Membranal Cholesterol Is Not Required for L-Selectin Adhesiveness in Primary Lymphocytes but Controls a Chemokine-Induced Destabilization of L-Selectin Rolling Adhesions

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Cholesterol-enriched lipid microdomains regulate L-selectin signaling, but the role of membrane cholesterol in L-selectin adhesion is unclear. Arrest chemokines are a subset of endothelial chemokines that rapidly activate leukocyte integrin adhesiveness under shear flow. In the absence of integrin ligands, these chemokines destabilize L-selectin-mediated leukocyte rolling. In the present study, we investigated how cholesterol extraction from the plasma membrane of peripheral blood T or B cells affects L-selectin adhesions and their destabilization by arrest chemokines. Unlike the Jurkat T cell line, whose L-selectin-mediated adhesion is cholesterol dependent, in primary human PBLs and in murine B cells and B cell lines, cholesterol depletion did not impair any intrinsic adhesiveness of L-selectin, consistent with low selectin partitioning into lipid rafts in these cells. However, cholesterol raft disruption impaired the ability of two arrest chemokines, CXCL12 and CXCL13, but not of a third arrest chemokine, CCL21, to destabilize L-selectin-mediated rolling of T lymphocytes. Actin capping by brief incubation with cytochalasin D impaired the ability of all three chemokines to destabilize L-selectin rolling. Blocking of the actin regulatory phosphatidylinositol lipid, phosphatidylinositol 4,5-bisphosphate, did not affect chemokine-mediated destabilization of L-selectin adhesions. Collectively, our results suggest that L-selectin adhesions are inhibited by actin-associated, cholesterol-stabilized assemblies of CXCL12- and CXCL13-binding receptors on both T and B lymphocytes. Thus, the regulation of L-selectin by cholesterol-enriched microdomains varies with the cell type as well as with the identity of the destabilizing chemokine. The Journal of Immunology, 2007; 179: 1030–1038.

Selectins are key lectins that mediate initial leukocyte capture and rolling of circulating leukocytes on lymphoid and inflamed endothelium (1). Selectin-mediated rolling is a dynamic process that involves the successive formation and breakage of adhesive tethers lasting for periods of subseconds (2). Because these tethers must resist disruptive shear forces, adhesiveness is favored by proper anchorage of both selectins and their ligands to the actin cytoskeleton (3, 4). Cholesterol- and glycosphingolipid-enriched lipid microdomains, also termed cholesterol rafts, are key signaling platforms enriched with cortical cytoskeleton proteins (5–7), some of which bind L-selectin (8). The disruption of these lipid rafts in immune cells by cholesterol extraction interferes with various integrin-mediated adhesive processes, including rapid activation by endothelial chemokines under shear flow (9–11), and could therefore potentially affect selectin-mediated adhesions.

L-selectin is expressed on most circulating leukocytes. Optimal L-selectin adhesiveness under shear flow depends on proper associations of its cytoplasmic tail with actin cytoskeletal adaptors such as ERM3 protein members and α-actinin (8, 12). L-selectin is also a costimulatory receptor that, upon ligation, can trigger T cell Src kinases, such as p56Lck (13), which is highly enriched in low-density cholesterol-stabilized lipid rafts (14). In Jurkat T cells, L-selectin partitions into low density lipid rafts and, upon ligation, undergoes rapid shedding by raft-associated machineries (15). Consequently, rolling adhesions mediated by L-selectin on Jurkat T cells are sensitive to disruption of cholesterol rafts by the cholesterol-depleting agent, methyl-β-cyclodextrin (MβCD) (16). We therefore speculated that cholesterol lipid rafts could also serve as key platforms for cytoskeletal associations and adhesive functions of L-selectin in freshly isolated human and murine PBL.

Chemokines are chemotactic cytokines implicated in immune cell motility and in situ modulation of adhesion receptors such as integrins and selectins (17). A subset of endothelial displayed chemokines, termed arrest chemokines (18), is presented to rolling leukocytes and promotes their shear-resistant arrest on target endothelial sites expressing integrin ligands (19). We have previously shown that α4 integrin activation by three major homeostatic chemokines, CXCL12 (stromal-derived factor-1α), CCL21 (secondary lymphoid tissue chemokine), and the B-lymphocyte-specific chemokine, CXCL13 (B cell attracting chemokine-1), takes place in cholesterol rafts of lymphocytes (11). A previous study

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Received for publication April 16, 2007. Accepted for publication April 30, 2007.

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1 This work was supported by the Israel Science Foundation and by the Minerva Foundation, Germany. R.A. is the Incumbent of the Linda Jacobs Chair in Immune and Stem Cell Research.

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3 Abbreviations used in this paper: ERM, Ezrin/Radixin/Moesin; GPCR, G protein-coupled receptor; MβCD, methyl-β-cyclodextrin; PIP_{2}, phosphatidylinositol 4,5-bisphosphate; PNAd, peripheral node addressin.

