inflammation.

Accumulation of leukocytes in the vasculature at inflammatory sites followed by immigration into the local tissue is a multistep process that requires selectins and their carbohydrate ligands, integrins and their ligands on endothelium and in the matrix, and chemoattractants and their receptors (56). Tethering of cells in vascular shear flow to the vessel wall is followed by rolling along the wall, then by development of firm adhesion, and finally by diapedesis. Selectins support the initial tethering and rolling steps. To date, the steps in leukocyte accumulation on endothelium have been viewed as a linear chain of events, with tethering of leukocytes to the vessel wall as the first event. We now provide evidence for a positive feedback loop that amplifies leukocyte accumulation and therefore modifies the view that accumulation is a linear process and show that leukocyte tethering to adherent leukocytes can precede tethering to and rolling on the vessel wall. These interleukocyte adhesion pathways are L-selectin dependent and provide evidence for cooperation between the selectin expressed on leukocytes, L-selectin, and the selectins expressed on vessel walls, the vascular selectins E- and P-selectin.

Previous studies have shown that L-selectin can functionally interact with E- and P-selectin but have been interpreted in the context of direct interactions between these molecules or between leukocytes and vessel walls. Neutrophil binding to stimulated endothelium expressing E-selectin and to E-selectin transfectants was found to be inhibited not only by mAb to E-selectin, but also by mAb to L-selectin or by removal of L-selectin from the cell surface (2, 27, 48). mAb to L-selectin was also found to inhibit binding of neutrophils to P-selectin transfectants (48). L-selectin on neutrophils bears sLe^x, a known E-selectin ligand; it was further found that purified neutrophil L-selectin, but not purified lymphocyte L-selectin that lacks sLe^x, binds E-selectin (48). L-selectin is preferentially localized to the tips of microvilli on leukocytes and was proposed to function as a specialized counterreceptor that presents sLe^x to E- and P-selectin in vitro (48) and in vivo (59). Functional dichotomy was revealed by the finding that mAb to L-selectin inhibited tethering in shear flow of neutrophils to E-selectin substrates but had no effect on rolling velocities or the strength of rolling adhesions on E-selectin; furthermore, the correlation among a panel of mAb to L-selectin between inhibition of lectin function and inhibition of tethering to E-selectin suggested the importance of the ligand binding site on L-selectin rather than covalently attached sLe^x (32). PSGL-1, the major P-selectin ligand, which is also decorated with sLe^x glycans and localized on microvilli (46), has also been suggested to serve, together with L-selectin, as a specialized tethering counterreceptor for E-selectin (47).

It is well established that L-selectin binds to carbohydrate

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ligands on multiple mucin-like molecules on high endothelial venules and is important in lymphocyte recirculation through peripheral lymph nodes and mucosa-associated lymphoid tissues (56). Further ligands for L-selectin are inducibly expressed on inflamed vascular endothelium in nonlymphoid tissues and support rolling adhesions in vivo (37, 59). These vascular ligands, as well as presentation by L-selectin of sLe^x to E- and P-selectin, have been suggested to account for the ability of L-selectin to support, augment, or synergize independently with E-selectin and P-selectin–mediated leukocyte rolling adhesions in vivo (35). Leukocytes in L-selectin–deficient mice show impaired rolling in inflamed vessels in vivo (4, 38). Putative vascular ligands for L-selectin also appear to be induced on stimulated endothelium in vitro (41, 54, 55).

Recently, Bargatze and co-workers have observed that adherent neutrophil monolayers express L-selectin ligand(s) that are capable of supporting rolling of circulating neutrophils in vitro (7). The same phenomenon is seen with the bovine γδ T cell subset (24). L-selectin and β2 are obligatory receptors for neutrophil aggregation in shear flow (52). *O*-sialoendopeptidase–sensitive ligands constitutively expressed on circulating neutrophils mediate the L-selectin–dependent phase of this homotypic interaction (10). Rolling velocities of neutrophils on adherent neutrophils are 5–20-fold faster than neutrophil rolling directly on stimulated endothelium or on a purified vascular selectin (7, 17). Rolling of leukocytes on one another requires "pavementing" of leukocytes on a vessel wall, which is only seen at later stages of inflammation in vivo. It is thought that after the vascular surface is saturated with accumulated leukocytes, this enables accumulation of still further leukocytes, and that leukocyte–leukocyte interactions are therefore important late in the inflammatory cascade. However, functional interactions between L-selectin and vascular selectins have been observed at the earliest step, tethering of leukocytes to the vessel wall (32), and we have therefore examined this step.

We show through analysis of accumulation of individual cells on purified ligands or on stimulated endothelium in vitro that L-selectin, through bidirectional interactions with specific sialylated ligand(s) on leukocytes, can mediate interleukocyte interactions between cells in flow and cells adherent to a vessel wall that nucleate subsequent rolling adhesions through other receptor-ligand pairs. Neutrophils, monocytes, and specific myeloid and lymphoid cell lines that coexpress functional L-selectin ligands (20) and L-selectin demonstrate this behavior. Interleukocyte tethers mediate nucleation of rolling on a wide variety of substrates, including E-selectin, P-selectin, L-selectin, the L-selectin ligand peripheral node addressin (PNAd),[1] VCAM-1, and activated endothelium. Interleukocyte tethers result in the formation of strings of rolling cells and account for the majority of cell accumulation on these substrates. L-selectin is not required for direct tethering or rolling on either E- or P-selectin. Our study demonstrates an additional step and a positive feedback loop in the cascade of events that promote leukocyte accumulation at inflammatory sites and synergy between L-selectin and endothelial adhesion molecules. This provides a novel regulatory mechanism for modulating leukocyte interactions with inflamed vessels.

1. *Abbreviations used in this paper*: CHO-E, E-selectin transfected CHO cells; FucTIII, α (1,3/1,4)-fucosyltransferase; HEV, high endothelial venules; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cells; PBMC, peripheral blood mononuclear cells; PNAd, peripheral node addressin.

Materials and Methods

mAbs and Reagents

The E-selectin mAb BB11 (9) was a generous gift from Dr. R. Lobb (Biogen, Cambridge, MA). Function-blocking anti–L-selectin mAbs DREG-56 and DREG-200 (26) were provided by Dr. T.K. Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Function-blocking anti–L-selectin mAb LAM1-3 and nonblocking mAb LAM1-14 (53) were provided by Dr. T. Tedder (Duke University Medical Center, Durham, NC). Function-blocking anti–PSGL-1 mAb PL-1 and nonblocking mAb PL-2 (46) were provided by Dr. R. McEver (University of Oklahoma, Oklahoma City, OK). mAb 187.1 (antimurine Ig κ chain) (63) was used as a control antibody. All of these mAbs were used as purified Ig. mAb CSLEX-1 (anti–sialyl-$Lewis^x$) (21) and X63, control myeloma IgG1, were used as spent culture supernatants. Anti-VLA-4 (α_4) mAb A4-PUJ1, kindly provided by Dr. M. Hemler (Dana Farber Cancer Institute, Boston, MA), was used as a 1:100 dilution of ascites.

P-selectin purified from platelets (44) was kindly provided by Dr. R. McEver. E-selectin was purified from detergent solubilized E-selectin transfected CHO cells (CHO-E) using a BB11 Sepharose immunoaffinity column, as previously described for purification of truncated soluble E-selectin (39), modified to incorporate detergent into the wash and elution buffers. L-selectin human IgG1 chimera (8, 61) was kindly provided by Dr. L. Lasky (Genentech, South San Francisco, CA). Affinity-purified, recombinant 7 domain, soluble VCAM-1 (62) was a generous gift of Dr. R. Lobb. Fucoidin, a plant-derived sulfated polyfucan that saturably blocks the lectin domains of L-selectin and P-selectin and not E-selectin (18), was obtained from Sigma Chemical Co. (St. Louis, MO). Plasmids encoding E-selectin, L-selectin, and P-selectin chimeras with the Fc region of human IgG1 (6), generously provided by Dr. Brian Seed (Massachusetts General Hospital, Boston, MA), were transiently transfected into COS cells as previously described (5). Filtered culture supernatants were stored at 4°C with 0.1% azide. L-selectin chimera plasmid was also cotransfected with an α (1,3/1,4)-fucosyltransferase (FucTIII) expression plasmid (28, 40) generously provided by Dr. J. Lowe (University of Michigan, Ann Arbor, MI). Filtered culture supernatant containing FucTIII-modified L-selectin chimera was collected and stored at 4°C. Protein A–precipitated FucTIII-modified L-selectin chimera was immunoblotted using standard methods (48) and found to be reactive with CSLEX-1 mAb, whereas unmodified chimera and control supernatants showed no reactivity (data not shown).

Preparation of Substrates

Purified P-selectin or E-selectin solubilized in octyl-glucoside were diluted in PBS buffered with 10 mM bicarbonate, pH 8.0, at 1 μg/ml and immediately spotted on a polystyrene plate (Nunc, Lab-Tek; Naperville, IL) for 3 h. sVCAM-1 was dissolved in PBS buffered with 10 mM bicarbonate, pH 9.1, at 15 μg/ml and similarly coated. The protein substrates were washed three times with PBS and blocked with 20 mg/ml human serum albumin (HSA) (Fraction V; Sigma Chemical Co.) in PBS for 2 h. All steps were performed at 24°C.

Selectin chimera substrates were generated by spotting polystyrene plates with 15 μl protein A (20 μg/ml in PBS, pH 9.1), and washing and quenching as described above for the sVCAM-1 substrate. Protein A–coated substrates were overlaid with 50 μl of purified L-selectin/IgG chimera (2 μg/ml in PBS, pH 7.4) or culture supernatants from selectin chimera transfected COS cells (1–2 μg chimera/ml).

Cells

T lymphoblastoid cell lines Jurkat and SKW3 were maintained in RPMI 1640 supplemented with 10% FCS and 5 μg/ml gentamicin. Human umbilical vein endothelial cells (HUVEC) were cultured as previously described (15) and stimulated with 200 U/ml of TNF-α or 10 U/ml IL-1α

(Genzyme, Boston, MA). Human E-selectin transfected CHO cells (14) were kindly provided by Dr. R. Lobb and maintained in α MEM supplemented with 10% FCS, 2 mM glutamine, and 5 μg/ml gentamicin.

Peripheral blood granulocytes and mononuclear cells (PBMC) were isolated from citrate anticoagulated whole blood after dextran sedimentation and density separation over Ficoll-Hypaque (16). Monocytes were isolated by Nycodenz (1.068) hyperosmotic gradient centrifugation of leukocyte rich plasma, as described (12), resulting in 90% monocytes (by CD14 staining) and 10% lymphocytes. The level of expression of L-selectin on isolated monocytes was comparable to that on unfractionated peripheral blood monocytes as shown by immunofluorescent flow cytometry (data not shown). T cells were purified by negative selection with mAb to CD14, CD11b, CD20, and CD16 and rat anti-mouse Ig coupled to magnetic beads (MACS; Miltenyi Biotec, Sunnyvale, CA) (15). Cells were maintained at 4°C at 5×10^6–10^7/ml in Ca^{2+}- and Mg^{2+}-free HBSS with 10 mm Hepes, pH 7.4 (H/H medium), and diluted at least 10-fold into binding medium at 24°C immediately before use in flow assays. Binding medium was H/H medium containing 2 mM Ca^{2+} and 2 mg/ml HSA for selectins, and H/H medium containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 2 mg/ml HSA for sVCAM-1.

