Endothelial Chemokines Destabilize L-selectin-mediated Lymphocyte Rolling without Inducing Selectin Shedding*[

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Chemokines presented on specialized endothelial surfaces rapidly up-regulate leukocyte integrin avidity and firm arrest through G-protein signaling. Here we describe a novel, G-protein-independent, down-regulatory activity of apical endothelial chemokines in destabilizing L-selectin-mediated leukocyte rolling. Unexpectedly, this anti-adhesive chemokine suppression of rolling does not involve L-selectin shedding. Destabilization of rolling is induced only by immobilized chemokines juxtaposed to L-selectin ligands and is an energy-dependent process. Chemokines are found to interfere with a subsecond stabilization of selectin tethers necessary for persistent rolling. This is a first indication that endothelial chemokines can attenuate in situ L-selectin adhesion to endothelial ligands at subsecond contacts. This negative feedback mechanism may underlie the jerky nature of rolling mediated by L-selectin in vivo.

Selectins mediate the reversible capture (tethering) of circulating leukocytes to vascular endothelium at numerous types of inflamed or lymphoid target tissues (1). Leukocyte tethers are short-lived and must be rapidly propagated into rolling adhesions, to allow the recruited leukocyte to survey the endothelial lining for additional stimulatory molecules, predominantly chemokines (2). Chemokines elicit rapid signals through binding to specific G-protein-coupled receptors (GPCR)† on tethered leukocytes, which trigger the avidity of leukocyte integrins to endothelial ligands, and thereby stabilize secondary leukocyte adhesion, arrest, and subsequent extravasation (3). Whether chemokine signals transduced to a rolling leukocyte can also modulate the adhesive properties of its selectin or selectin ligands has not been demonstrated. Soluble chemotactants have been shown, on the other hand, to trigger L-selectin shedding by cell surface endoproteolysis, implicating blood-borne chemokines as potential down-regulators of selectin-mediated rolling (4, 5). Selectin rolling is a highly dynamic process that depends on sub-second coupling of tethers successively formed and broken at the cell front and trailing edge under disruptive shear forces (6, 7). L-selectin rolling adhesions can be mediated by single tethers preferentially formed at microvillar surface projections where L-selectin is preferentially localized (8, 9). Notably, L-selectin-mediated leukocyte rolling in vivo is extremely fast and jerky in nature (10), even at endothelial sites expressing high levels of L-selectin ligands such as the peripheral lymph node high endothelial venules (HEV) (10, 11). Stabilization of L-selectin-mediated rolling, characterized by smooth rather than jerky motion, is critically dependent on the number of bonds simultaneously formed at each microvillar contact site (12). We therefore speculated that the jerky nature of L-selectin-mediated rolling in various in vivo settings might be caused by reduced L-selectin adhesiveness on leukocytes interacting with endothelium-displayed L-selectin ligands. In the present in vitro study, we found that several key chemokines, shown to be displayed on endothelial surfaces in vivo, are capable of strongly destabilizing the rolling activity of L-selectin in different types of leukocytes. Notably, this suppression of rolling was mediated by immobilized rather than soluble chemokines. Leukocyte capture to ligand, although normal, allowed the rapid encounter of immobilized chemokines co-displayed with L-selectin ligands, thereby eliciting the in situ reduction of L-selectin tether avidity to ligand. Surprisingly, destabilization of rolling was not the result of proteolytic L-selectin shedding. Furthermore, chemokine-mediated suppression of selectin rolling, although dependent on metabolic energy, did not involve intracellular signaling through the chemokine receptor. This is a first demonstration that endothelial chemokines may regulate selectin-mediated leukocyte rolling through a nonproteolytic G-protein-independent process, prior to and independent of their triggering of integrin adhesiveness.