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from our laboratory also indicated that these arrest chemokines, as well as CXCL8, an arrest chemokine specific for neutrophils, interfere with a subsecond stabilization of adhesive L-selectin tethers on major endothelial ligands, when coimmobilized with these ligands on the same adhesive surface (20). Without this critical stabilization of L-selectin adhesions, L-selectin-mediated rolling cannot persist, and captured cells rapidly detach back to the circulation (20). These findings collectively raised the possibility that whereas arrest chemokines can trigger integrin adhesiveness, they can also destabilize L-selectin-mediated rolling interactions when integrin ligands are scarce or absent (20). Whether these opposite functions of arrest chemokines take place in shared or distinct membranal microdomains remained an open issue.

We now report that cholesterol extraction from the plasma membrane of primary T and B lymphocytes as well as from a pre-B cell line does not impair intrinsic adhesive activities of L-selectin at physiological shear stresses. These results are in contrast to the effect of cholesterol extraction from Jurkat T cells (16) and reflect the low partition of L-selectin into raft fractions in the cellular models tested in the present study. Cholesterol extraction impairs, however, the ability of the two major homeostatic arrest chemokines, CXCL12 and CXCL13, to destabilize L-selectin-mediated rolling adhesions. Notably, a third major homeostatic chemokine, CCL21, a ligand of the CCR7 G protein-coupled receptor (GPCR), can destabilize L-selectin-mediated rolling even in cholesterol-depleted lymphocytes. Destabilization of rolling by all chemokines tested requires intact actin cytoskeleton, but does not depend on the presence of the phosphatidylinositol 4,5-bisphosphate (PIP2) phosphatidylinositol, a regulator of attachments of the actin cytoskeleton to the plasma membrane (21). Collectively, our results suggest that the arrest chemokines, CXCL12 and CXCL13, deliver an inhibitory signal to L-selectin that takes place in actin-stabilized cholesterol rafts. L-selectin adhesiveness, in contrast, is far less sensitive to cholesterol raft disruption than recently suggested (16). Regulation of L-selectin-mediated rolling by cholesterol raft components and chemokines is therefore dependent both on the cell type and the particular chemokine engaged at the L-selectin adhesive contact.

Materials and Methods

Abs and reagents

The anti-L-selectin mAbs, DREG-200 (22); CA21, directed against the C terminus of the cytoplasmic tail of L-selectin (23); and JKV23, a polyclonal rabbit anti-human L-selectin Ab, directed against the extracellular domain of L-selectin (23), were provided by T. Kishimoto and J. Kahn (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Peripheral node addressin (PNAd) purified from human tonsil lysates by MECA-79 mAb affinity chromatography (24) was a gift from E. Berg (Protein Design Labs, Mountain View, CA) and J. Campbell (Children’s Hospital, Boston, MA), and was stored in 1% octyl glucoside/PBS solution at 4°C. All tested chemokines were obtained from R&D Systems. The anti-CXC4R4 mAb, 12G5, was purchased from BD Pharmingen. The anti-CCR7 mAb 150503 was purchased from R&D Systems. BSA (fraction V), Ficol-Hypaque 1077, MjCJ, and Ca2+ and Mg2+-free HBSS were obtained from Sigma-Aldrich. Human serum albumin (fraction V) and cytochalasin D were obtained from Calbiochem. The rhodamine-labeled PIP2-binding peptide PBPI0 (rhodamine B-QRLFQVKGGR) corresponding to gelsolin residues 160–169 and the control peptide (rhodamine B-QRL) was a gift from P. Janmey (University of Pennsylvania, Philadelphia, PA), were prepared by solid-phase peptide synthesis, as previously described (25).

Cells

Human PBL (obtained from healthy donors) were isolated from citrate-anticoagulated whole blood, as described (26), and consisted of >90% CD19+ T lymphocytes. The only CXCR12 receptor detected on these lymphocytes was CXCR4 (data not shown). The Jurkat and CEM T cell lines were cultured, as previously described (11). Murine B lymphocytes were derived from fresh splenocytes by positive immunoselection with mAb B220, followed by MACS purification, as described (27). The murine pre-B 300.19 cell line, stably expressing native human L-selectin (28), was a gift from G. Kansas (Northwestern University, Chicago, IL). Clones were maintained in RPMI 1640, supplemented with antibiotics, 10% FCS, 2 mM glutamine, and 0.1 μM 2-ME. The pre-B cells bound both CXCL12-Fc and CCL19-Fc fusion proteins, gifts from J. Cyster (University of California, San Francisco, CA).

Immunofluorescence flow cytometry and microscopy

Indirect immunofluorescence was performed on washed cells that were suspended in PBS and 10% bovine serum. Cells were incubated with primary Ab or with preimmune mouse IgG (10 μg/ml) for 30 min at 4°C, washed, and incubated with secondary Ab for an additional 30 min at 4°C. Cells were washed and analyzed immediately on a FACScan flow cytometer (BD Biosciences). L-selectin stained with the polyclonal rabbit anti-human L-selectin Ab JK923 was ligated by Cy3-conjugated anti-rabbit IgG, and GM1 patching was induced by Alexa 488-conjugated cholera toxin subunit B (Molecular Probes). Colocalization of ligated L-selectin and patched GM1 was analyzed by confocal microscopy (Zeiss).