Cell Treatments

For mAb inhibition studies, granulocytes (5×10^5–1×10^7/ml) were preincubated at 4°C for 5 min in H/H medium with 30 μg/ml mAb. The cell suspension was diluted in a 10-fold volume of binding medium at 24°C and immediately perfused into the flow chamber. To assess the effects of activation on cell binding to the different selectin substrates, cells were preincubated for 3 min at 24°C in binding medium in the presence of 10 nM fMLP (Calbiochem-Novabiochem, San Diego, CA). To remove L-selectin ligands from the leukocyte surface, cells were incubated with 0.1 U/ml *Vibrio Cholera* neuraminidase (Calbiochem-Novabiochem) for 30 min at 25°C in H/H medium with 2 mM Ca^{2+}. Cleavage was terminated by washing the cells twice with H/H medium with 5 mM EDTA. Removal of surface sialic acid was monitored by staining cells with mAb CSLEX-1, which defines a neuraminidase-sensitive epitope (21). Chymotrypsin treatment of neutrophils was performed by incubating 10^7 cells/ml in H/H medium with the enzyme (Calbiochem-Novabiochem) at 20 U/ml for 30 min at room temperature, followed by washing the cells twice with H/H medium supplemented with 10 mg/ml HSA. For metabolic inhibition studies, neutrophils (5×10^6/ml) were incubated at room temperature with H/H medium with 0.1% NaN_3 and 50 mM 2-deoxyglucose for 30 min. Cells were diluted 1:10 in binding medium and immediately assayed.

Laminar Flow Assays

The plastic slide on which adhesion molecules were adsorbed, or the 150-mm tissue culture dish on which endothelial cells were cultured, was incorporated as the lower wall in a parallel wall flow chamber and mounted on the stage of an inverted phase-contrast microscope (Diaphot-TMD; Nikon Inc., Garden City, NY) as previously described (30, 31). All flow experiments were performed at 24°C. The wall shear stress was calculated as previously described (30). A 1 ml volume of cell suspension (10^6 cells/ml in binding medium, unless otherwise indicated) was perfused through the flow chamber with an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side, and the cells interacting with the field of view (with a 10× objective the field of view was 0.43 mm^2) during flow were quantitated by analysis of images videotaped with a video camera (TEC-470 CCD; Optronics Engineering, Goleta, CA) and recorder (Hi 8 CVD-1000; Sony, Park Ridge, NJ). Cellular accumulation in the field was defined as cells that tethered and remained rolling or stationary in the field for at least 3 s. At the end of each observation period, residual adherent cells were removed by perfusion with H/H medium with 5 mM EDTA.

Quantitation of Primary and Secondary Cellular Accumulation

Cells that tethered directly to the substrate were quantitated as primary cell accumulation, and cells that tethered to the substrate after first interacting with an adherent cell through an interleukocyte tether were quantitated as secondary accumulation. For quantitation, the adherent cells present at the end of the observation period were first identified. Then the video tape was played backward and forward several times in slow motion, and each cell was scored for accumulation through a primary or secondary tether. Interleukocyte tethers could be visualized as brief (one to a few video frames) cell–cell contacts accompanied by transient or jerky motion of the newly tethered cell along the top or edge of the adherent cell and followed by transfer of the tethered cell to the substrate downstream of the adherent cell. To facilitate analysis, any cell that adhered to the substrate within 10 cell diameters downstream of a previously adherent cell was operationally defined as a secondary tether. In general, the majority of secondarily attached cells accumulated immediately downstream or within one or two cell diameters of previously tethered cells. Because the distinction between primary and secondary accumulation became difficult as cells became more crowded on the substrate, usually only early time points in accumulation were scored. To avoid scoring tethering events modulated by adherent cells upstream from the field of view, the observation field was located at the upstream edge of the spot of adsorbed protein and included a portion of the unadsorbed surface to which no cell binding occurred (see Fig. 2 *A*). On selectin substrates all tethered cells remained adherent and subsequently rolled on the substrate, as visualized either immediately after tethering or as the shear stress was further increased. On VCAM-1 substrates, T cell lines and the majority of monocytes arrested after tethering or came to full arrest after short periods of rolling. On PNAd, both rolling leukocytes as defined above and transiently rolling leukocytes, i.e., cells that traveled at a mean velocity 5–10-fold lower than the hydrodynamic velocity for <3 s, were observed. Secondary tethers were scored only downstream of rolling leukocytes but were scored regardless of whether the secondarily tethered cells were rolling or transiently rolling. For all comparison studies within an experiment, the same fields of view were used to ensure that the results reflected identical distribution of the immobilized adhesive proteins.

Shear Resistance Measurements

Detachment assays were performed on cells after they had bound to the substrate for 30–60 s. The shear flow was increased every 10 s to a maximum of 35 dyn/cm^2 in 1.5–2-fold increments, and the number of cells remaining bound at the end of each 10-s interval was determined. Rolling velocities were measured by following cell displacements over 5–10-s intervals, as previously described (31).

Results

L-Selectin Synergizes with Vascular Selectins and PNAd in Augmenting Cell Accumulation in Shear Flow

We studied the role of L-selectin in leukocyte tethering and accumulation in shear flow on vascular selectins. Neutrophil accumulation on both E-selectin (Fig. 1 *A*) and P-selectin (Fig. 1 *B*) was reduced in the presence of function-blocking L-selectin mAbs DREG-56 and LAM1-3 but not the control nonblocking L-selectin mAb LAM1-14. However, the inhibitory effect of blocking with L-selectin mAb was negligible during the first 5–10 s of cell accumulation. Moreover, the rate of neutrophil accumulation in the absence of L-selectin blockade accelerated after 5–10 s, but in the presence of L-selectin blocking mAbs, it was linear (Fig. 1, *A* and *B*, and data not shown). These data suggest that neutrophil accumulation on E- and P-selectin is a cooperative process and that L-selectin cooperates at later but not early time points in the accumulation of neutrophils on vascular selectin substrates.

When following the pattern of accumulation of individual neutrophils, we observed cells in flow to preferentially accumulate downstream of previously tethered, rolling cells on P-selectin (Fig. 2 *A*) and E-selectin substrates (Fig. 2 *B*). Thus, "strings" of rolling cells, aligned on the substrate in the direction of flow, were formed. In the presence of blocking mAb to L-selectin, these strings did not form on P-selectin (Fig. 2 *A*) or E-selectin substrates (not

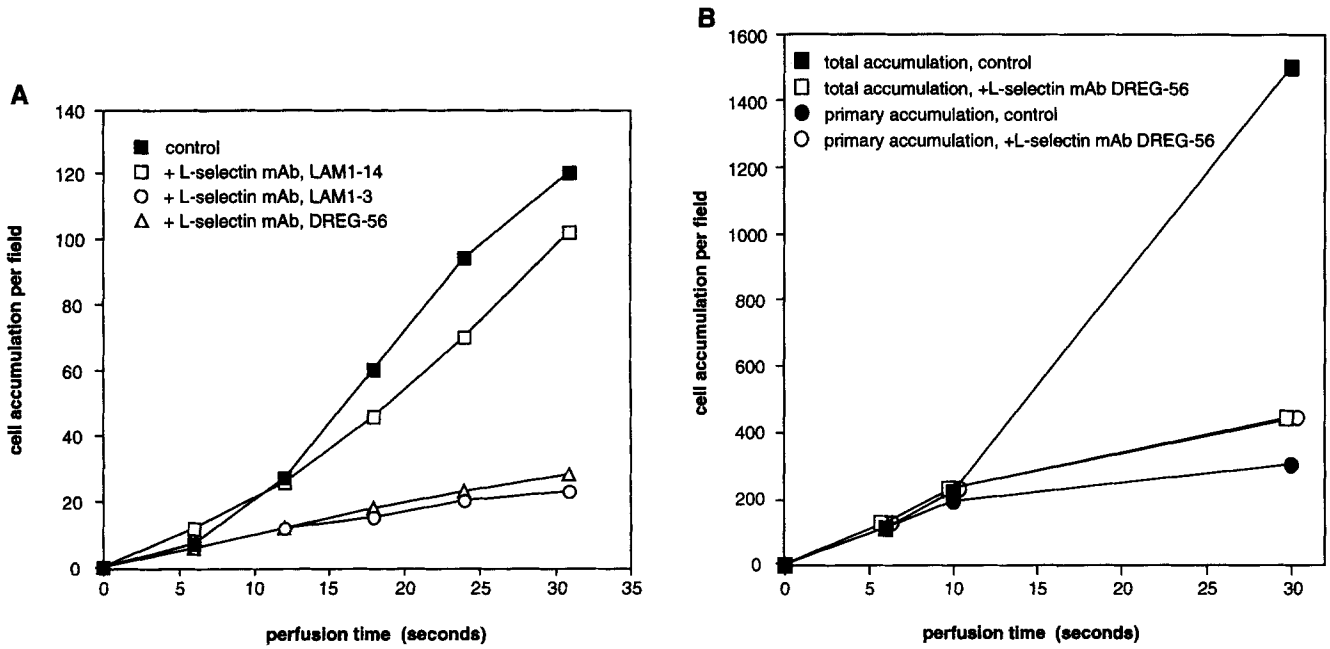


Figure 1. L-selectin accelerates accumulation of neutrophils on E- and P-selectin in shear flow. (*A*) Kinetics of cell accumulation on E-selectin in the presence of L-selectin mAbs. Neutrophils were perfused at 5×10^5/ml at a wall shear stress of 3.0 dyn/cm^2 on E-selectin substrates. (*B*) Kinetics of cell accumulation in presence of L-selectin mAb on P-selectin. Neutrophils were perfused at 5×10^6/ml at a shear stress of 2.25 dyn/cm^2 on substrates bearing P-selectin–Ig chimera bound to protein A. Both total cell accumulation and primary accumulation defined as direct binding to the substrate independent of interaction with any previously bound cells on the substrate were quantitated. *A* and *B* are representative of six independent experiments.

shown). In the presence of mAb to L-selectin, cells accumulated randomly over the substrate, and there was substantially less accumulation (Fig. 2 *A*). Close examination of video tapes revealed that accumulation of cells in strings was the result of neutrophil–neutrophil interactions. A neutrophil in flow touched an adherent cell and remained in contact a few video frames, typically for <0.15 s. The newly adherent cell then either rolled along the surface of the previously adherent cell and directly transferred to rolling on the substrate (Fig. 2 *B*, *arrows* at 6 and 15 s and leftmost two *arrows* at 27 s) or sometimes appeared to let go and then tether to the substrate a few cell diameters downstream of the previously adherent cell (Fig. 2 *B*, *arrows* at 12 and 18 s and rightmost *arrow* at 27 s). During the formation of strings (Fig. 2 *B*), secondary cells transferred from one adherent cell to another along the length of the string and finally began rolling on the substrate part way along or at the end of the string. Transfer from one cell to another along the string was readily distinguished by its faster velocity from neutrophil rolling on E- or P-selectin. Thus, adherent leukocytes formed nucleation sites for the adhesion of further leukocytes and accelerated the accumulation process in shear flow. Adhesion of leukocytes to the substrate will alter hydrodynamic streamlines and lower the hydrodynamic drag forces acting on cells within a cell diameter or two of an adherent cell. This may contribute to but cannot account for the nucleation of secondary tethers and formation of strings, as these were abolished by specific L-selectin mAbs (Fig. 2 *A*).