EXPERIMENTAL PROCEDURES
Antibodies and Reagents

The anti-L-selectin mAb, DREG-200 (13), was provided by Dr. T. K. Kishimoto (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). The anti-very late antigen 4 (VLA-4) mAb, HP1/2 (14), was a gift from Dr. Roy Loeb (Biogen Inc., Cambridge, MA). The anti-glycoprotein cell adhesion molecule 1 (GlyCAM-1), purified from mouse serum by immunoaffinity chromatography (15), was a gift from Dr. S. D. Rosen (University of California, San Francisco, CA). P-selectin glycoprotein ligand 1 (PSGL-1) was affinity-purified from human neutrophil lysates, was a
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generous gift from Dr. R. P. McEver (University of Oklahoma, Oklahoma City, OK), and was stored frozen in 1% n-octyl-β-D-glucopyranoside/PBS. Peripheral node addressin (PNAd) purified from human tonsil lysates by MECA-79 mAb affinity chromatography (16), a generous gift from Drs. E. L. Berg (Protein Design Laboratories, Mountain View, CA) and J. J. Campbell (Children’s Hospital, Boston, MA), was stored in 1% octyl glucose/PBS solution at 4 °C. Chemokines were obtained from R&D Systems (Minneapolis, MN), except for BCA-1, a gift from Dr. P. Loetscher (University of Bern, Bern, Switzerland). Chemokines were functionally inactivated by brief heat inactivation for 5 min at 100 °C as described previously (17). Biotin-labeled stromal cell-derived factor-1α (SDF-1α) derivatives (modified either at the COOH or NH2 terminus of the chemokine) were a kind gift from Dr. F. Baleux (Institute Pasteur, Paris, France) and Dr. N. Fujii (Kyoto University, Kyoto, Japan). Both derivatives exhibited similar chemotactic activity toward T cells in Transwell chemotaxis assays. Biotin-labeled PSGL-1-derived sialyl Lewisα (sLeα)-decorated glycopeptide and a nonfucosylated control peptide, both corresponding to the 19-residue NH2 terminus of human PSGL-1, and each containing a single biotin group at its COOH terminus (18), were a gift from Dr. R. T. Camphausen (Genetics Institute, Cambridge, MA). Neutralize avidin (19) was a gift from Dr. E. A. Bayer, (Weizmann Institute of Science, Rehovot, Israel). Bovine serum albumin (fraction V), Ca2+/Mg2+-free Hank’s balanced salt solution, Ficoll-Hyphaque 1077, and phorbol 12-myristate 13-acetate were obtained from Sigma-Aldrich. Human serum albumin (HSA, fraction V), pertussis toxin (PTX), and tyrphostin AG490 were obtained from Calbiochem (La Jolla, CA). The protease inhibitors Ro31-9790 (20) and KD-IX-73-4-21 (21) were obtained from Dr. P. Altevogt (German Cancer Research Center, Heidelberg, Germany) and T. K. Kishimoto, respectively.

Cells

Human peripheral blood lymphocytes (PBL; obtained from healthy donors) were isolated from citrate-anticoagulated whole blood by dextran sedimentation and density separation over Ficoll-Hyphaque (23). Murine B lymphocytes were derived from fresh splenocytes (24). An equimolar mixture of biotin-labeled SDF-1α and either intact or heat-inactivated chemokine encounter by rolling lymphocytes on the dynamic adhesive substrate lasting at least 3 s after initial tethering. Transient tethers were cells that attached only briefly to the substrate (< 0.2 s). Frequency of each category of tethers was expressed in %; 1% unit measured at a 1.75 dyn/cm2 corresponding to tethering rate of 5.25 × 10−3 event × cell−1 min−1 s−1. To block GPCRs on target leukocytes, cells suspended in binding medium were preincubated for 45 min at 37 °C with 0.5 μg/ml soluble chemokines and perfused into the chamber. For blocking G protein signaling, PBL were cultured for 15 h at 37 °C in culture medium alone or in the presence of 100 ng/ml PTX. For blocking JAK/STAT pathway stimulation by chemokines, lymphocytes were preincubated for 2 h at 37 °C with 150 μM JAK inhibitor, tyrphostin AG490, or with control MeSO solution (0.1%, v/v). To inhibit metabolic energy without interfering with intact L-selectin signaling, rolling activity (rolling events/5 min × 106 cells) was determined by computerized cell motion tracking. Adhesive interactions of transiently tethered cells were also manually analyzed as described (27). The frequency of rolling cells was defined as the number of cells out of the cell flux that initiated persistent rolling on the adherent substrate lasting at least 3 s after initial tethering. Transient tethers were cells that attached only briefly to the substrate (< 0.2 s). Frequency of each category of tethers was expressed in %; 1% unit measured at a 1.75 dyn/cm2 corresponding to tethering rate of 5.25 × 10−3 event × cell−1 min−1 s−1.

Indirect immunofluorescence was performed on washed cells that were suspended in PBS supplemented with 5% FCS and 5 mM EDTA. Cells were incubated at 4 °C either with 10 μg/ml L-selectin mAb DREG-200 or with pre-immune mouse IgG. Cells were washed, stained with fluorescein isothiocyanate-conjugated goat anti-mouse Ig, resuspended in H/H medium containing 10 mM HEPES, pH 7.4, supplemented with 2 mg/ml bovine serum albumin (HSA, fraction V), pertussis toxin (PTX), and tyrphostin AG490, or with control MeSO solution (0.1%, v/v). To inhibit metabolic energy without interfering with intact L-selectin signaling, rolling activity (rolling events/5 min × 106 cells) was determined by computerized cell motion tracking. Adhesive interactions of transiently tethered cells were also manually analyzed as described (27). The frequency of rolling cells was defined as the number of cells out of the cell flux that initiated persistent rolling on the adherent substrate lasting at least 3 s after initial tethering. Transient tethers were cells that attached only briefly to the substrate (< 0.2 s). Frequency of each category of tethers was expressed in %; 1% unit measured at a 1.75 dyn/cm2 corresponding to tethering rate of 5.25 × 10−3 event × cell−1 min−1 s−1.