Isolation and analysis of membrane low-density lipid fractions

Cells (3 × 10^7) were lysed in 10 mM Tris-HCl (pH 8.0) containing 1% Brij-96 or 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, and 1 μg/ml aprotinin. The lysate was mixed with an equal volume of 85% sucrose (w/v in TBS buffer), and 0.5 ml of lysate was transferred to a centrifuge tube. A step gradient was prepared by overlaying 3.5 ml of 35% sucrose in TBS, followed by a final layer of 0.5 ml of 5% sucrose in TBS, as previously described (9). The sucrose gradient was centrifuged for 1 h at 200,000 × g using a Beckman SW 55Ti or SW60 rotor. Fractions of 0.5 ml were collected from the top of the gradient and were each precipitated with chloroform/methanol (29). Samples were then dissolved in SDS-sample buffer and analyzed by blotting with a polyclonal anti-L-selectin Ab, followed by ECL detection, as described (30). For spingomyelin GM1 detection, an aliquot of each gradient fraction was mixed with an equal volume of SDS-sample buffer and analyzed with biotin-conjugated cholera toxin subunit B (Sigma-Aldrich), followed by streptavidin-peroxidase and ECL detection.

Lymphocyte motility

PBLs were resuspended in cation-free H/H medium (cation-free HBSS containing 10 mM HEPES (pH 7.4)) and pretreated with either the PIP2-binding peptide PBPI0, or the control peptide, QRL (see above), each at 40 μM, for 5–10 min at 37°C. Peptide-loading and -blocking activities were assessed by live fluorescence microscopy (Delta Vision Spectra RT; Applied Precision). Microslide chambers (Ibidi) were coated with 2 μg/ml CXCL12 overnight at 4°C, washed, and blocked with 2 mg/ml human serum albumin. Peptide-loaded lymphocytes were immediately injected into the chemokine-coated chambers, and their interaction with the substrate was recorded using Softworx 3.5 (Applied Precision) for -5 to 10 min at 6 frames/min using a ×200/0.95 NA differential interference contrast objective. Cells were classified as motile based on their ability to locomote for at least three cell diameters during a 10-min period of tracking.

Laminar flow assays and leukocyte treatments

Aliquots of PNAed were diluted in coating medium (PBS, supplemented with 20 mM bicarbonate (pH 8.5)) and adsorbed onto polystyrene plates, as previously described (31). Washed substrates were adsorbed with 4 μg/ml either intact or heat-inactivated chemokines for 3 h at 4°C. The anti-CXCR4 or anti-CCR7 mAbs were each coated onto a polystyrene plate for 2 h at 4°C. The polystyrene plate was assembled in a parallel plate laminar flow chamber, as previously described (20). Leukocyte populations were washed in H/H medium (HBSS/10 mM HEPES (pH 7.4), supplemented with 2 mg/ml BSA) containing 5 mM EDTA, resuspended in cation-free H/H medium supplemented with 2 mM CaCl2, and perfused through the flow chamber at a wall shear stress of 0.1 dyn/cm 2. Once the cells reached the upstream side of the adhesive test substrate, the fluid rate was elevated to generate a shear stress of 1.75 dyn/cm 2, and all cellular interactions were visualized at two different fields of view (one each 0.17 mm 2 in area) using the ×10 objective of an inverted phase-contrast microscope (Diaphot 300; Nikon).

Cholesterol raft disruption in human PBL and murine 300.19 pre-B cells was performed by pretreating cells for 10 min at room temperature, in cell-binding medium with 10–15 mM MβCD, followed by immediate introduction of cells into the flow chamber. These treatments were found to deplete up to 65% of the entire cholesterol content in both cultured 300.19 pre-B cells and Jurkat T cells (11); treatment with 30 mM MβCD did not
further deplete cellular cholesterol. MβCD did not affect lymphocyte viability within a 30-min period after cholesterol extraction. Mild disruption of the actin cytoskeleton was induced by pretreating cells for 1 min at room temperature with 20 μM cytochalasin D, followed by immediate introduction into the flow chamber with binding medium containing 2 μM cytochalasin D. In a control experiment, we found that 1-min exposure of T cells migrating over ICAM-1 and CXCL12 to 20 μM cytochalasin D was sufficient to block lymphocyte motility (data not shown).

Analysis of instantaneous velocities of leukocytes was performed using the WSCAN-Array-3 cell tracking software (Galai), as previously described (31). Adhesive interactions of transiently tethered cells (cells that attached for <0.2 s to the substrate) and of rolling cells (cells that rolled at least 3 s after initial tethering) were manually analyzed. Frequency of each category of tethers was expressed in percentage units.