We grouped cells that accumulated on the substrate in shear flow in two categories: cells that bound directly to the substrate were termed primary cells and the event was considered a primary or direct tether, and cells that accumulated in strings downstream of and after interaction with an adherent cell were termed secondary cells and the event was considered a secondary tether. At early time points and at all densities of E- and P-selectin examined, all tethers were primary and random, i.e., uniformly scattered on the substrate (data not shown). Secondary tethers accounted for 70% of neutrophil accumulation on E- and P-selectin (Fig. 2, *C* and *D*). Strikingly, L-selectin blocking mAb eliminated only the secondary leukocyte tethers and had little or no effect on primary tethers to E-selectin (Fig. 2 *C*) and P-selectin (Fig. 2 *D*). Fucoidin, an inhibitor of L- and P-selectin but not E-selectin (18), blocked secondary tethers but not primary tethers to E-selectin (Fig. 2 *C*). The proportion of secondary tethers was higher at later than early time points (Fig. 2 *D*), and L-selectin–dependent secondary tethers accounted for the acceleration in the rate of leukocyte accumulation with time (Figs. 1 *B* and 2 *D*). In general on a range of substrates, the proportion of accumulation inhibited by L-selectin mAb increased with time. No effect of L-selectin mAb on rolling velocity or shear resistance of neutrophils was observed on either E- or P-selectin substrates (data not shown), in agreement with previous findings (32, 47). Thus, L-selectin synergizes with both E- and P-selectin to augment tethering to these vascular selectins in shear flow and is not required for direct interaction with E- and P-selectin in shear flow.

To test if L-selectin–dependent interleukocyte interactions also contribute to L-selectin–mediated leukocyte tethering to endothelial L-selectin ligands, rolling of neutrophils and lymphocytes on immunopurified PNAd, the major L-selectin ligand on high endothelial venules (HEV)

(11, 33) was examined. On PNAd, in contrast to E- and P-selectin, maximal leukocyte accumulation on the substrate was reached very rapidly and neutrophils rolled out of the field of view soon after tethering. Nevertheless, neutrophils rolling through the field of view were observed to nucleate downstream tethering events (Fig. 2 *E*). Neuraminidase treatment of neutrophils had little effect or augmented the rate of primary tethering of neutrophils to PNAd (data not shown) but completely eliminated interleukocyte tethers (Fig. 2 *E*), in agreement with the finding that L-selectin ligands on neutrophils are sialylated (7, 20). Jurkat cells express L-selectin at similar levels as neutrophils, but lack L-selectin ligand (20). Jurkat cells formed primary tethers and rolled on PNAd but failed to nucleate secondary tethers by other Jurkat cells (Fig. 2 *E*). These results show that even with an L-selectin ligand as the substrate, interleukocyte interactions dependent on L-selectin can nucleate rolling adhesions.

L-Selectin and PSGL-1 Are Not Major E-Selectin Counterreceptors on Neutrophils

Direct (primary) tethering of neutrophils to E-selectin was evaluated in the presence of different mAb to L-selectin and PSGL-1. Three L-selectin function blocking mAbs that blocked secondary tethers had little effect on primary neutrophil tethers to E-selectin (Figs. 2 *C* and 3 *A*) or P-selectin (Figs. 1 *B* and 2 *D*). Similarly, whereas fucoidin completely blocked secondary tethers of neutrophils on E-selectin, it had no effect on primary tethers to E-selectin (Fig. 2 *C*).

PSGL-1, the major leukocyte P-selectin ligand (45, 50) is decorated with sLe^x carbohydrates and can bind E-selectin (46, 47). PSGL-1 has been suggested to be an E-selectin tethering counterreceptor in shear flow as shown by inhibition with PL-1 mAb to PSGL-1 of HL-60 cell accumulation on E-selectin (47). To assess a possible contribution of PSGL-1 to neutrophil accumulation on E-selectin, we analyzed primary and secondary neutrophil accumulation in the presence of different PSGL-1 mAbs. mAb PL-1 that blocks the P-selectin recognition site on PSGL-1 and completely abrogates neutrophil accumulation or rolling on P-selectin (46, 47, and data not shown) had no effect on either primary or secondary neutrophil accumulation or string formation on E-selectin (Fig. 3 *A*). These findings suggest that on neutrophils, this binding site on PSGL-1 is not a major counterreceptor for either E- or L-selectin. Consistent with these observations, removal of PSGL-1 from neutrophils with *O*-glycoprotease resulted in a complete loss of P-selectin binding in shear flow or stasis but had no effect on direct neutrophil tethering to E-selectin (data not shown).

L-selectin bears multiple N-linked glycans, and on neutrophils these are decorated with sLe^x and have E-selectin ligand activity (48). It has further been proposed that neutrophil L-selectin contributes to interactions with vascular selectins by presenting sLe^x to E- and P-selectin (48). To test if L-selectin mAbs that block L-selectin function can partially mask these carbohydrate ligands, we generated a chimeric L-selectin Ig fusion protein in COS cells cotransfected with fucosyl transferase III cDNA (57), as a model for neutrophil L-selectin. The recombinant L-selectin is decorated with sLe^x as shown in immunoblot with CSLEX-1 mAb (see Materials and Methods), and when immobilized on a substrate, efficiently supports tethering and rolling of CHO cells transfected with E-selectin (Fig. 3 *B*). DREG-56 mAb and fucoidin had no effect on the ability of the sLe^x-bearing L-selectin chimera to support E-selectin interactions in shear flow (Fig. 3, *B* and *C*). Therefore, sLe^x presentation by L-selectin is not affected by antagonists that block L-selectin ligand-binding function. Control experiments showed that neuraminidase treatment of the L-selectin Ig chimera on the substrate abolished its ability to support E-selectin–mediated rolling adhesions (Fig. 3 *B*); however, the substrate retained L-selectin ligand-binding function (data not shown).

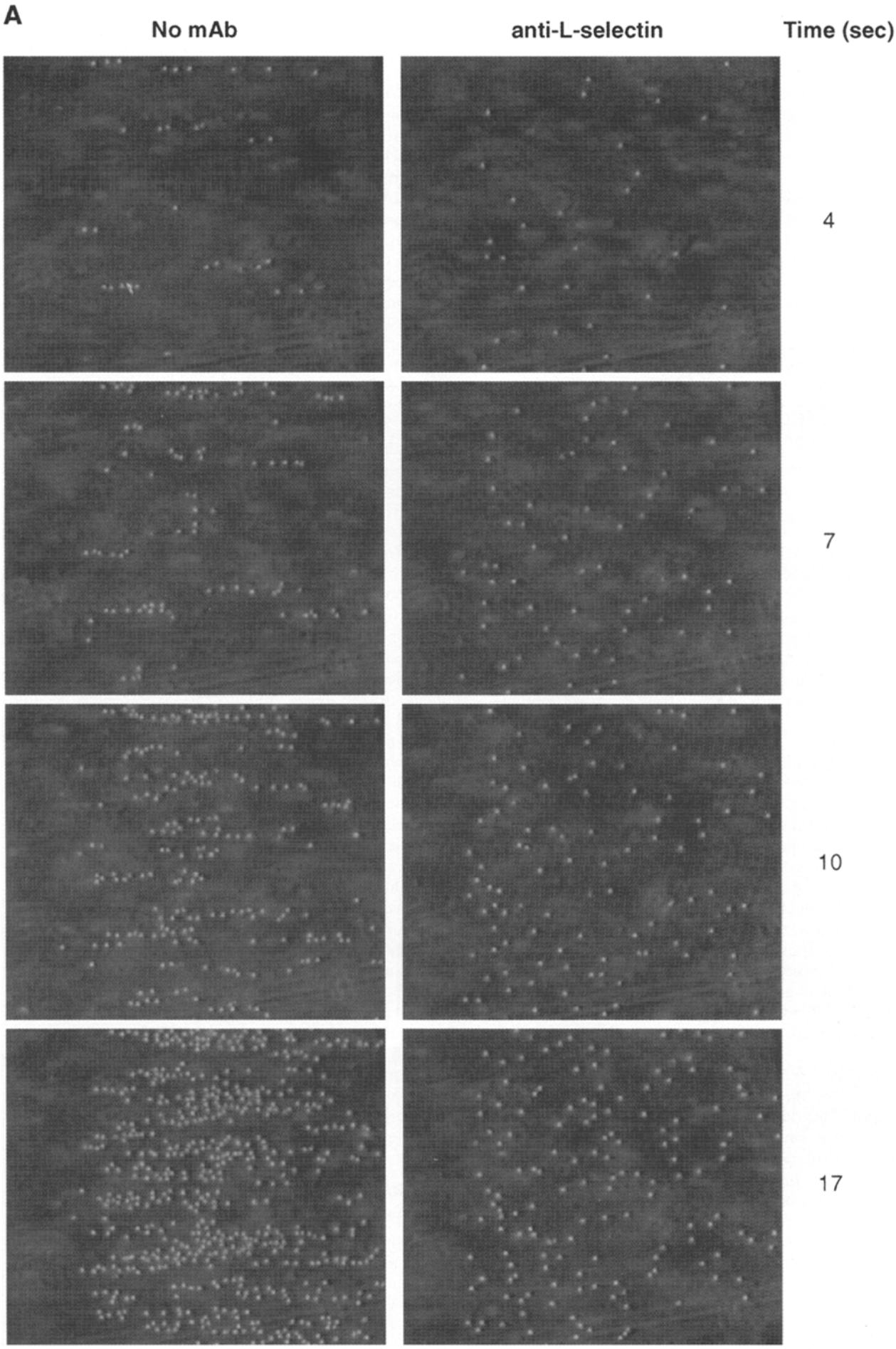
L-selectin can be proteolytically removed by mild chymotrypsin treatment (48). Chymotrypsin treatment removed 80% of L-selectin from neutrophils as shown by immunofluorescence flow cytometry and abolished almost all secondary tethers (Fig. 4 *A*) and all strings (not shown) on E-selectin at 4 dyn/cm^2 wall shear stress. Similar results were obtained at a shear stress of 2.25 dyn/cm^2 (not shown). The same chymotrypsin treatment completely removed the PL-1 mAb epitope on PSGL-1 as shown by 95% reduction in fluorescence intensity and abolished P-selectin binding (not shown). Qualitatively identical results were obtained with trypsin (data not shown). Nevertheless, even when both L-selectin and PSGL-1 were removed from the neutrophil surface, primary tethering to E-selectin was only marginally affected.