Immunoﬂuorescence Flow Cytometry

To block GPCRs on target leukocytes, cells suspended in binding medium were preincubated for 45 min at 37 °C with 0.5 μg/ml soluble chemokines and perfused into the chamber. For blocking G protein signaling, PBL were cultured for 15 h at 37 °C in culture medium alone or in the presence of 100 ng/ml PTX. For blocking JAK/STAT pathway stimulation by chemokines, lymphocytes were preincubated for 2 h at 37 °C with 150 μM JAK inhibitor, tyrphostin AG490, or with control MeSO solution (0.1%, v/v). To inhibit metabolic energy without interfering with intact L-selectin signaling, rolling activity (rolling events/5 min × 106 cells) was determined by computerized cell motion tracking. Adhesive interactions of transiently tethered cells were also manually analyzed as described (27). The frequency of rolling cells was defined as the number of cells out of the cell flux that initiated persistent rolling on the adherent substrate lasting at least 3 s after initial tethering. Transient tethers were cells that attached only briefly to the substrate (< 0.2 s). Frequency of each category of tethers was expressed in %; 1% unit measured at a 1.75 dyn/cm2 corresponding to tethering rate of 5.25 × 10−3 event × cell−1 min−1 s−1.

Preparation of Ligand-coated Substrates

Aliquots of GlyCAM-1, PNAd, or PSGL-1 were diluted in coating medium (PBS, supplemented with 20 mM boricarbonate, pH 8.5) and adsorbed onto polystyrene plates as described previously (27). Washed substrates were adsorbed with 0.1–4 μg/ml amount of either intact or heat-inactivated chemokines for 3 h at 4 °C. The anti-L-selectin mAb DREG-200 was mixed with either intact or heat-inactivated chemokines in the presence of 2 μg/ml HSA and coated onto polystyrene plates overnight at 4 °C. Neutralize avidin was diluted in PBS, 40 μM bicarbonate, pH 9.0, and adsorbed onto a polystyrene plate overnight at 4 °C, followed by HSA blocking at 4 °C. An equimolar mixture of biotin-labeled DREG-200 and either biotin-labeled SDF-1α or an inactive biotin-labeled control PSGL-1 peptide was diluted in cell binding medium (see below) and adsorbed for 4 h at 4 °C on the avidin-coated plate. Substrates coated with avidin complexed with inactive biotin-labeled glycopolypeptides lacked any adhesive activity to all L-selectin-expressing leukocytes tested.

Laminar Flow Assays

Cell Tethering and Rolling Measurements—The polystyrene plate, on which purified cells were adsorbed, was assembled in a parallel plate laminar flow chamber as described previously (28). Various leukocyte populations were washed in H/H medium (Hanks’ balanced salt solution, 10 mM HEPES, pH 7.4, supplemented with 2 mg/ml bovine serum albumin) containing 5 mM EDTA, resuspended in cell binding medium (H/H medium supplemented with 2 mg/ml CaCl2 at 2 × 105 cells/ml, and perfused at room temperature through the flow chamber at a rate generating wall shear stress of 0.3 dyn/cm2, as described (27). Once reaching the upstream side of the test adhesive substrate, the flow rate was elevated to generate a shear stress of 0.75, 1, or 1.75 dyn/cm2, and all cellular interactions were visualized at two different fields of view (each one of 0.17 mm2 area) using a 10× objective of an inverted phase contrast microscope (Diaphot 300, Nikon Inc., Tokyo, Japan). An imaging system was used for analysis of instantaneous velocities of HSA, or PMA, WSCAN-Array-3 (Galul, Migdal-Ha’emek, Israel) as described previously (27). Accumulation of rolling leukocytes on the test fields was determined by computerized cell motion tracking. Adhesive interactions of transiently tethered cells were also manually analyzed as described (27). The frequency of rolling cells was defined as the number of cells out of the cell flux that initiated persistent rolling on the adherent substrate lasting at least 3 s after initial tethering. Transient tethers were cells that attached only briefly to the substrate (< 0.2 s). Frequency of each category of tethers was expressed in %; 1% unit measured at a 1.75 dyn/cm2 corresponding to tethering rate of 5.25 × 10−3 event × cell−1 min−1 s−1.