Results

Cholesterol extraction from lymphocytes does not affect intrinsic L-selectin adhesiveness and rolling

To test the involvement of cholesterol rafts in the regulation of L-selectin-mediated lymphocyte rolling, freshly isolated human PBL were perfused on a substrate coated with PNAd, a prototypic L-selectin ligand (32). At physiological shear stresses, freshly isolated human lymphocytes (11), had no effect on either PBL attachment to or rolling on PNAd (Fig. 1A). In contrast to PBL, the Jurkat T lymphoblastoid line retains a significant portion of its L-selectin in lipid rafts (16). MβCD pretreatment of these cells was found to reduce L-selectin-mediated Jurkat rolling on P-selectin glycoprotein ligand 1 (16). In agreement with these results, the MβCD protocol, which had no functional impact on L-selectin adhesiveness in PBL, strongly impaired L-selectin-mediated Jurkat rolling on PNAd (Fig. 1, D and E). The MβCD protocol also abolished L-selectin adhesiveness in a second T lymphoblastoid cell line, CEM (data not shown). Thus, L-selectin, although requiring intact cholesterol rafts in T lymphoblastoid cell lines, is excluded from lipid rafts in primary circulating PBL, and membranal cholesterol is not

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required to maintain the inherent ability of L-selectin in these lymphocytes to interact with endothelial ligands under physiological conditions of shear flow.

These results could suggest that L-selectin dependence on cholesterol rafts is restricted to immortalized lymphocytes such as Jurkat. Nevertheless, and similar to PBL, L-selectin ectopically expressed in another immortalized line, the murine 300.19 pre-B cell line, was also unaffected by cholesterol extraction (Fig. 2. A and B). Notably, a small fraction of L-selectin in this pre-B cell line (<15%) still partitioned within low-density lipid fractions (Fig. 2C), but MβCD did not interfere with this residual L-selectin partition (data not shown).

A recent study on integrins suggested that these adhesion proteins translocate into rafts upon ligation (10). We therefore reasoned that L-selectin might also translocate into these domains following ligation. Aggregated GM1-containing low-density lipid rafts can be visualized by fluorescence microscopy of cholera toxin B binding, a high-affinity multivalent receptor for GM1 (34). Only partial overlap was found between Ab-ligated L-selectin and GM1 (data not shown), suggesting that L-selectin is excluded from GM1 rafts even after it is artificially ligated. Thus, L-selectin is expressed on the pre-B cell line primarily in high-density non-raft domains, with a small fraction present in low-density cholesterol-independent membranal domains. Consequently, extraction of cholesterol from this line, as from primary PBL, did not affect intrinsic adhesiveness of L-selectin in these two cell types. Thus, L-selectin regulation by cholesterol rafts is cell type dependent.

Cholesterol extraction reverses the destabilization of L-selectin-mediated rolling induced by surface-bound CXCL12 and CXCL13

Major arrest chemokines, including CXCL12, CCL21, CXCL13, and CCL8, strongly destabilize L-selectin-mediated lymphocyte or neutrophil rolling on various L-selectin ligands (20). This destabilization is induced only by immobilized chemokines juxtaposed to the L-selectin ligands (20). We therefore next determined

FIGURE 2. L-selectin shows low partitioning into low-density lipid fractions in pre-B lymphocytes, but its adhesiveness is insensitive to cholesterol extraction. A, Accumulation at 1.75 dyn/cm² 300.19 pre-B cells transfected with wild-type human L-selectin on a substrate containing PNAd (coated at 100 ng/ml). For MβCD pretreatment, pre-B cells were preincubated with the cholesterol chelator (10 mM) for 10 min. Mean velocities of rolling cells ± SEM are indicated near the accumulation plots. Data are representative of three independent experiments. B, Effect of a 10-min treatment with MβCD (10 mM) on the surface expression of L-selectin on 300.19 pre-B cells probed with the anti L-selectin mAb, DREG-200. C, Wild-type human L-selectin-transfected 300.19 pre-B cells were lysed and fractionated, as described in Fig. 1C. Fractions were analyzed by SDS-PAGE and immunoblotted with anti-Lyn and anti-L-selectin Abs. The fractions are numbered from 1 to 9 according to their position in the gradient.

FIGURE 3. Cholesterol extraction completely relieves suppression of rolling mediated by immobilized CXCL12. Effect of a 10-min pretreatment with MβCD on accumulation of human PBL (A) or L-selectin-transfected pre-B cells (C) on PNAd (coated at 100 ng/ml) coimmobilized with either inactivated or active CXCL12 at 4 μg/ml. For MβCD pretreatment, both types of cells were preincubated with the cholesterol chelator alone or in the presence of cholesterol (2 μg/ml) for 10 min. Mean rolling velocities ± SEM are indicated near respective accumulation plots. B and D, Effects of MβCD pretreatment on transient tethering and rolling of either human PBL (B) or of L-selectin-transfected pre-B cells (D) interacting with the PNAd/CXCL12 substrates used in A and C. Experiment pairs in A, B and C, D are each representative of five independent experiments.
whether and how destabilization of L-selectin rolling by immobilized chemokines is affected by cholesterol extraction, in both human PBL and murine pre-B lymphocytes. Strikingly, the suppression of L-selectin rolling by immobilized CXCL12 was entirely reversed by MβCD pretreatment of PBL (Fig. 3, A and B). Furthermore, the ability of MβCD to rescue adhesion was abolished in the presence of exogenous cholesterol (Fig. 3, A and B), consistent with cholesterol depletion, accounting for the effect of MβCD in this assay. Thus, interference with the integrity of cholesterol rafts disrupts the suppressive effects of immobilized CXCL12 on L-selectin-mediated rolling. Notably, our PBL preparations contained >90% CD3 T cells and were found negative for expression of the newly identified CXCL12 receptor, RDC1 (CXCR7) (35) (T. Hartmann, manuscript in preparation). Thus, the entire responsiveness of these cells to CXCL12 was assumed to be mediated by CXCR4.