Immobilized L-selectin supports tethering and rolling of neutrophils in shear flow (20). L-selectin on a substrate serves as a surrogate for L-selectin presented to cells in flow by adherent leukocytes. We assessed if cells adherent on L-selectin substrates could nucleate adhesion of flowing cells to the L-selectin substrate. Neutrophils accumulated in strings on the L-selectin substrate and a substantial proportion of tethers was secondary (Fig 4 *B*). Neutrophil activation by fMLP, which removed >90% of L-selectin as shown by flow cytometry, abolished all strings on L-selectin substrates, as well as on P- and E-selectin substrates (data not shown), but had no effect on direct tethering to L-selectin substrates (Fig. 4 *B*). Cell activation was not associated with removal of L-selectin ligands from neutrophils because primary tethers to L-selectin substrates were not affected. Therefore, strings and secondary cell accumulation were abolished after neutrophil activation because of L-selectin shedding and not because of L-selectin ligand shedding or down-regulation.

Metabolic energy is not required for selectin function (31). Treatment of neutrophils with a combination of sodium azide and 2-deoxyglucose had no effect on the extent of string formation or primary or secondary tethers on E-, P-, and L-selectin substrates (data not shown), suggesting that metabolic energy is also not required for L-selectin–dependent interleukocyte tethering interactions in shear flow.

Shear Stress Regulates Ability of Adherent Leukocytes to Nucleate Rolling Cells

A wall shear stress above a threshold level is required to support rolling of neutrophils on PNAd and on a monolayer of adherent neutrophils (17). Consistently, at wall shear


A
No mAb
anti-L-selectin
Time (sec)
4
7
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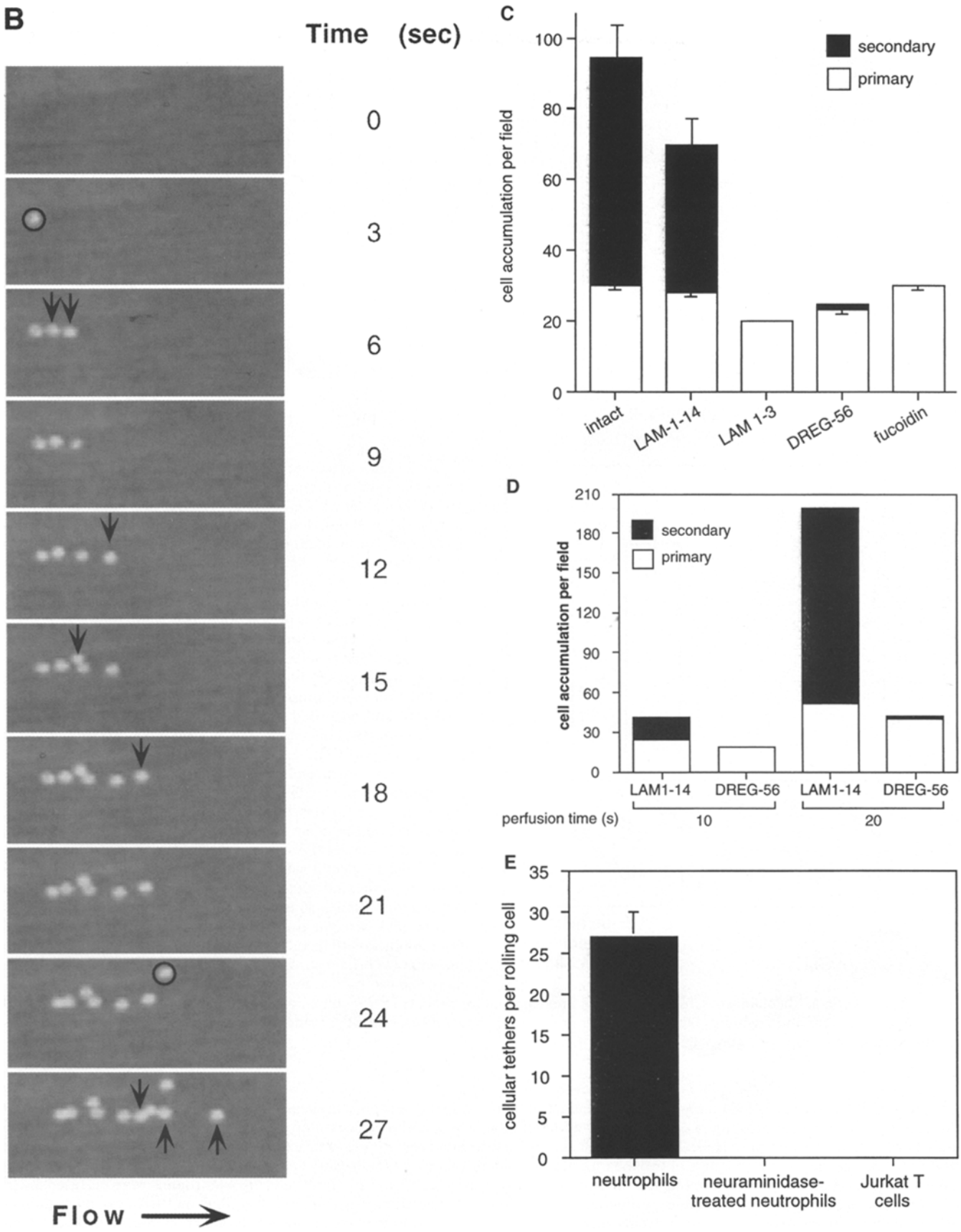

Figure 2. L-selectin mediates interleukocyte tethering resulting in string formation and secondary cell accumulation on E-selectin, P-selectin, and PNAd substrates. (*A*) Video images of neutrophil accumulation on a P-selectin substrate. Images show about 2/3 of the field of view from the experiment described in Fig. 1 *B*. The field of view is selected so that the left 1/5 is uncoated and the right 4/5 is present within the spot of P-selectin chimera. Accumulation of cells, in the absence and presence of the L-selectin blocking mAb DREG-56, is shown at parallel time points. (*B*) Formation of an individual string of neutrophils on an E-selectin substrate at 1.5 dyn/cm^2. Cells are marked in the first frame in which they appear with a circle (primary tethers) or arrow (secondary tethers). Direction of flow in *A* and *B* is left to right. Results are representative of six experiments. Both selectins were immobilized at comparable site densities. (*C*) Primary

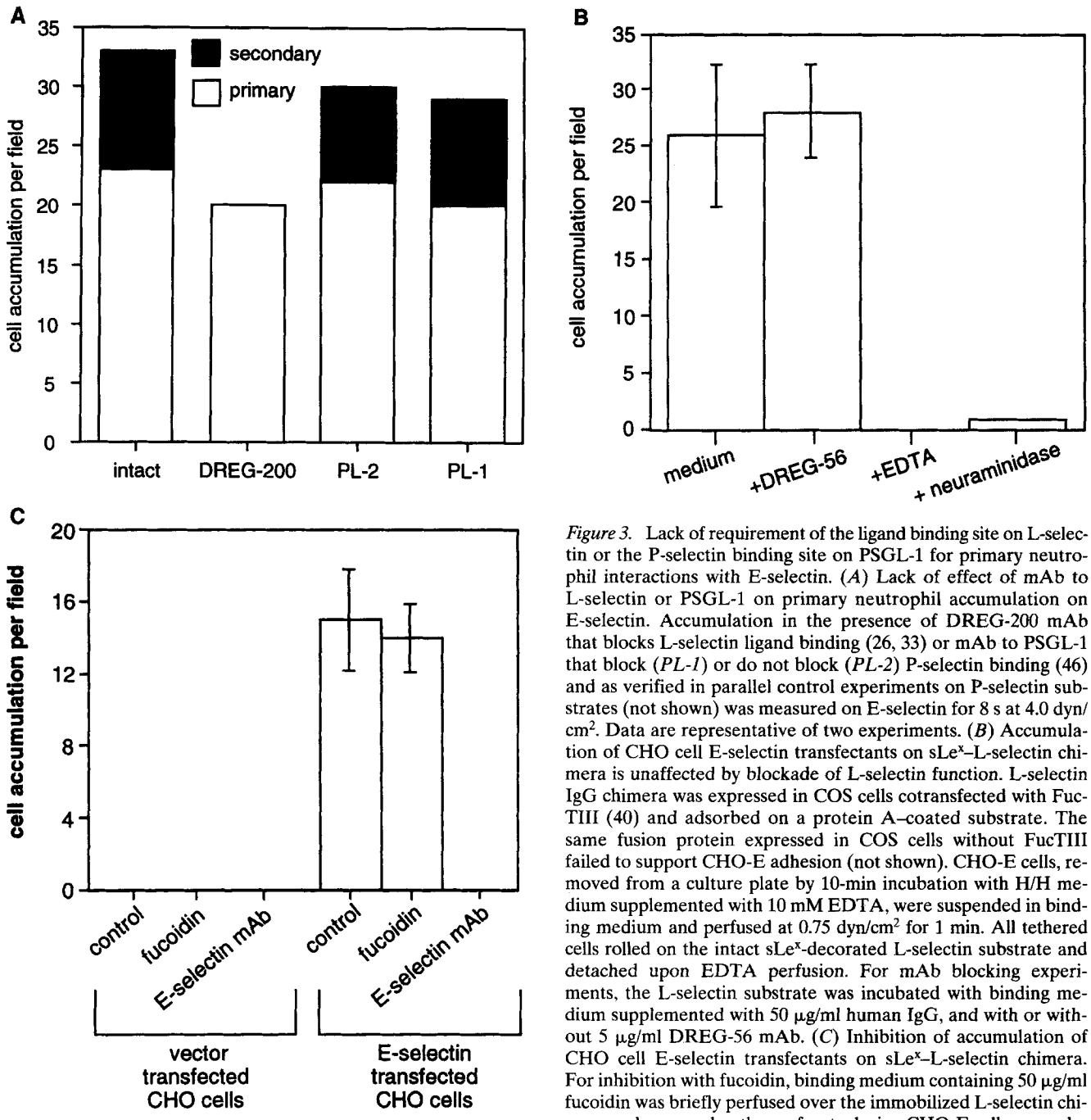

Figure 3. Lack of requirement of the ligand binding site on L-selectin or the P-selectin binding site on PSGL-1 for primary neutrophil interactions with E-selectin. (*A*) Lack of effect of mAb to L-selectin or PSGL-1 on primary neutrophil accumulation on E-selectin. Accumulation in the presence of DREG-200 mAb that blocks L-selectin ligand binding (26, 33) or mAb to PSGL-1 that block (*PL-1*) or do not block (*PL-2*) P-selectin binding (46) and as verified in parallel control experiments on P-selectin substrates (not shown) was measured on E-selectin for 8 s at 4.0 dyn/cm^2. Data are representative of two experiments. (*B*) Accumulation of CHO cell E-selectin transfectants on sLe^x–L-selectin chimera is unaffected by blockade of L-selectin function. L-selectin IgG chimera was expressed in COS cells cotransfected with FucTIII (40) and adsorbed on a protein A–coated substrate. The same fusion protein expressed in COS cells without FucTIII failed to support CHO-E adhesion (not shown). CHO-E cells, removed from a culture plate by 10-min incubation with H/H medium supplemented with 10 mM EDTA, were suspended in binding medium and perfused at 0.75 dyn/cm^2 for 1 min. All tethered cells rolled on the intact sLe^x-decorated L-selectin substrate and detached upon EDTA perfusion. For mAb blocking experiments, the L-selectin substrate was incubated with binding medium supplemented with 50 μg/ml human IgG, and with or without 5 μg/ml DREG-56 mAb. (*C*) Inhibition of accumulation of CHO cell E-selectin transfectants on sLe^x–L-selectin chimera. For inhibition with fucoidin, binding medium containing 50 μg/ml fucoidin was briefly perfused over the immobilized L-selectin chimera and was used as the perfusate during CHO-E cell accumulation. For E-selectin mAb inhibition, CHO-E cells were preincubated for 5 min in H/H medium with E-selectin mAb and then diluted 1:20 into binding medium and perfused. Data in *B* and *C* are average of two measurements ± range and are representative of three experiments. Accumulation was for 1 min at 0.9 dyn/cm^2.