RESULTS

Immobilized Chemokines Suppress Rolling Activity of L-selectin on Peripheral Blood Lymphocytes—To study the effect of chemokine encounter by rolling lymphocytes on the dynamic stability of their selectin contact, the ability of L-selectin-expressing leukocytes to tether to and roll on surfaces bearing both purified L-selectin ligands and chemokines was investigated in a parallel plate flow chamber. Human PBL were perfused on a substrate coated with PNAd or GlyCAM-1, pro-
Chemokine Destabilization of L-selectin Rolling Does Not Involve Selectin Shedding or G-protein Signaling—L-selectin rolling velocity can be affected by proteolytic shedding of the selectin by a cell surface protease (21). Interestingly, although soluble chemotaxtrants induce L-selectin shedding in myeloid cells (5), soluble SLC or SDF-1α failed to induce L-selectin shedding in PBL (Fig. 3A). Furthermore, the suppressive activity of SLC on rolling of T cells pretreated with the potent protease inhibitor Ro31-9790, confirmed to significantly block PMA-induced L-selectin shedding in lymphocytes (Fig. 3A, right panel), could not be rescued by the inhibitor (Fig. 3B) or by another hydroxamic acid-based L-selectin sheddase inhibitor, KD-IX-73-4 (data not shown). The protease inhibitor also did not augment L-selectin-mediated rolling of PBL on substrate coated with GlyCAM-1 alone (Fig. 3B), suggesting that spontaneous L-selectin shedding in lymphocytes does not take place during rolling on purified ligand, as reported previously for neutrophils (21, 32). Indeed, L-selectin lacking a protease recognition site has been reported to retain wild type activity when expressed in a lymphocyte line (33). L-selectin suppression was also induced by immobilized SDF-1α, and, as with SLC, suppression could not be rescued by inhibition of L-selectin shedding (Fig. 3B). Thus, chemokine destabilization of L-selectin-mediated rolling did not involve in situ L-selectin shedding on tethered lymphocytes.

Immobilized chemokines signal to target lymphocytes within subseconds of contact by binding their seven spanner receptors and activating their associated heterotrimeric G-proteins (2). Surprisingly, however, PTX inactivation of these G-proteins on PBL, which did not interfere with intrinsic L-selectin adhesive activity (Fig. 3C), also had no effect on chemokine suppression of L-selectin rolling (Figs. 3C and 4A). Nevertheless, destabilization of L-selectin-mediated rolling by chemokine required metabolic energy, because lymphocyte pretreatment with low levels of sodium azide could rescue chemokine-induced suppression of L-selectin rolling, whereas it did not affect spontaneous L-selectin rolling (Fig. 4A).

Taken together, these results suggest that chemokine destabilization of L-selectin-mediated rolling, although receptor-medi-
ated and energy-dependent, did not involve Gβγ-protein signaling by the chemokine receptor and was not the result of L-selectin shedding.

Leukocyte tethering to immobilized mAbs is inefficient under flow and is not followed by rolling adhesions, but serves as a sensitive measure of antigen density or availability on the surface of the tethered leukocyte at subsecond contact sites (9, 17). To investigate whether chemokine suppression of L-selectin rolling involves interference with local surface density of L-selectin at these tether sites, SDF-1α was co-immobilized with an anti-L-selectin mAb and its effect on lymphocyte tethering to the mAb was determined. Consistent with such interference, immobilized SDF-1α and SLC (data not shown), but not their soluble counterparts, strongly suppressed the ability of T lymphocytes to tether to anti-L-selectin mAb (Fig. 4B). In contrast, SDF-1α dramatically augmented lymphocyte tethering to an immobilized α4 integrin-specific mAb (Fig. 4B), consistent with its ability to induce in situ VLA-4 clustering at lymphocyte-substrate contact zones (17). Similar to the effects of SDF-1α on suppression of L-selectin rolling on authentic ligand (Fig. 4A), intact signaling capacity through the GPCR was not essential for the chemokine to suppress L-selectin binding to mAb, because suppression was insensitive to PTX pretreatment and could be induced by the non signaling SDF-1α mutant, P2G (Fig. 4B). Thus, lymphocyte binding to both authentic carbohydrate ligands and to an L-selectin-binding mAb was similarly sensitive to suppression by immobilized chemokines.

To further understand the specific requirement for immobilized chemokines in destabilization of L-selectin rolling, anti-CXCR4 mAb was immobilized alone or with SDF-1α (Fig. 4C). Specificity of the assay was confirmed by the ability of SDF-1α, but not of SLC, to augment CXCR4-dependent lymphocyte adhesion to immobilized anti-CXCR4 mAb (Fig. 4C). Notably and consistent with its inability to destabilize L-selectin-mediated rolling (Fig. 2), soluble SDF-1α did not augment or suppress CXCR4-dependent mAb adhesion in this assay (Fig. 4C). On the other hand, and consistent with its suppressive effects on L-selectin rolling and L-selectin mAb binding (Fig. 4, A and B), P2G could significantly augment CXCR4-dependent PBL adhesion in this assay (Fig. 4C). SDF-1α-augmented PBL adhesion to anti-CXCR4 mAb was also PTX-insensitive (Fig. 4C) and azide-sensitive (data not shown), reminiscent of SDF-1α suppression of L-selectin rolling (Fig. 4A). Thus, the ability of SDF-1α to destabilize L-selectin rolling of PBL correlated with the ability to augment CXCR4-dependent rolling of these PBL to immobilized CXCR4-binding mAb. Assuming that this binding depends on locally elevated densities of CXCR4 on the PBL surface at the site of immobilized CXCR4 ligand, SDF-1α, it appears that local clustering of CXCR4, induced by immobilized, but not by soluble SDF-1α, underlies the ability of SDF-1α to suppress L-selectin-mediated adhesion at subsecond contacts.