As was observed with PBLs, CXCL12 coimmobilized with PNAd strongly suppressed L-selectin-mediated rolling of the pre-B cell line without interfering with initial L-selectin-mediated tethers (Fig. 3, C and D). This suppression was entirely rescued by MβCD pretreatment (Fig. 3, C and D), and the effect of MβCD was reversed in the presence of exogenous cholesterol (Fig. 3, C and D). L-selectin destabilization in primary murine B splenocytes by a second B cell-specific chemokine, CXCL13, was also completely abolished by cholesterol extraction by MβCD (Fig. 4). As in human PBL and the murine pre-B cell 300.19 line, extraction of cholesterol from murine B splenocytes did not affect the inherent ability of their L-selectin to stabilize rolling on PNAd (data not shown). Thus, although cholesterol-dependent lipid rafts are not required for intrinsic adhesiveness of L-selectin in either primary T or B lymphocytes, they are necessary for immobilized CXCL12 and CXCL13 to deliver their destabilization signals to L-selectin, and abolish rolling adhesions on PNAd.

Cholesterol extraction does not rescue destabilization of L-selectin-mediated rolling by the CCR7 ligand, CCL21

To further extend these observations, we next tested whether cholesterol extraction would also rescue L-selectin destabilization induced by a third homeostatic arrest chemokine, the major CCR7 ligand CCL21. Human PBL perfused over substrates containing PNAd coadsorbed with functional CCL21 failed to accumulate on the ligands (Fig. 5A, left) and CCL21 binding to tethered T cells suppressed both L-selectin-mediated rolling as well as transient L-selectin tethers (Fig. 5A, right). Strikingly, CCL21-mediated

FIGURE 4. Cholesterol extraction from primary murine B lymphocytes relieves suppression of rolling mediated by immobilized CXCL13. A, Effect of MβCD pretreatment of murine B splenocytes on their L-selectin-mediated rolling on PNAd alone or coimmobilized with CXCL13 at 4 µg/ml. Mean rolling velocities ± SEM are indicated at the appropriate points on the accumulation plots. B, Effect of MβCD pretreatment on transient tethering and rolling of B splenocytes on the PNAd/CXCL13 substrate described in A. Data points are the means ± range of measurements taken in two fields of view. Experiments in A and B are representative of three.

FIGURE 5. Cholesterol extraction does not rescue suppression of L-selectin rolling by immobilized CCL21 (secondary lymphoid tissue chemokine). Accumulation of human PBL (A) or murine B splenocytes (B) perfused over PNAd (100 ng/ml) coimmobilized with either inactivated or active CCL21 at 4 µg/ml. Right, Frequency and type of tethers formed by the PBL (A) or the murine B splenocytes (B) during the accumulation periods shown in the left panels. Pretreatment of CCR7 on the PBL with soluble CCL19 (0.5 µg/ml, in binding medium for 45 min) rescued the effect of CCL21 on rolling adhesions. Data points represent the means ± range of measurements taken in two fields of view. The experiment shown is representative of three independent experiments.
suppression in PBL was insensitive to cholesterol extraction (Fig. 5A), although it was inhibited by pre-exposure of the lymphocytes to soluble CCL19 (EBV-induced molecule 1 ligand chemokine; Fig. 5A), a second ligand of CCR7. When immobilized CCL19 was used instead of immobilized CCL21, destabilization of L-selectin in these two cell types remained insensitive to cholesterol extraction (data not shown). Results did not change also when CCL21 or CCL19 was immobilized on endothelial-like monolayers expressing L-selectin ligands (data not shown). Similar findings were observed in murine B lymphocytes interacting with PNAd and CCL21 (Fig. 5B). Thus, whereas CXCL12- and CXCL13-mediated destabilization of L-selectin-mediated rolling is fully eliminated upon cholesterol raft disruption, in human PBL, murine B lymphocytes, and 300.19 pre-B cells (Figs. 3 and 4), suppression of L-selectin rolling by immobilized CCR7 ligands is retained after cholesterol extraction (Fig. 5).