and secondary cell accumulation on E-selectin. Accumulation of individual neutrophils in shear flow was followed frame-by-frame, and direct interaction with the substrate, i.e., primary cell accumulation, was quantitated separately from tethering to the substrate immediately downstream of adherent cells, i.e., secondary cell accumulation. Data are from the same experiment described in Fig. 1 *A* after 24 s perfusion and are the average ± range of two measurements on identical fields. (*D*) Primary and secondary neutrophil accumulation on P-selectin. Accumulation was quantitated at a shear stress of 4 dyn/cm^2 after 10 and 20 s on P-selectin. Neutrophils were perfused at 10^6 cells/ml. Data are representative of four experiments. (*E*) Neutrophils but not Jurkat T lymphoblasts rolling on PNAd can nucleate secondary tethers. Interleukocyte tethering events that resulted in tethering downstream of previously adherent rolling leukocytes on PNAd at 1.5 dyn/cm^2 were quantitated over a 20-s period and divided by the total number of leukocytes that rolled through the field of view. Cells were treated with neuraminidase as described in Materials and Methods. Jurkat T lymphocytes formed direct tethers and rolled on the substrate at comparable velocities to neutrophils (not shown). The number of direct neutrophil tethers on PNAd was not decreased by neuraminidase. Data are average of two experiments ± range and are representative of three experiments.

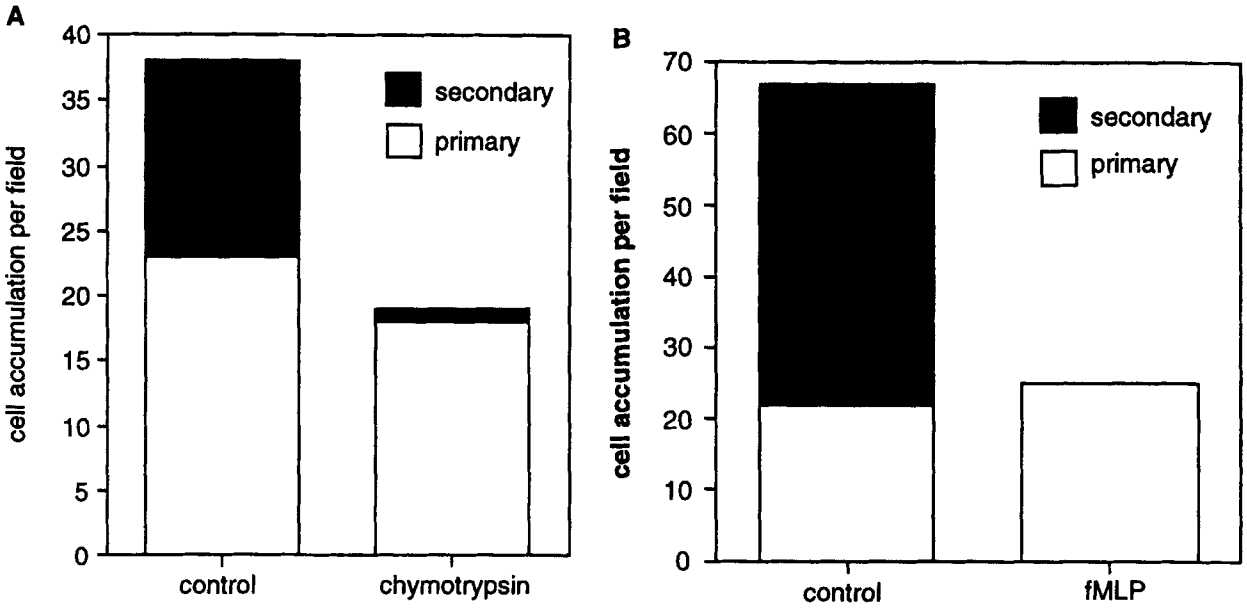

Figure 4. Removal of L-selectin from neutrophils abolishes secondary and not primary accumulation on E-selectin and L-selectin substrates. (*A*) Effect of chymotrypsin on accumulation on E-selectin. Neutrophils were treated with or without chymotrypsin and allowed to accumulate at 4.0 dyn/cm^2 for 25 s on a substrate with E-selectin IgG chimera bound at 2 μg/ml on protein A. Similar results were obtained at 2.2 dyn/cm^2, and the small effect of chymotrypsin on primary tethers at 4.0 dyn/cm^2 was not seen at 2.2 dyn/cm^2. Chymotrypsin-treated cells failed to bind to P-selectin substrates (data not shown). (*B*) Effect of fMLP on accumulation on L-selectin. Neutrophils stimulated with or without fMLP were allowed to accumulate at 3 dyn/cm^2 on a substrate with L-selectin IgG chimera bound at 2 μg/ml to protein A. fMLP activation reduced mean fluorescence intensity of staining with L-selectin mAb by >90%. *A* and *B* are representative of three experiments.

stresses ≤0.70 dyn/cm^2, neutrophils attached to P-selectin solely by primary tethers with the substrate, and no secondary tethers or strings were observed (Fig. 5 *A*). As shear was increased above this threshold, the amount of cell accumulation through secondary tethers increased, and the amount of cell accumulation through primary tethers was little changed until 3.8 dyn/cm^2, where it declined (Fig. 5 *A*). Consequently, a greater proportion of cells accumulated through secondary tethers and strings became more evident as shear was increased. Similar results were obtained on E-selectin substrates, and on high density L-selectin chimera substrates. At a wall shear stress of 0.7 dyn/cm^2, 95% of neutrophil accumulation was through primary tethers, whereas at ≥3.5 dyn/cm^2, almost all neutrophil accumulation was through secondary tethers with formation of strings on E- and L-selectin chimera substrates (data not shown). At a wall shear stress of 4.0 dyn/cm^2 and above, no direct tethers or cell accumulation occurred on E-, P-, and L-selectin substrates. However, when a small number of neutrophils were allowed to accumulate at lower shear, and shear was then rapidly increased, the adherent neutrophils nucleated string formation, and efficient accumulation of neutrophils occurred on all three selectin substrates at wall shear stresses up to 6 dyn/cm^2 (data not shown).

Cells that detached from the substrate at shear stresses of 6–15 dyn/cm^2 could not retether to the selectin substrate, but still efficiently interacted with adherent neutrophils through transient interleukocyte tethers and transferred from one adherent cell to another (not shown). Interleukocyte L-selectin–mediated interactions were more tolerant to high shear than primary tethers through any selectin–counterreceptor pair, at all site densities tested.

Reducing the concentration of cells in the perfusion medium delayed but did not prevent the formation of strings and secondary cell accumulation (Fig. 5 *B*). String formation was seen at all cell concentrations examined, ranging from 0.1 × 10^6/ml (Fig. 5 *B*) to 5 × 10^6/ml (Fig. 1 *B*).

L-Selectin Ligands on Adherent Neutrophils Nucleate Leukocyte Attachment on Stimulated Endothelium

We further examined the accumulation of individual neutrophils on IL-1–stimulated HUVEC in shear flow (Fig. 6 *A*). Strings formed, and the majority of neutrophil accumulation at both 15 and 30 s was through secondary tethers. As on purified selectin substrates, secondary but not primary interactions were eliminated by blocking L-selectin function with DREG-56 mAb. Fucoidin also almost completely eliminated secondary tethers. A small decrement in direct tethers by fucoidin but not L-selectin mAb may suggest a small contribution to direct tethers by P-selectin. Consistent with little inhibition by fucoidin of primary tethers, the majority of direct tethering was mediated by E-selectin, as it was efficiently blocked by HUVEC pretreatment with E-selectin mAb (data not shown). Thus, blockade of L-selectin did not inhibit primary tethering of neutrophils to IL-1–stimulated HUVEC but inhibited cell accumulation by blocking all secondary accumulation events. Shear resistance and rolling velocities of neutro-

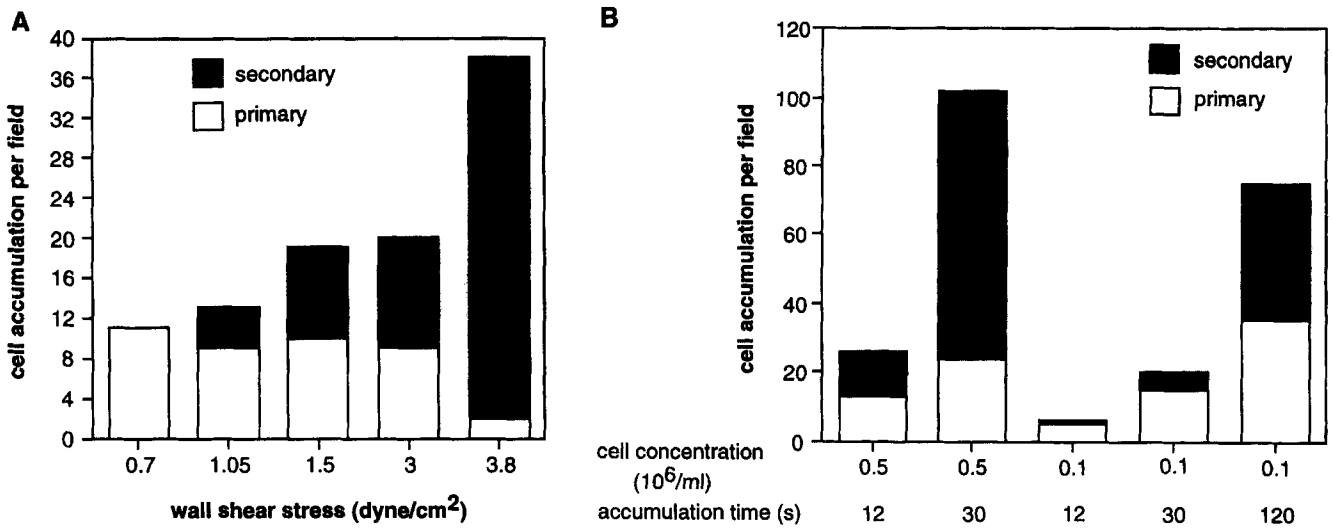

Figure 5. Effect of wall shear stress and cell concentration on interleukocyte tethers and secondary cell accumulation. (*A*) Accumulation of neutrophils on P-selectin at different shear stresses. Similar numbers of neutrophils (10–12 per field) were allowed to adhere for 5 s at low shear (0.15 dyn/cm^2) to P-selectin to provide nucleation sites for neutrophils in flow, and shear flow was then abruptly increased to the indicated values. Accumulation of new cells after the increase in wall shear stress was quantitated as primary or secondary over a 15-s period. (*B*) Extent of neutrophil primary and secondary accumulation as a function of cell concentration in the perfusate and accumulation time. Neutrophils were perfused at 3.0 dyn/cm^2 in the presence of the nonblocking L-selectin mAb, LAM 1-14, on a substrate with purified E-selectin. *A* and *B* are representative of three experiments.

phils on stimulated HUVEC were not altered by DREG-56 treatment (not shown), consistent with a lack of participation of L-selectin in direct interactions with HUVEC. Lack of a detectable effect of the PL-1 mAb on interleukocyte tethers in the present study suggests that the P-selectin binding site on PSGL-1 is not predominant, but it does not rule out a contribution by PSGL-1 to L-selectin ligand activity. These data suggest that an L-selectin ligand was not induced on the stimulated HUVEC studied here. Indeed Jurkat cells, which express functional L-selectin, tethered to stimulated HUVEC solely through VLA-4/VCAM-1 interactions (not shown). We therefore conclude that even in the absence of an endothelial ligand for L-selectin, L-selectin plays an important role in leukocyte accumulation on stimulated endothelium.