**Chemokine Suppression of L-selectin Rolling Is Shared among Different Leukocytes and Requires GPCR Recognition**—The destabilizing effects of chemokines on L-selectin-dependent rolling were not restricted to human PBL. The ability of murine B lymphocytes to establish rolling following tethering to GlyCAM-1 was similarly abolished in the presence of immobilized SDF-1α or B cell attracting chemokine 1 (BCA-1, CXCL13), both potent B cell chemokines (Fig. 5, A and B). As in PBL, soluble chemokines did not suppress L-selectin rolling in these cells, but could selectively rescue suppression of rolling by immobilized counterparts (Fig. 5B and data not shown). The suppressive effects of chemokines on L-selectin rolling were also not restricted to endothelial L-selectin ligands or to lymphoid cells, as they could be also observed with neutrophils interacting with a nonendothelial L-selectin ligand, PSGL-1 (Fig. 5C). Interleukin 8 (IL-8, CXCL8), a key chemokine implicated in neutrophil recruitment to inflamed endothelial sites (35), strongly suppressed neutrophil rolling on PSGL-1 (Fig. 5C). PSGL-1 immobilized with inactive chemokines or with SLC supported efficient rolling of neutrophils (Fig. 5C), consistent with low expression levels of the SLC GPCR, CCR7, on these leukocytes (data not shown). SLC strongly suppressed, however, L-selectin-mediated rolling of PBL on PSGL-1 (data not shown). Thus, the correct GPCR on a target leukocyte is required for its ligand to suppress L-selectin-mediated rolling.

Interestingly, the L-selectin rolling activity of PSGL-1 did not depend on its native mucin scaffold, because short PSGL-1-derived peptides, corresponding to the NH2-terminal selectin-binding region of PSGL-1, supported efficient L-selectin-mediated lymphocyte rolling when immobilized through a biotin spacer on an avidin-coated substrate (Fig. 6A), compa-
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Fig. 3. Neither L-selectin shedding nor G-protein signaling is involved in chemokine-suppression of L-selectin-mediated rolling. A, left panel, effects of PBL treatment with saturating levels of soluble SDF-1α (0.5 μg/ml; solid black line) or SLC (0.5 μg/ml; gray line) for 45 min on their surface L-selectin, determined by immunostaining with the L-selectin-specific mAb DREG-200; right panel, flow cytometry analysis of L-selectin on PMA-activated PBL. PBL preincubated with carrier solution (gray solid line) or with the protease inhibitor (S.I., sheddase inhibitor), Ro31-9790 (solid black line) were treated for 15 min with 100 ng/ml PMA and subsequently stained with anti-L-selectin mAb DREG-200. In both panels, the dashed black line indicates L-selectin staining of intact lymphocytes; the dotted line indicates PBL staining with an isotype matched control mAb. All experiments were carried out at 25°C. B, effect of blocking L-selectin shedding with the protease inhibitor Ro31-9790 on the frequency and type of tethers formed by lymphocytes perfused over GlyCAM-1 coated at 100 sites/μm² and co-immobilized with inactivated SDF-1α (−) or active SLC or SDF-1α (each at 4 μg/ml). C, effect of a 45-min PBL exposure to saturating levels of soluble chemokines (0.5 μg/ml) or of PBL pretreatment with PTX on rolling and transient tethering to GlyCAM-1 at 100 sites/μm². The ligand was coated alone or in the presence of SLC. Adhesion assays depicted in B and C were performed at a shear stress of 1.75 dyn/cm² and are each representative of five independent experiments with different donor PBL.

rable with that of native PSGL-1 (Fig. 6B). Notably, when SDF-1α was co-adsorbed with the biotinylated PSGL-1 peptide on an avidin-coated substrate through its non GPCR-binding COOH terminus, it could efficiently suppress L-selectin-dependent PBL rolling on the PSGL-1 peptide, through its functionally exposed NH₂ terminus (Fig. 6A, N°-ter). In contrast, SDF-1α, coupled to the avidin-coated substrate via its NH₂-terminal CXCR4 binding site, lost its ability to destabilize L-selectin-mediated PBL rolling (Fig. 6A). Thus, a functionally intact GPCR binding domain is required for immobilized SDF-1α to suppress L-selectin-mediated rolling, whereas the mode of chemokine presentation, either direct, by a plastic surface (Fig. 6B) or by an avidin scaffold (Fig. 6A), does not affect the capacity of the chemokine to suppress L-selectin rolling.