**FIGURE 6.** Cholesterol extraction impairs CXCR4 adhesiveness to surface-bound anti-CXCR4 mAb, but not CCR7 binding to surface-bound anti-CCR7 mAb under shear flow. Left, Frequency and type of tethers measured at 0.5 dyn/cm² of intact or MβCD-treated PBL interacting with surface-bound anti-CXCR4 mAb, coated at low or high density (0.2 or 1 µg/ml, respectively). Filled bars denote stable (arrested) tethers. Right, Tethering frequency, measured at 0.5 dyn/cm², of intact or MβCD-treated PBL interacting with surfaces coated with low or high densities of anti-CCR7 mAb. Values represent mean ± range of measurement in two fields of view. Adhesion experiments shown are representative of three experiments.

**FIGURE 7.** Actin filament capping with cytochalasin D retains L-selectin adhesiveness, but interferes with both CXCL12- and CCL21-mediated suppression of L-selectin rolling. A, PBL were pretreated for 1 min with 20 µM cytochalasin D at room temperature. Accumulation of intact or cytochalasin D-treated PBL was tested at a shear stress of 1.75 dyn/cm² on substrates coated with PNAd at 100 ng/ml. As a control, where indicated, PBL were treated with 20 µM cytochalasin D for 20 min at room temperature to globally disrupt the actin cytoskeleton. B, Effect of 1 min pretreatment of PBL with 20 µM cytochalasin D on accumulation at a shear stress of 1.75 dyn/cm² on PNAd coimmobilized with either inactive or active CXCL12 each at 4 µg/ml. Data points represent the means ± range of measurements taken in two fields of view. C, Effect of the 1 min cytochalasin D pretreatment on PBL accumulation on PNAd coimmobilized with either inactive or active CCL21 each at 4 µg/ml. Data points represent the means ± range of two measurements. The experiments shown in A–C are each representative of three independent experiments.

Cholesterol is necessary for CXCR4, but not for CCR7 engagement by surface-bound mAbs under shear flow

Imobilized chemokines destabilize L-selectin-mediated rolling during subsecond-lived adhesive contacts but their soluble counterparts fail to suppress rolling (20). We therefore hypothesized that successful engagement of the respective GPCR by its cognate immobilized ligand under shear force may be required for the engaged GPCR to destabilize L-selectin-mediated adhesions. Leukocyte GPCR associations with immobilized mAbs serve as a sensitive measure of the cytoskeletal anchorage of the GPCR and its force resistance at subsecond encounters (3, 36). We therefore tested whether CXCR4 and CCR7, the main GPCRs for the chemokines CXCL12 and CCL21 on PBL, require intact cholesterol to engage with their respective surface-immobilized mAbs under shear flow. When PBL were perfused over diluted surface-bound anti-CXCR4 mAb (12G5), PBL adhesion to anti-CXCR4 was largely resistant to cholesterol raft disruption by MβCD (Fig. 6, left). Nevertheless, CXCR4-mediated PBL adhesion to high-density anti-CXCR4 mAb was substantially suppressed by cholesterol extraction from the cells (Fig. 6), indicating that multivalent engagement of CXCR4 requires intact cholesterol rafts. CCR7-mediated PBL adhesion to anti-CCR7 mAb immobilized at low and high density was, in contrast, entirely resistant to MβCD treatment (Fig. 6, right). A CXCR5-specific mAb failed to support B lymphocyte adhesion in this assay, and therefore the effect of MβCD treatment on this GPCR could not be compared with the other two GPCRs (data not shown).

Intact actin filaments are required for all types of chemokine-mediated suppression of L-selectin

Assemblies of GPCRs are regulated by actin cytoskeleton networks (37). Because general disruption of the actin cytoskeleton strongly interferes with intrinsic selectin adhesiveness (3, 28) (Fig. 7A), we used a modified approach in which cells were treated for 1 min with the actin-capping drug, cytochalasin D (38), and were then immediately analyzed, unwashed, for their L-selectin adhesiveness to ligand in the presence or absence of destabilizing chemokines. Strikingly, this short cytochalasin D pretreatment, unlike a prolonged pretreatment, did not alter L-selectin adhesiveness under shear flow (Fig. 7A). However, it completely rescued the suppression of L-selectin rolling mediated by either CXCL12, CXCL13, or CCL21 (Fig. 7, B and C, and data not shown). At this high dose and short exposure period, cytochalasin D binds to both...
cell diameters during the assay period is shown. Lymphocytes within each experimental group that migrated at least three immediately analyzed on a substrate coated with CXCL12. The fraction of selectin.

were preloaded with the PBP10 or control peptides as in

FIGURE 8. Blocking membranal PIP2 in PBL does not affect L-selectin adhesiveness and does not impair CXCL12-mediated suppression of L-selectin-rolling adhesions. A. Incorporation of the rhodamine B-labeled PIP2-binding peptide (PBP10) and its rhodamine-labeled control peptide (QRL) into PBL. Lymphocytes were presuspended with the peptides, each at 40 μM for 20 min, and visualized by live fluorescence microscopy. DIC = differential interference contrast. B. Effect of PIP2, blocking on chemokine-triggered PBL motility. PBL were preloaded as in A and immediately analyzed on a substrate coated with CXCL12. The fraction of lymphocytes within each experimental group that migrated at least three cell diameters during the assay period is shown. C. PBP10, blocking does not reduce L-selectin-mediated capture or rolling adhesions on PNAAd and does not rescue CXCL12-mediated suppression of L-selectin adhesions. PBL were preloaded with the PBP10 or control peptides as in A and B, and immediately perfused over PNAAd coimmobilized with either inactive or active CXCL12, as in Fig. 7. The results shown in A–C are each representative of three independent experiments.