As on purified selectin substrates, the extent of string formation on stimulated endothelium and the percentage of cell accumulation through secondary tethers was time dependent (Fig. 6, *A* and *B*) and was tightly regulated by wall shear stress (Fig. 6 *B*). Consistent with the shear threshold requirement for string formation observed on purified selectins, no strings and few or no secondary tethers were observed when neutrophils interacted with the stimulated HUVEC at 0.7 dyn/cm^2. Neutrophils that were rolling or firmly adherent on the endothelium were similarly efficient in nucleating tethering of cells in shear flow. The extent of strings was similar when Mg^{2+} was added to promote integrin-dependent arrest of the rolling leukocytes on the stimulated endothelium (data not shown).

L-Selectin Augments Accumulation of other Leukocytes Expressing L-Selectin Ligands

We have recently found that peripheral blood monocytes and certain T cell lines express functional L-selectin ligands that support tethering and rolling on L-selectin–coated substrates (20). Since these cells also express L-selectin, we examined whether they may tether to one another in shear flow through L-selectin and use interleukocyte tethers to enhance cell accumulation on substrates coated with selectins or integrin ligands. Peripheral blood T lymphocytes, immunomagnetically negatively selected with CD11b, CD14, CD16, and CD20 mAb, and >95% $CD3^+$, express L-selectin, but failed to accumulate in strings on either P- or E-selectin at any shear tested (data not shown), consistent with the absence of L-selectin ligands on these cells (20). PBMC were studied because they contain monocytes that express both L-selectin and L-selectin ligand. PBMC accumulation on E-selectin was significantly augmented by strings forming downstream of adherent monocytes rolling on E-selectin (Fig. 7 *A*). Strings and the contribution of secondary tethers to leukocyte accumulation were completely blocked by DREG-56 mAb to L-selectin. Similar to the results with neutrophils, inhibitory mAb to L-selectin had no effect on direct tethers to E-selectin. Shear strongly modulated the extent of string formation by PBMC. Similar to neutrophils, no strings were observed at 0.7 dyn/cm^2. The proportion of cells that accumulated in strings increased with increasing shear stress, 22% at 1.5 dyn/cm^2 and 55% at 2.25 dyn/cm^2. The upper shear limit in which interleukocyte tethers took place was 3 dyn/cm^2 in PBMC versus 6 dyn/cm^2 in granulocytes (not shown).

HL-60 cells express E-, P-, and L-selectin ligands but lack L-selectin and did not accumulate on selectin substrates in strings (data not shown). Both HL-60 and T lymphocyte accumulation on selectins is significantly lower than neutrophil accumulation, in particular at high shear. Lymphocyte subsets have, however, comparable rolling strength (shear resistance to detachment) to neutrophils.

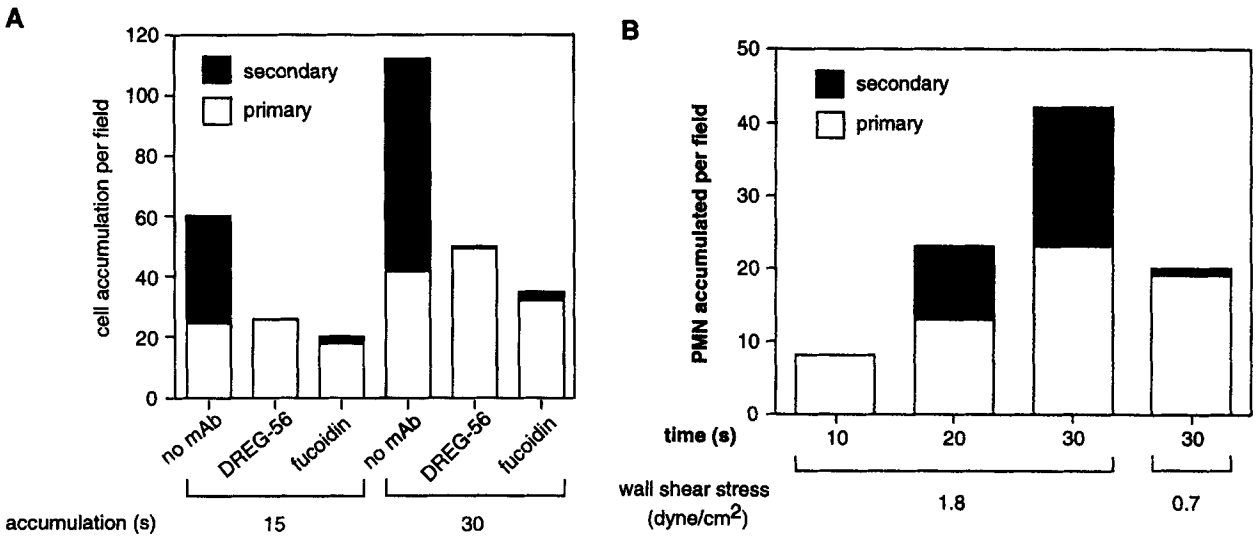

Figure 6. Adherent neutrophils nucleate, through interleukocyte L-selectin–dependent tethers, secondary accumulation of neutrophils on stimulated endothelium. (*A*) L-selectin and time dependence. Neutrophils preincubated with and in continued presence of DREG-56 mAb or fucoidin were allowed to accumulate on HUVEC substrate at a shear stress of 2.25 dyn/cm^2 for the indicated periods. HUVEC were stimulated with 10 U/ml IL-1 for 6 h. Representative of three experiments. (*B*) Dependence on wall shear stress and time. Neutrophil accumulation at a wall shear stress of 1.8 or 0.7 dyn/cm^2 was quantitated after various periods of perfusion. HUVEC were stimulated with 200 U/ml TNFα for 6 h. Representative of three experiments.

Monocytes can tether through VLA-4 in shear flow to purified or endothelial VCAM-1 (41). Fucoidin inhibited overall accumulation of monocytes on VCAM-1 and blocked secondary but not primary tethers on VCAM-1 (Fig. 7 *B*). In contrast to results with monocytes, peripheral blood T cells preadhered to VCAM-1 failed to nucleate secondary tethers of T cells on VCAM-1 (not shown). PBMC also accumulated through L-selectin–dependent strings on IL-4–stimulated HUVEC (not shown).

Jurkat and SKW3 T cells coexpress L-selectin and the integrin VLA-4. VLA-4 on these cells is activated and supports tethering and firm adhesion on purified VCAM-1 as well as on stimulated HUVEC (3). SKW3 but not Jurkat cells express L-selectin ligands that support rolling on L-selectin substrates (20). We compared accumulation in flow of these T cell lines on VCAM-1. At 2 dyn/cm^2, SKW3 cells accumulated in strings on VCAM-1, and fucoidin selectively abolished string formation but not primary interactions with the VLA-4 ligand (Fig. 7 *C*). Jurkat cells tethered to VCAM-1 in a random distribution, and fucoidan and L-selectin mAb had no effect on their rate of accumulation (Fig. 7 *C* and data not shown). At 0.5 dyn/cm^2, SKW3 cells failed to form strings and their attachment to VCAM-1 was essentially identical to Jurkat cells (not shown). L-selectin–dependent strings also accounted for the majority (70%) of SKW3 cell accumulation on IL-4– and IL-1–stimulated HUVEC; accumulation on this substrate was VLA-4 dependent (not shown). When Mn^{2+} was added to increase VLA-4 avidity and the number of tethers to VCAM-1 (3), a similar fraction of SKW3 accumulation on VCAM-1 was L-selectin dependent (data not shown).

Discussion

We have defined a novel mechanism for accelerating the accumulation of leukocytes in shear flow (Fig. 8). L-selectin–dependent interactions occur between leukocytes in shear flow and adherent leukocytes on a wide range of substrates, including E-selectin, P-selectin, L-selectin, PNAd, VCAM-1, and cytokine-stimulated endothelial cells. These L-selectin–dependent interactions between adherent cells and cells in shear flow nucleate secondary tethering and rolling of leukocytes on the substrate downstream of the primary adherent cells. The accumulations of secondary leukocytes are built up as strings of rolling cells. Cells that were recruited to the substrate as secondary tethers can bind cells in shear flow and recruit further accumulation of secondary cells. Therefore, the mechanism we have described can act as a positive feedback loop. Secondary tethers account for as much as 80% of leukocyte accumulation on the substrate. A wide range of leukocytes including neutrophils, monocytes, myeloid and T cell lines, but not peripheral blood T lymphocytes, formed homotypic interleukocyte tethers that nucleated rolling. L-selectin therefore can amplify a wide range of vascular adhesive interactions of leukocytes in shear flow. The ability of adherent leukocytes to amplify subsequent leukocyte recruitment through L-selectin is analogous to that of adherent platelets, which can amplify leukocyte recruitment through P-selectin (13, 30).