Chemokine Suppresses Stable Rolling but Not Transient Tethers—To destabilize L-selectin rolling, chemokines must be properly displayed on endothelial surfaces. We next tested the ability of cell surface determinants to display SDF-1α in a conformation capable of suppressing L-selectin-mediated rolling. In light of low ligand expression on endothelial-derived cell lines, we made use of a human umbilical vein endothelial cell-derived line, ECV-304, stably transfected with α-1,3-fucosyltransferase and N-acetylgalactosamine 6-O-sulfotransferase (26), two key regulators of L-selectin ligand biosynthesis on lymph node HEV and inflamed endothelia (36, 37). A murine pre-B lymphocytic cell line expressing CXCR4 but lacking endogenous L-selectin and transfected with human L-selectin cDNA could establish persistent rolling on these ligand-expressing ECV-304 cells under physiological shear flow (Fig. 7A). As observed with purified proteins, SDF-1α immobilized on the ECV-304 cell surface strongly suppressed L-selectin-mediated rolling of the L-selectin transfected B cells, without interfering with initial capture (Fig. 7A and inset). SDF-1α also strongly destabilized L-selectin rolling of these cells on purified GlyCAM-1 (Fig. 9A). In contrast to the pre-B transfectants, PBL captured only transiently to the same ligand-expressing ECV-304 monolayers and failed to establish rolling (Fig. 7A, inset). Strikingly, transient PBL tethers, although L-selectin-dependent, were completely resistant to SDF-1α suppression (Fig. 7A, inset). This result suggested that chemokines do not interfere with transient tethers mediated by weak L-selectin-ligand interactions. To further delineate this observation, we next tested how the suppressive effect of a chemo-
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Fig. 4. Immobilized but not soluble SDF-1α reduces effective L-selectin density on tethered lymphocytes at local adhesive contacts while increasing CXCR4 density. A, effect of signaling capacity and context of presentation of SDF-1α on the frequency and type of tethers formed by PBL interacting with PNAd at a shear stress of 1.75 dyn/cm². PNAd was coated at 100 ng/ml, and either SDF-1α or the nonsignaling SDF-1α derivative, P2G, were coimmobilized with it. Where indicated, PBL were pretreated with either soluble SDF-1α (0.5 μg/ml) or 0.05% azide, as described under “Experimental Procedures.” B, frequency and type of tethers mediated by PBL perfused over substrates coated with L-selectin-specific mAb DREG-200 (at 0.1 μg/ml) in the presence of inactive (−) or active SDF-1α or P2G. Tethers were measured at 1 dyn/cm². For comparison, effect of SDF-1α on frequency of PBL tethers formed with surface-bound α₄ integrin-specific mAb HP1/2 is shown. C, frequency and type of tethers mediated by PBL perfused at 0.75 dyn/cm² over surface-bound anti-CXCR4 mAb (0.1 μg/ml) coated with inactive (−) or active SDF-1α or the P2G derivative (each at 2 μg/ml). Note that co-immobilized SLC does not induce any adhesive activity in this setting. The scheme depicts a CXCR4-bearing microvillus tethered to a substrate coated with anti-CXCR4 mAb and SDF-1α. The experiments shown in A–C are each representative of three.

kine varies with the density of L-selectin ligand on the substrate. Reminiscent of the inability of SDF-1α to suppress transient PBL tethering (Fig. 7A, inset), immobilized SLC effectively suppressed L-selectin rolling of PBL observed on high or medium physiological GlyCAM-1 densities, but could not suppress either the formation or the duration of transient
L-selectin-mediated PBL tethers to low density GlyCAM-1 (Fig. 7B). Taken together, these findings suggest that chemokine binding to a tethered leukocyte selectively suppresses L-selectin-mediated rolling adhesions without interfering with transient L-selectin tethers.