ends of actin filaments and can also sequester G-actin from elongating existing filaments (39). Thus, intact actin cytoskeletal assemblies are required by all the chemokines tested to destabilize L-selectin adhesions at subsecond-lived adhesive contacts under shear flow.

Polyphosphoinositides affect the organization and activities of many actin-modulating proteins (40). The lipid PIP2 plays a key role in activating adaptors that strengthen the adhesion of the actin cytoskeleton and the plasma membrane (41). A cell-permeable 10-residue peptide, PBP10, derived from the PIP2-binding region of the actin-capping protein gelsolin, was previously shown to block cell motility in various cell types and disassemble actin filaments nearby the plasma membrane (25). This peptide efficiently penetrated primary T lymphocytes (Fig. 8A) and was highly effective in inhibiting their CXCL12-triggered motility (Fig. 8B). Nevertheless, and in contrast to the actin-capping reagent, cytochalasin D, the PIP2-blocking peptide, could not rescue the suppression of L-selectin rolling by subsecond-lived contacts with CXCL12, nor did it interfere with inherent L-selectin activities (Fig. 8C). These results collectively suggest that all GPCRs involved in subsecond destabilization of L-selectin require intact polymerized actin, but not membranal PIP2, to deliver their destabilizing signals to the selectin.

Discussion

Lymphocytes rolling on L-selectin ligands can incorporate local signals from endothelial-presented chemokines and juxtaposed integrin ligands within a fraction of 1 s (42–44). In contrast to integrins, which undergo rapid activation by homeostatic and inflammatory arrest chemokines, L-selectin-mediated rolling adhesions are subjected to negative regulation by these same chemokines in multiple types of primary and immortalized leukocytes (20). The present study was aimed at elucidating the involvement of membranal cholesterol, a key component of lipid rafts, in this in situ regulation of multivalent L-selectin contacts by chemokines, because cholesterol rafts were recently implicated in signaling to lymphocyte integrins (11) as well as in L-selectin signaling (15). A recent study also suggested that the inherent adhesive activity of L-selectin in both neutrophils and the T cell line, Jurkat, is sensitive to cholesterol extraction from the cell surface, but did not address a similar role of cholesterol in L-selectin adhesiveness in primary lymphocytes (16). Our results indicate that cholesterol is dispensable for the intrinsic adhesiveness of L-selectin under shear flow in human PBL, primary murine B lymphocytes, and a pre-B cell line. L-selectin is indeed largely excluded in these cells from low-density lipid rafts (this study) (11). Notably, even mAb-induced colocalization of L-selectin into microdomains enriched with the raft marker GM1 did not require membranal cholesterol, consistent with poor partition of L-selectin into cholesterol rafts. Thus, the previously reported partition of L-selectin into cholesterol-enriched lipid rafts and the dependence of L-selectin adhesiveness on these rafts (16) are cell type dependent and cannot be extrapolated to all leukocytes. A possibility that remains to be addressed is whether L-selectin partitions into cholesterol rafts in subsets of activated T or B cells, reminiscent of its partition in Jurkat cells, which represent a subtype of activated lymphocytes. As activated/effector lymphocytes generally express low levels of L-selectin, they use endothelial selectins as their main capturing and rolling receptors (45). It would be interesting to test how specific selectin ligands partition into cholesterol rafts and how assemblies of P-selectin and E-selectin ligands vary with different subsets of effector/activated lymphocytes. Nevertheless, even in those cellular environments in which cholesterol lipid rafts are dispensable for L-selectin adhesiveness, these very rafts are essential for the ability of both CXCL12 and CXCL13 chemokines to destabilize L-selectin-rolling adhesions. Thus, the ability of both chemokines to deliver a suppressive signal to L-selectin requires cholesterol-stabilized assemblies. Notably, although L-selectin ligation was reported to drive the selectin into raft microdomains and induce shedding (15), the chemokine-mediated suppressive signals studied in our system are not associated with L-selectin shedding, because chemokine-mediated suppression of L-selectin rolling cannot be rescued by inhibition of L-selectin shedding (20). Ab-driven dimerization of the ectodomain of L-selectin known to augment L-selectin adhesiveness under shear flow (46) was also unable to rescue chemokine-mediated destabilization of rolling (20). Our data are therefore consistent with the possibility that chemokine-driven GPCR assemblies do not interfere with the ability of L-selectin to dimerize, but rather inhibit key cytoplasmic associations of L-selectin necessary for adhesion stabilization under external forces (47).