Elsewhere, we characterize the ligand on leukocytes for L-selectin using rolling on substrates bearing purified L-selectin or L-selectin chimera as the assay (20). Sialic acid and fucose are required for ligand activity, but lack of the MECA-79 sulfation-associated epitope (22) distinguishes the leukocyte L-selectin ligand from that expressed on HEV (20). Using rolling on purified L-selectin to define cells that express the L-selectin ligand, together with the results here, shows that expression of both L-selectin and its ligand, but not L-selectin alone or the ligand

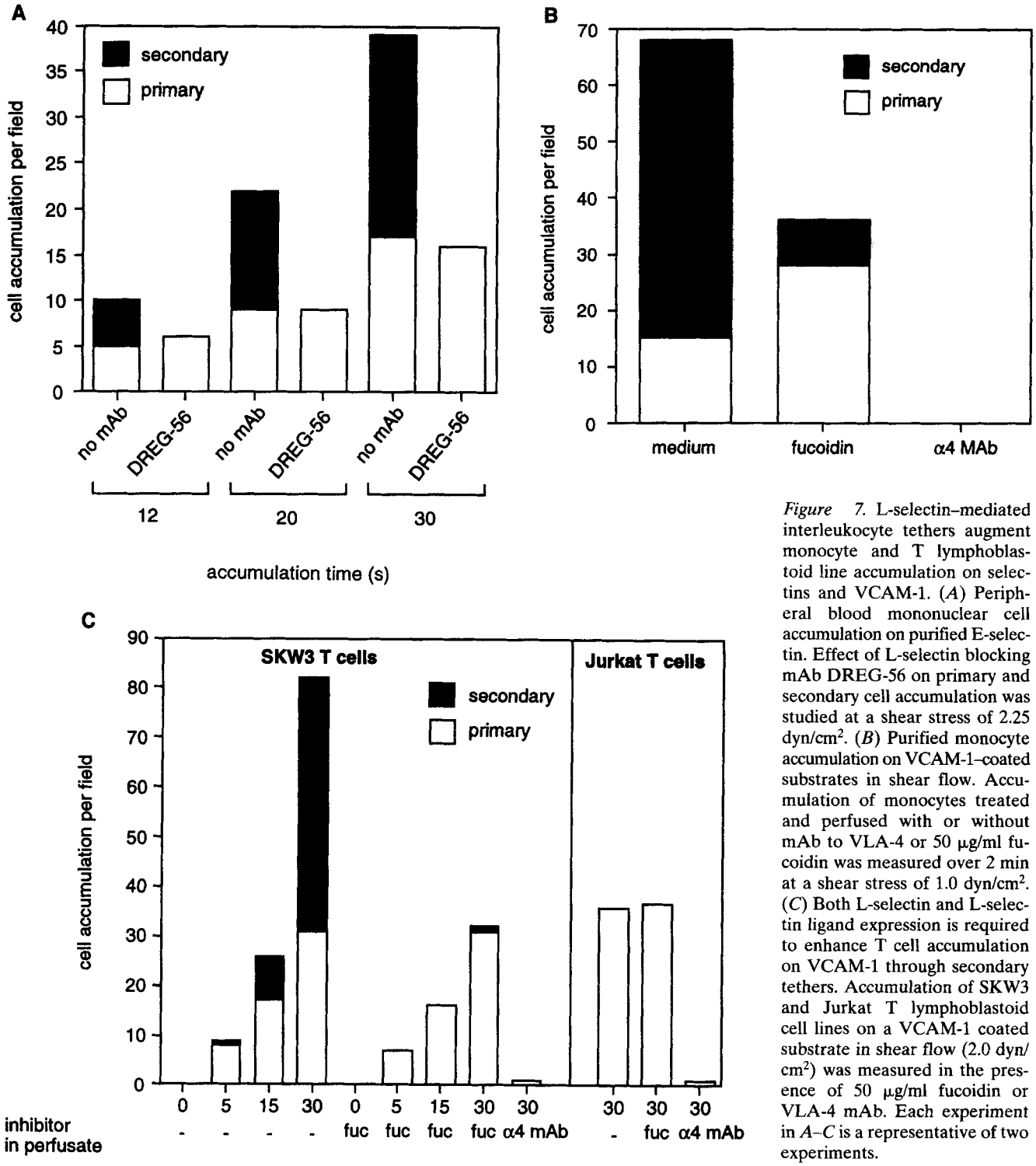

Figure 7. L-selectin–mediated interleukocyte tethers augment monocyte and T lymphoblastoid line accumulation on selectins and VCAM-1. (*A*) Peripheral blood mononuclear cell accumulation on purified E-selectin. Effect of L-selectin blocking mAb DREG-56 on primary and secondary cell accumulation was studied at a shear stress of 2.25 dyn/cm^2. (*B*) Purified monocyte accumulation on VCAM-1–coated substrates in shear flow. Accumulation of monocytes treated and perfused with or without mAb to VLA-4 or 50 μg/ml fucoidin was measured over 2 min at a shear stress of 1.0 dyn/cm^2. (*C*) Both L-selectin and L-selectin ligand expression is required to enhance T cell accumulation on VCAM-1 through secondary tethers. Accumulation of SKW3 and Jurkat T lymphoblastoid cell lines on a VCAM-1 coated substrate in shear flow (2.0 dyn/cm^2) was measured in the presence of 50 μg/ml fucoidin or VLA-4 mAb. Each experiment in *A–C* is a representative of two experiments.

alone, are sufficient for the formation of interleukocyte tethers and strings.

Many lines of evidence support the occurrence of L-selectin–dependent interleukocyte tethers, their importance in accumulation of cells through secondary tethers, and their distinction from primary tethers on vascular substrates. mAb to L-selectin did not inhibit leukocyte accumulation at the earliest time points, when leukocytes were bound randomly across the substrate, but inhibited at later time points. At later time points, untreated cells accumulated in strings, but mAb to L-selectin completely inhibited string formation and resulted in the accumulation of a reduced number of cells that were randomly distributed across the substrate. Separate enumeration of accumulation of primary cells, i.e., cells that tethered directly to the substrate, and secondary cells, i.e., cells that tethered to an adherent cell before tethering to the substrate downstream, showed that secondary cell accumulation and not primary cell ac-

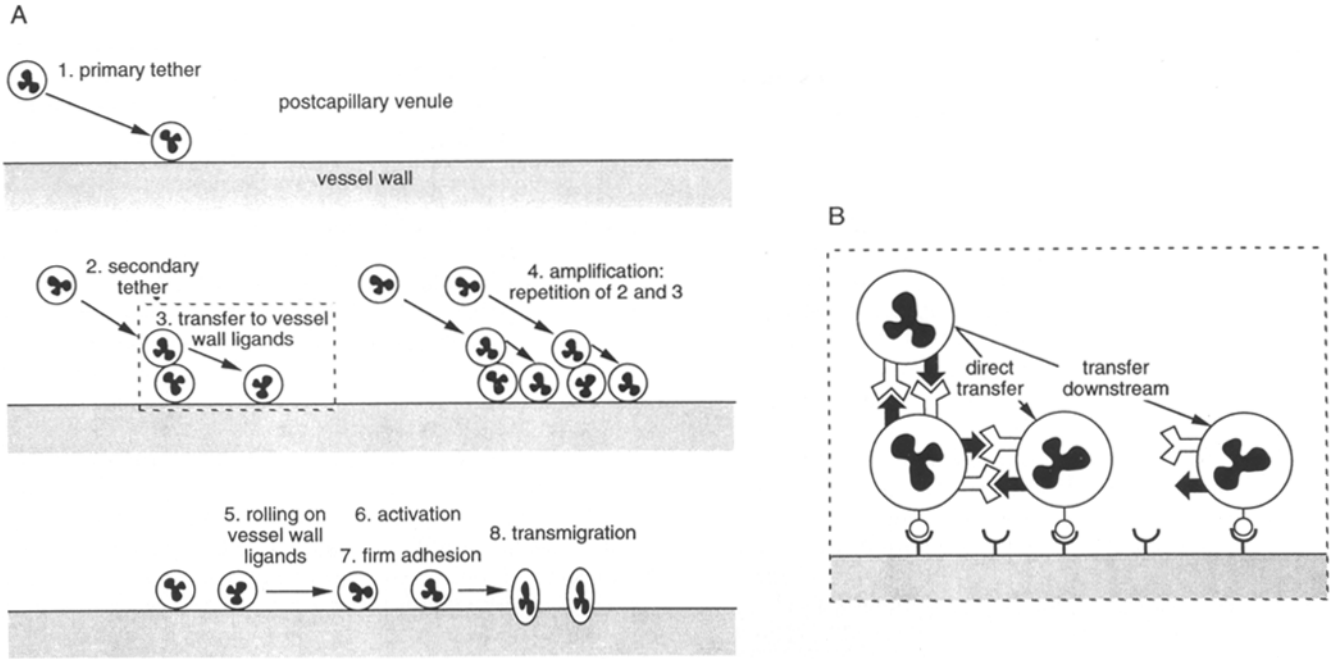

Figure 8. Leukocyte–leukocyte tethers through L-selectin: a positive feedback loop and additional step in the cascade of leukocyte accumulation in inflammation. (*A*) The steps in accumulation. (*B*) Transfer of a leukocyte from an interleukocyte tether to the vessel wall. A leukocyte expressing both L-selectin and L-selectin ligands can use either or both to interact with counterreceptors on an adherent leukocyte. Transfer to ligands on the vessel can either occur directly, while the leukocytes remain tethered (*direct transfer*), or after the two leukocytes appear to dissociate (*transfer downstream*).

cumulation was L-selectin–dependent with a wide range of substrates and cell types. Fucoidan and mAb that block L-selectin function, but not nonblocking mAb to L-selectin, inhibited secondary cell accumulation and the pattern of cell accumulation in strings. In a system where neuraminidase treatment of neutrophils did not inhibit primary tethers, i.e., L-selectin–dependent tethering to PNAd on the substrate, neuraminidase completely abolished secondary tethers. This is in agreement with abolishment by neuraminidase of L-selectin ligand function on neutrophils, as assessed both with rolling of neutrophils on one another (7) and rolling of neutrophils on L-selectin substrates (20). L-selectin–dependent interleukocyte tethers are transient, lasting less than 0.2 s. This is similar to the lifetime of neutrophil transient tethers through L-selectin on low densities of peripheral node addressin (0.15 s) or through the L-selectin ligand on neutrophils on low densities of L-selectin (Alon, R., S. Chen, K.D. Puri, E.B. Finger, and T.A. Springer, manuscript in preparation).

Newly secondarily tethered cells either transiently rolled along the surface of previously adherent cells or briefly touched adherent leukocytes and then tethered to the substrate, either immediately adjacent to or downstream of the adherent cell, or at a nearby site up to 10 cell diameters away. In the latter case, the interleukocyte tether appeared to dissociate before formation of the secondary tether to the substrate. Secondary cells could also transfer from one adherent cell to another along the length of the string, in an L-selectin–dependent manner, before finally tethering to the substrate part way along or at the end of the string. Transfer along the length of a string was much more rapid than rolling on vascular selectin substrates or on cytokine-stimulated endothelium, consistent with the rapid velocity of L-selectin–dependent neutrophil rolling on neutrophils (7, 17) and on peripheral node addressin (33).

In the presence of mAb to L-selectin, cells in shear flow were observed to bump into adherent cells, but the duration of cell contact was much shorter, and such contact did not promote tethering to the substrate. Adherent cells will alter streamlines in their vicinity, and cells within a cell diameter or two of one another will hydrodynamically interact by lowering the shear forces on one another. This may facilitate the formation of strings but could not by itself result in their formation, as shown by complete inhibition with L-selectin mAb. Previous studies that noted a functional interaction between L-selectin and vascular selectins (2, 27, 32, 48) did not examine the history of individual cell accumulation, but only the final amount of accumulation. Together with the short lifetime of interleukocyte tethers, this may explain why this important phenomenon has not previously been noted.

Primary cell accumulation on E- and P-selectin substrates, i.e., accumulation that was not preceded by interleukocyte tethers, was not reduced when L-selectin function was abolished. Furthermore, L-selectin antagonists did not reduce primary cell accumulation on VCAM-1 or cytokine-stimulated endothelium that expressed VCAM-1 and E-selectin. Moreover, shedding of L-selectin from the neutrophil surface by fMLP-induced activation had a negligible effect on primary interactions between neutrophils and E- or P-selectin–coated substrates. L-selectin antagonists also did not inhibit accumulation in shear flow of CHO cells transfected with E-selectin on substrates where sLe^x was presented by L-selectin. Thus, L-selectin, despite

its expression of sLe^x glycans and its specialized distribution on tips of microvilli (48) does not appear to be a primary counterreceptor for either of the vascular selectins in shear flow, as previously suggested (2, 27, 48). Although differing in interpretation, the data on the final amount of cell accumulation in these previous studies is in excellent agreement with the present data.