**Chemokine Suppresses a Subset of High Avidity L-selectin Tethers Independently of Cytoskeletal Association of the Selectin**—To gain further insights into the kinetics of GPCR-mediated suppression, the motion of individual T lymphocytes interacting with medium density GlyCAM-1 and SLC (Fig. 7B) was next compared by computerized image analysis (27). The duration of L-selectin tethers comprising leukocyte rolling motions was shown to be progressively shorter at reduced bond numbers within each tether (12). Thus, tether duration can serve as a sensitive reporter of effective L-selectin avidity at the contact zone. Upon initial tethering to the ligand-coated surface, captured lymphocytes continued to roll on the ligand through engagement of closely spaced successive tethers (Fig. 6A, right panel). Over 80% of these tethers dissociated from the ligand with a first order dissociation rate corresponding to a $t_{1/2}$ of 19.5 ms (Fig. 8A, left panel, open circles). The remaining tethers lasted significantly longer, with a $t_{1/2}$ of 43 ms (Fig. 8A, left panel, closed circles). As reported earlier, lymphocytes interacting with GlyCAM-1 in the presence of immobilized SLC could not establish rolling (Fig. 8B, right panel) and engaged with the L-selectin ligand exclusively through short-lived tethers (Fig. 8B, left panel; $t_{1/2}$ of 17.8 ms). PBL pretreated with soluble SLC engaged with GlyCAM-1 co-immobilized with SLC with microkinetics nearly identical to that of intact PBL interacting with GlyCAM-1 in the absence of SLC (Fig. 8, A and C, left panels). Thus, persistent PBL rolling on GlyCAM-1 is mediated by a subset of prolonged tethers with a $t_{1/2}$ of 45 ± 2 ms, which make up about one fifth of all L-selectin-mediated tethers (Fig. 8, A and C). Chemokine suppression of rolling adhesions in this system is therefore associated with the ability to suppress this subset of nascent tethers by reducing their lifetime to a $t_{1/2}$ shorter than 20 ms.

The cytoplasmic domain of L-selectin has been shown to regulate leukocyte rolling under shear flow through stabilizing association of L-selectin with the cell actin cytoskeleton (25, 38). An L-selectin mutant lacking the carboxyl-terminal 11 residues of the cytoplasmic domain, with deficient cytoskeletal association, expressed on pre-B cells, can establish weak but persistent rolling on high density L-selectin ligands (39). Notably, SDF-1α could fully destabilize the rolling adhesions mediated by this tail-truncated L-selectin mutant on high density GlyCAM-1, despite of the very fast rolling mediated by this mutant (Fig. 9, A and B). Thus, chemokine destabilization of
L-selectin-mediated rolling does not require an intact cytoplasmic domain of L-selectin, suggesting that the suppressive signals exerted by chemokines do not require L-selectin association with the cytoskeleton.

**DISCUSSION**

Selectin-mediated tethering and rolling are prerequisite for leukocytes to survey the endothelial lining for proadhesive and promigratory signals, primarily apical chemokines (3, 10). Rolling leukocytes must integrate chemokine signals within sub-second contacts along the direction of flow (17, 40, 41). Here we suggest that apically displayed endothelial chemokines may not merely transmit integrin-activating signals to rolling leukocytes, but in fact directly modulate the rolling process itself, through an in situ reduction of selectin tether stability. Thus, rolling adhesions that allow a captured leukocyte to sample the endothelium for specific chemokines are subjected to a negative feedback mechanism by these very chemokines. Rather than being discrete and sequential events (42), reversible selectin interactions and chemokine receptor occupancy events appear to simultaneously operate at particular adhesive zones bearing immobilized chemokines juxtaposed to L-selectin ligands. As a result, selectin-mediated rolling, which has been predicted to increase encounter of endothelium-displayed chemokines (43), is in fact attenuated by this encounter. Attenuation of rolling is predicted to be more robust at sites of leukocyte interaction with high densities of L-selectin ligand, probably at endothelial regions within lymph node HEV enriched with L-selectin ligands (11). In addition, because the local density of chemokine on endothelial surfaces is heterogeneous (35), this attenuation mechanism may result in multiple dynamic outcomes. In regions of low chemokine density, L-selectin-mediated rolling is expected to be accelerated (Fig. 2), whereas L-selectin-mediated rolling on specific regions expressing high density chemokine is expected to be strongly suppressed (Fig. 2). This would cause a rolling leukocyte to detach from such sites, while allowing it to jerk and rebind ligand at an adjacent downstream sites. Furthermore, chemokine distribution on individual endothelial cells is nonuniform, as chemokines can be found in clusters on endothelial microvilli (35). These domains could be preferential sites of chemokine destabilization of L-selectin rolling. The jerky nature of L-selectin rolling is not controlled solely by chemokines. Anti-adhesive glycoproteins like CD43 (44), topological heterogeneity of both leukocyte and endothelial surfaces (45), as well as intrinsic properties of L-selectin bonds (46, 47) can each contribute to the jerky nature of L-selectin-mediated rolling of leukocytes along various blood vessels. The existence of such multiple mechanisms for attenuating L-selectin rolling suggests that the jerky nature of L-selectin-mediated rolling is of major physiological significance. One possible outcome of such suppression of rolling could be to attenuate direct integrin activation by L-selectin, a process that depends on L-selectin ligation by ligand and bypasses chemokine regulation of leukocyte arrest on integrin ligands (48–50).