Indeed, all L-selectin-suppressing chemokines tested were very sensitive to a short lymphocyte pretreatment with the actin-capping compound, cytochalasin D, a treatment that on its own did not impair L-selectin adhesiveness. These data raise the possibility that an available pool of actin nearby the plasma membrane, which is antagonized or competed by chemokine-occupied GPCRs, is necessary for L-selectin to optimally stabilize adhesion upon binding to its endothelial ligand. L-selectin is constitutively linked to the cortical cytoskeleton via associations with both ERM proteins and α-actinin (8, 12). Upon ligation, L-selectin can further incorporate
into the detergent-insoluble cytoskeleton via a domain recognized by α-actinin (48). Truncation of this domain while retaining the ERM binding site strongly reduces L-selectin tether stabilization (3), reminiscent of the effects triggered by immobilized chemokines. GPCR assemblies induced by homeostatic chemokines may therefore mimic the effect of L-selectin tail truncation in that they may rapidly sequester actin filaments from L-selectin tails and thereby reduce the ability of L-selectin to assemble these actin cytoskeleton components. We thus propose that lymphocyte GPCRs, when assembled by their surface-immobilized chemokines, rapidly interfere with the ability of ligand-occupied L-selectin to assemble actin and undergo postligand stabilization under shear forces. Ligand-induced selectin stabilization (49) is likely to benefit from proper selectin anchorage to the cortical cytoskeleton (50). Stabilization following ligand binding may also involve multivalent contact formation, which could be sterically blocked by chemokine-GPCR-cytoskeletal bonds. Without such selectin-mediated contact stabilization, a process completed on average within 0.3–0.5 s (20), L-selectin tethers cannot progress into stable rolling adhesions under shear flow, and transiently captured lymphocytes are readily released back to the circulation. Contact stabilization involves, however, distinct lipid microdomains, depending on the chemokine type. Thus, whereas chemokine-occupied CXCR4 and CXCR5 require cholesterol to block L-selectin adhesion stabilization, chemokine-occupied CCRL7 does not. Chemokine-mediated suppression of L-selectin via CCRL7 is also more potent than the suppression mediated by CXCR4 and CXCR5 (20). Thus, it is conceivable that chemokine-occupied CCRL7 directly interferes with the earliest L-selectin bond stabilization events (within a time frame of <0.1 s of initial tethering), whereas CXCR4 and CXCR5 interfere with a later L-selectin adhesion stabilization event (within a time frame of 0.3 s and longer) (20).

Notably, chemokine suppression of leukocyte rolling does not involve proteolytic L-selectin shedding and is most robust on high-density ligand, suggesting that it interferes with the stabilization of multivalent L-selectin contacts critical for stable leukocyte-rolling adhesions on high-density endothelial ligands (20). As the destabilizing chemokine can trigger integrin-mediated arrest if juxtaposed to the integrin ligand, interference with L-selectin adhesions is likely only at regions high in L-selectin ligand and low in integrin ligands. Such interference may underlie the jerky nature of rolling mediated by L-selectin in subsets of high endothelial venules (51, 52). The jerky nature of L-selectin rolling can also arise from anti-adhesive glycoproteins such as CD43 (53) as well as from topological heterogeneity of both the leukocyte and the endothelial surfaces (54). Chemokine destabilization of L-selectin adhesions may serve as a negative feedback means to render L-selectin rolling more labile because faster rolling has been shown to attenuate activation of integrin-mediated arrest (55). The existence of multiple mechanisms for modulating the strength of L-selectin rolling supports the notion that the dynamics of these adhesions indeed control the efficiency of subsequent integrin activation steps. Chemokine-accelerated L-selectin-mediated rolling could thus serve to counterbalance excessive chemokine activation of integrin-mediated stoppage.

Notably, the cholesterol dependence of a given GPCR-chemokine pair that interferes with L-selectin rolling is conserved among all cell types tested. This finding suggests that GPCRs capable of transmitting inhibiting signals to L-selectin pre-exist in unique membranal assemblies, stabilized by specific actin cytoskeletal linkers and possibly by membranal partners (56, 57). These complexes may be preferentially enriched in microvilli, the major sites for rapid generation of adhesive contacts under shear flow (58). Future ultrastructural microscopic and cross-linking studies will be required to shed more light on such putative GPCR assemblies on the plasma membrane. Identification of these platforms may shed light not only on the mechanisms underlying the selectin destabilization by chemokines. Such studies are also expected to resolve how a given chemokine occupancy of a GPCR may act as a switch, transmitting either a suppressive signal to L-selectin, or an integrin-activation signal at short-lived endothelial contacts. Future studies should also elucidate how this switch is fine-tuned by the molecular composition of various leukocyte-endothelial contacts as well as by the tensile forces experienced by the leukocyte at these contacts.

Acknowledgments

We thank Dr. S. Schwarzbbaum for editorial assistance and Dr. P. A. Janney (University of Pennsylvania, Philadelphia, PA) for providing cell-permeant peptides.

Disclosures

The authors have no financial conflict of interest.

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