Previously, it was found that mAb to L-selectin inhibited neutrophil accumulation on E-selectin but had no effect on binding under static conditions, resistance to detachment in shear, or rolling velocity on E-selectin (32). We have confirmed this and found no effect of L-selectin antagonists on rolling velocity or shear resistance on P-selectin, VCAM-1, and cytokine-stimulated endothelium. Our finding that L-selectin contributes indirectly to tethering to these substrates, and has no role in direct tethering, is in excellent agreement with the lack of effect of antagonism of L-selectin on rolling velocity or strength of adhesion on these substrates and provides the underlying explanation. One recent study suggested that PSGL-1 on neutrophils and HL-60 cells was important for optimal HL-60 cell accumulation on E-selectin in shear flow (47). The PL-1 mAb to PSGL-1 shown to inhibit in the previous study had no effect on neutrophil accumulation on E-selectin in the current study. Lack of a detectable effect of the PL-1 mAb on interleukocyte tethers in the present study suggests that the P-selectin binding site on PSGL-1 is not the predominant ligand on leukocytes for L-selectin but does not rule out a contribution by PSGL-1 to L-selectin ligand activity. Proteolytic removal of both PSGL-1 and L-selectin from neutrophils had little effect on primary accumulation on E-selectin, showing that neither molecule is a major tethering counterreceptor for E-selectin.

The shear tolerance of interleukocyte tethers and the projection of adherent leukocytes into the flow stream are features that are likely to be important in vivo, and enhance the importance of leukocyte–leukocyte compared to leukocyte–endothelial tethers. Interleukocyte tethers occurred and nucleated string formation and leukocyte accumulation at wall shear stresses as high as 6 dyn/cm^2, whereas no direct tethers occurred at or above 4 dyn/cm^2 on E-, P-, or L-selectin substrates. A direct comparison showed that tethering of neutrophils to L-selectin substrates was not more efficient than to P-selectin substrates at high shear stresses (20). This argues that L-selectin ligands are not more shear tolerant per se than P-selectin ligands on neutrophils and that other features may be important. Adherent neutrophils project into the flow stream, making them highly accessible for binding. This may be compared to the clustering of L-selectin and PSGL-1 on tips of microvilli on neutrophils, which makes them more accessible (46, 48, 60). The gap in our flow chamber is 260 μm, whereas postcapillary venules, the sites of leukocyte accumulation in vivo, range from 10 to 100 μm in diameter and in typical intravital microscopy preparations are 20 to 50 μm in diameter. An adherent neutrophil 8.5 μm in diameter therefore presents a substantial profile to the flow-stream in a postcapillary venule. Furthermore, a head-on or sideways collision between two leukocytes may be more favorable than a glancing collision with the vessel wall for formation of an adhesive contact. Both the distractive forces on the region of contact and flattening or other distortion of the shape of the cell surface at the region of collision, a function of cellular viscoelasticity, would differ. Moreover, for leukocytes that coexpress L-selectin and L-selectin ligands, interleukocyte tethers can be bidirectional as discussed below, whereas interactions with vascular selectins are unidirectional.

Interleukocyte tethers and secondary accumulation required a wall shear stress above a threshold of 0.7 dyn/cm^2. This extends previous observations that rolling of neutrophils on one another and rolling on peripheral node addressin require a shear threshold (17). The shear threshold requirement may help prevent inappropriate leukocyte accumulation in vessels with inherently low wall shear stresses, or in hypoperfusion.

The ability of adherent leukocytes to accelerate the accumulation of further leukocytes through interleukocyte tethers introduces a positive feedback loop into the paradigm for leukocyte emigration at inflammatory sites (Fig. 8). This amplification mechanism may be important to make leukocyte mobilization more rapid and focal. Almost all positive amplification mechanisms in biology are accompanied by a negative regulatory mechanism; such a down-regulatory mechanism may be the most important biological function of L-selectin shedding. L-selectin is quantitatively shed within minutes of neutrophil activation by chemoattractants (25). Shedding is highly selective, since E-selectin ligands (32), PSGL-1 (46), and L-selectin ligands (20) (and as shown here) are not shed. We found that chemoattractant stimulation of neutrophils accompanied by L-selectin shedding completely down-regulated secondary accumulation and string formation; however, in this case, both the adherent cells and cells in shear flow had shed L-selectin. Neutrophils can roll on L-selectin substrates (20); if we view L-selectin on a substrate as a surrogate for an adherent leukocyte, this suggests that unidirectional interactions, where the L-selectin ligand is on the cell in shear flow and L-selectin is on the adherent cell, could be functionally important. Furthermore, the finding that pretreatment of adherent neutrophils with mAb to L-selectin inhibits secondary accumulation of untreated neutrophils on E- and P-selectin substrates by 60 to 70% (data not shown), suggests that L-selectin on the adherent leukocyte can make an important contribution to interleukocyte tethers; therefore, shedding of L-selectin from the adherent neutrophil is predicted to down-regulate accumulation. Previously, it was reported that L-selectin functioned only on the cell in flow in neutrophil rolling on adherent neutrophils (7); however, the adherent neutrophils had been in contact with the substrate for 10 min before neutrophils in flow were introduced, and likely had already shed their L-selectin. This system probably models events late in leukocyte accumulation in the inflammatory cascade, whereas our system models early events.

Ligands for L-selectin are also expressed on vascular endothelium. Ligands on high endothelial venules that are important in lymphocyte trafficking into lymphoid tissues have been characterized by binding of lymphocytes and of L-selectin chimera to tissue sections, by immunoaffinity isolation and determination of the structure of the carbohydrates and the mucin-like glycoproteins on which they are expressed, and by reconstitution of rolling with purified ligands in vitro (29, 33, 49). No such studies are avail-

able on L-selectin ligands on vascular endothelium from nonlymphoid tissues, although intravital microscopy has shown that L-selectin contributes to rolling in these vessels (36, 58), and L-selectin contributes to exudation from such vessels (4, 34). The relative contribution of L-selectin ligands on endothelium and on adherent leukocytes to rolling interactions in nonlymphoid vessels and on cultured endothelium will be an important topic for future research because previous studies have not examined the histories of interactions of individual rolling cells with adherent leukocytes and endothelium. Several studies have shown inhibition by mAb to L-selectin of monocyte and neutrophil accumulation on cytokine-stimulated cultured endothelial cells (1, 2, 41, 54, 55). By contrast, under similar conditions mAb to L-selectin have no effect on accumulation of purified T lymphocytes on cytokine-stimulated endothelium (23, 42). This is a surprising inconsistency, because T lymphocytes, neutrophils, and monocytes express similar densities of L-selectin (51) and bind through L-selectin to peripheral node addressin in shear flow with similar efficiency (33). The ligand for L-selectin is expressed on neutrophils and monocytes but not on peripheral blood T lymphocytes (20); therefore, the ability of neutrophils and monocytes but not lymphocytes to form L-selectin–dependent interleukocyte tethers as shown here may provide an explanation for this conundrum. In our own studies on IL-4–, TNF–, and IL-1–stimulated endothelium, we have failed to observe any primary neutrophil accumulation that was blocked by mAb to L-selectin, although secondary accumulation was readily inhibited. However, conditions appear to exist under which L-selectin ligands can be induced on cultured endothelium, as suggested by one study in which neuraminidase treatment of the endothelium inhibited L-selectin–dependent accumulation (54).

Knockout studies suggest that L-, E-, and P-selectin all contribute to rolling of neutrophils in nonlymphoid vessels, with L- and P-selectin the most important in acute inflammation (19, 38, 43). These studies show that L-selectin makes little or no contribution to leukocyte rolling at early time points after surgery and preparation of the microcirculation for observation, when P-selectin plays the dominant role. Beginning at 30 min and continuing past 60 min, both P- and L-selectin are important. P-selectin is rapidly mobilized from Weibel-Palade bodies, the secretory granules of endothelial cells, and the differing kinetics of L- and P-selectin functional activity make it unlikely that an L-selectin ligand could be mobilized from the same compartment. Synthesis of an L-selectin ligand could be induced de novo, although the kinetics appear rapid for this. It is plausible that leukocytes recruited to the vessel wall at early time points when P-selectin is functionally dominant could be an important source of the L-selectin ligand activity that is important for leukocyte rolling at later time points, together with ligand expressed directly on the vessel wall. Both types of ligand could contribute to tethering and rolling of L-selectin transfectants in vivo (38, 58), since there is accumulation of host leukocytes on vessel walls in these studies (see Fig. 3 in 37). At later time points, post capillary venules in typical intravital preparations contain one firmly adherent leukocyte per 9–15 μm of vessel length, and a considerably greater density of rolling cells (19, 58). Both firmly adherent and rolling cells could tether leukocytes in shear flow through L-selectin, and nucleate rolling on the vessel wall through adhesive ligands of which P-selectin appears the most important. This could account in part for the observed synergy in vivo between L- and P-selectin (4, 19, 36, 38, 43, 58). It should be noted that in intravital microscopy, the number of cells that roll through a vessel segment is quantitated. Tethering to the vessel wall is not quantitated and would be more difficult to observe than in the present study because the area within the focal plane of a vessel in vivo is more limited, and most tethering may occur in vessel segments that are upstream and outside the area of observation.

Our findings introduce a new step into the multistep model for leukocyte accumulation at inflammatory sites. In this model, leukocytes first tether to vascular ligands expressed on postcapillary venules in inflamed tissue. L-selectin and L-selectin ligands presented by adherent leukocytes can then interact with their counterreceptors on leukocytes in the flow stream and promote interleukocyte tethers. These tethers amplify leukocyte transfer to and subsequent rolling on adhesive ligands on the vessel wall. Leukocyte rolling is followed by integrin activation, firm adhesion, and finally diapedesis into the inflamed tissue. Recruitment of leukocytes from the flow stream can be down-regulated by shedding of L-selectin from adherent leukocytes. This introduces a novel L-selectin–dependent mechanism for modulation of accumulation of leukocytes at inflammatory sites. Notably, our study shows that contribution of L-selectin to leukocyte accumulation on endothelium in shear flow does not require the presence of vascular L-selectin ligands on the endothelium, but our study does not exclude contributions by L-selectin ligands expressed directly on vascular endothelium. We have studied purified leukocyte populations, but in vivo different populations can interact with one another through interleukocyte tethers. It is interesting that the cells that appear first at inflammatory sites, neutrophils and monocytes, express ligands for L-selectin, whereas lymphocytes that appear later do not express the ligand. Thus, the earlier appearance of neutrophils and monocytes may help recruit lymphocytes that express L-selectin to the vessel wall. Furthermore, the abundant expression of L-selectin ligands on HEV in lymphoid organs that function in lymphocyte recirculation may compensate for the inability of lymphocytes to form interlymphocyte tethers because of their lack of the L-selectin ligand, and thus the inability of this mechanism to augment lymphocyte binding to HEV. The synergy between leukocyte–leukocyte and leukocyte–endothelial interactions described here provides an additional mechanism for combinatorial regulation of leukocyte emigration in inflammation.

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