Spontaneous and chemoattractant-induced proteolytic shedding of L-selectin was traditionally proposed as a major negative feedback mechanism of L-selectin-mediated leukocyte rolling (21, 51). However, the G-protein-independent chemokine destabilization of L-selectin rolling studied here did not involve L-selectin shedding, previously shown to involve activation of GPCR signaling (4, 51). The insensitivity of chemokine suppression of rolling to PTX blockage of G, signaling, demonstrated here, also rules out the possibility that chemokines suppress L-selectin rolling through G-protein-dependent phosphorylation of the L-selectin cytoplasmic tail (52). Indeed, even lymphocytes expressing tail-truncated L-selectin were sensitive to chemokine suppression of rolling. Similar to our finding of an accelerated L-selectin rolling induced by low level chemokine (Fig. 2), Campbell and co-authors (40) reported 2-fold faster L-selectin-dependent rolling of murine lymphocytes on PNAa co-immobilized with chemokines. The study attributed the accelerated rolling to chemokine blockage of L-selectin.
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binding carbohydrates on the substrate. Our evidence that chemokines suppress L-selectin binding to mAb, an L-selectin-binding protein that lacks any selectin-binding carbohydrates, rules out the possibility of L-selectin ligand masking by chemokine. Furthermore, our finding that chemokines fail to suppress L-selectin adhesion to low density ligand (Fig. 7B) also rules out a direct blockage of ligand activity by chemokine. Instead, our data strongly suggest that chemokines induce rapid redistribution of both chemokine receptors and L-selectin at adhesive contact sites, possibly through extracellular or membranal associations of their receptors with juxtaposed L-selectin molecules. Recent electron microscopic analysis of the chemokine receptors for SDF-1α and RANTES (regulated on activation normal T cell expressed and secreted), CXCR4 and CCR5, respectively, in PBL has demonstrated that these GPCRs localize to lymphocyte microvilli (53), where L-selectin as well as α4 integrins are preferentially co-expressed (8, 9, 54, 55). These observations suggest that these chemokine receptors and probably other GPCRs of endothelial chemokines, including CCR7, CXCR5 and CXCR1/2, receptors for SLC/ELC, BCA-1, and IL-8 or Groα (CXCL1), respectively, may also be found on leukocyte microvilli. We propose a model whereby, upon binding immobilized chemokines juxtaposed to endothelial L-selectin ligands, the leukocyte-based GPCRs may cluster in proximity to L-selectin molecules, sterically hindering L-selectin clustering with high density ligand (Fig. 4C, inset). Such selectin/ligand clusters appear essential to stabilize a newly formed tether at subsecond endothelial contacts (12, 56–58). We have previously demonstrated that PBL GPCR occupancy by immobilized but not by soluble chemokines, induces rapid α4 integrin avidity at adhesive contacts containing chemokine and integrin ligand (17). The present study suggests the reverse activity of GPCR occupancy by immobilized chemokines, i.e., interference with L-selectin avidity to ligand. The ability of chemokine to suppress L-selectin rolling is closely correlated with the ability of immobilized chemokine to augment binding of its GPCR to a GPCR-binding mAb on a countersurface (Fig. 4C). This suggests that the local density of the leukocyte GPCR at sites containing immobilized chemokine

![Diagram](image-url)
must be rendered high to effectively destabilize selectin avidity to respective ligands. Notably, to suppress selectin rolling, immobilized chemokines do not need to activate G-protein signaling or trigger GPCR internalization. We considered that chemokine-induced GPCR clustering could activate JAK/STAT signaling cascades independent of G-protein signaling (59). However, blockage of JAK activation with the specific inhibitor tyrphostin AG490 did not block chemokine suppression of L-selectin rolling in PBL (data not shown). Notably, suppression of L-selectin rolling appeared to require specialized GPCR-associated machinery, because engagement of a non-GPCR cytokine receptor, IL-2 receptor, by high level IL-2 co-immobilized with L-selectin ligand had no effect on L-selectin-mediated rolling of PBL. Nevertheless, suppression of L-selectin-mediated rolling by chemokine-occupiedGPCRs required metabolic energy, consistent with an involvement of active cellular machinery in chemokine suppression of L-selectin function.

The new notion that chemokines can reciprocally modulate selectin and integrin adhesiveness predicts that a proper balance and coordinated timing of these opposite chemokine activities at leukocyte/endothelial contacts must be maintained to allow optimal conversion of selectin-mediated rolling into integrin-mediated leukocyte arrest. Chemokine recognition by a rolling leukocyte poses a regulatory hurdle in the propagation of multistep L-selectin initiated adhesive cascades, not previously realized. This novel activity of chemokines and its unique G-protein-independent mode of action should be considered when using specific chemokine antagonists to attenuate pathological L-selectin-mediated leukocyte recruitment at various target tissues.

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